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miR-23a and miR-27a Promote Human Granulosa Cell Apoptosis by Targeting *SMAD5*[1]

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ABSTRACT

In mammals, follicular atresia can be partially triggered by granulosa cell apoptosis. However, very little is known about the functions of miRNAs in granulosa cell apoptosis. We previously reported that hsa-mir-23a (miR-23a) and hsa-mir-27a (miR-27a) were highly expressed in the plasma of patients with premature ovarian failure, but the action of these two miRNAs in follicular development was unclear. In this study, we explored the roles of miR-23a and miR-27a in the granulosa cells of women undergoing in vitro fertilization/embryo transfer. Using Hoechst staining, we found that miR-23a and miR-27a promoted apoptosis in human granulosa cells. In addition, the Western blotting results suggested that the miR-23a/miR-27a-mediated apoptosis occurred via the FasL-Fas pathway. Based on the results of a luciferase-reporter assay and quantitative RT-PCR and Western blotting analyses, we found that *SMAD5* is a target gene of both miR-23a and miR-27a. Furthermore, knocking down *SMAD5* expression increased the rate of apoptosis, as well as the levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3 protein. Taken together, these data suggest that miR-23a and miR-27a target *SMAD5* and regulate apoptosis in human granulosa cells via the FasL-Fas pathway. These findings provide an improved understanding of the mechanisms underlying granulosa cell apoptosis, which could potentially be used for future clinical applications.

apoptosis, granulosa cells, miR-23a, miR-27a, ovary, SMAD5

INTRODUCTION

The mammalian ovary produces mature oocytes and synthesizes hormones that are essential for reproduction. An enormous number of follicles are present in the ovary, and each follicle contains an oocyte surrounded by one or several layers of granulosa cells. Communication between the oocytes and granulosa cells plays an important role in follicular development. Before ovulation, 99% of follicles undergo atretic degeneration at various stages of development [1], and granulosa cell apoptosis is the primary trigger of follicular atresia [2]. An abnormally high rate of atresia can result in pathological ovarian consequences, such as premature ovarian insufficiency, premature ovarian failure (POF), and infertility [3]. Multiple factors, including hormones, cytokines, and growth factors, are crucial for the regulation of granulosa cell apoptosis. Although the mechanisms of follicular atresia have not been precisely defined, recent reports have demonstrated an association between the growth of follicles and the levels of certain miRNAs that are essential for the regulation of these factors [4, 5].

MicroRNAs are noncoding, single-stranded, small RNAs consisting of 22–24 nucleotides (nt) that regulate gene expression at the posttranscriptional level. Pri-miRNAs are primary transcripts several kilobases in length that undergo substantial processing in the nucleus, resulting in the generation of a 70- to 90-nt stem-loop precursor miRNA (pre-miRNA). The RNase III-containing enzyme dicer is necessary for processing miRNA precursors into mature miRNAs. Mature miRNAs become incorporated into the RNA-induced silencing complex through complementary interactions with target genes, resulting in sequence-specific translational repression or mRNA degradation [6]. Through this mechanism, miRNAs influence the outcomes of various cellular activities in both normal and disease states. MicroRNAs are involved in many cellular processes, including cell proliferation, differentiation, and death, under both physiological and pathological conditions [7].

More than 1000 miRNAs are encoded by the human genome, targeting 60% of the genes [8, 9]. Hundreds of miRNAs are present in the ovary, and they are associated with the regulation of the expression of genes involved in many processes, including the cell cycle, cellular growth, proliferation, differentiation, angiogenesis, steroidogenesis, and atresia [10]. In mammalian cells, Lei and colleagues [11] reported that the conditional inactivation of Dicer1 in mouse granulosa cells resulted in an increased pool of primordial follicles and accelerated the rate of early follicle recruitment and follicular degeneration, implicating miRNAs in follicular development. In pig ovaries, miR-92a regulates granulosa cell apoptosis by targeting *SMAD7* directly [12]. Liu et al. [13] also demonstrated that miR-26b functioned as a proapoptotic factor in porcine follicular granulosa cells by targeting *SMAD4*.

The cluster miR-23a ~ 27a ~ 24-2, which encodes a pri-miRNA transcript consisting of three miRNAs—miR-23a, miR-27a, and miR-24-2—induces caspase-dependent and caspase-independent apoptosis via the c-Jun N-terminal kinase pathway in human embryonic kidney (HEK293T) cells [14]. We previously observed that the levels of miR-23a and miR-27a were upregulated in the plasma of POF patients compared with those of normally cycling women and that miR-23a

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TABLE 1. General characteristics of the study population.*

Characteristics	Mean ± SD
Age, yr	28.82 ± 1.93
BMI, kg/m^2	21.58 ± 4.36
Duration of infertility, yr	2.49 ± 1.56
Basal FSH, mIU/ml	7.02 ± 3.02
Basal LH, mIU/ml	5.58 ± 1.82
Basal E_2, pg/ml	39.03 ± 7.62
Basal P, ng/ml	0.83 ± 0.37
Basal PRL, ng/ml	17.33 ± 5.85
Basal T, ng/dl	19.46 ± 7.02
Antral follicle number	8.59 ± 3.56
Cause of infertility, %	
Male factor	38
Tubal factor	62
hCG injection day	
E_2, pg/ml	2639.71 ± 1561.69
LH, mIU/ml	1.48 ± 0.79
P, ng/ml	1.11 ± 0.47
Gn dosage, IU	2758.26 ± 1121.43
No. of oocytes retrieved	9.82 ± 3.27

* BMI, body mass index; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; E_2, estradiol; P, progesterone; PRL, prolactin; Gn, gonadotropins; T, testosterone.

induced apoptosis in human granulosa cells by decreasing the level of X-linked inhibitor of apoptosis protein (XIAP) and increasing the level of caspase-3 cleavage [15]. In addition, Kim et al. [16] found that transfection with an miR-27a mimic sequence decreased the oocyte maturation rate of mouse follicles. In cows, miR-23b, miR-24, and miR-27a have been found to be associated with follicular development and fertility [10]. In ovarian cancer cells, miR-27a has been found to be a proto-oncogene that plays an important role in growth and metastasis, and an inhibitor of miR-27a has been shown to suppress the growth and migration of tumor cells [17]. However, the exact roles of miR-23a and miR-27a in human granulosa cells have not yet been fully elucidated.

In the present study, we investigated miR-23a and miR-27a to explore their functions in human granulosa cell apoptosis and to elucidate the underlying mechanisms. Our results demonstrated that miR-23a and miR-27a targeted *SMAD5* directly and had similar functions in promoting apoptosis via the FasL-Fas pathway in granulosa cells in vitro. These findings could help to explain the roles of miRNAs in granulosa cell apoptosis and facilitate controlling follicular growth and rescuing oocytes in future clinical applications.

MATERIALS AND METHODS

Study Participants

This study was approved by the Human Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Informed consent was obtained from each patient. Overall, 74 patients undergoing their first in vitro fertilization (IVF) or intracytoplasmic sperm injection-embryo transfer (ICSI-ET) due to tubal or male factors were involved in the study. Patients with a history of polycystic ovarian syndrome (PCOS) and/or endometriosis were excluded. The baseline characteristics are summarized in Table 1. All of the participants were subjected to the midluteal gonadotropin-releasing hormone agonist downregulation protocol. A total of 250 μg of human chorionic gonadotropin was administered to induce final maturation when the largest follicle was at least 18 mm in diameter or when two follicles were at least 17 mm in diameter. Oocytes were retrieved 34–36 h later.

Isolation and Culture of Granulosa Cells

Human granulosa cells were collected from the follicular fluid of patients undergoing IVF/ICSI, as described previously by Shi et al. [18]. Cells from more than one patient were pooled at a density of 3×10^4 to 4×10^4 cells per well on 24-well plates. Briefly, granulosa cells were separated from red blood cells by centrifugation through 50% Percoll (Sigma, St. Louis, MO) for 15 min at 1000 × *g*. The granulosa cells in the interface layer were harvested. Following centrifugation and washing with PBS, the cells were resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (penicillin G, streptomycin, and amphotericin B; Sigma) and were cultured at 37°C in 5% CO_2.

MicroRNA Transfection

The granulosa cells were seeded in 24-well plates to ensure that they attained 40%–50% confluence by the next day. The cells were transfected with pre-miR-23a, pre-miR-27a, or a negative control (NC; Ambion Inc., Austin, TX), or with an miR-23a inhibitor or miR-27a inhibitor, as well as their NCs (Exiqon, Vedbaek, Denmark), using Lipofectamine2000 (Invitrogen, Carlsbad, CA) at a final concentration of 100 nM, according to the manufacturer's instructions. The NC was a scrambled nonspecific sequence that has been extensively tested in human cell lines and tissues and has been shown to produce no identifiable effects on known miRNA functions. RPMI-1640 containing 10% FBS was added after incubating the samples in Opti-MEM (Invitrogen) for 6 h. A fluorescein isothiocyanate-labeled inhibitor control was included to demonstrate the efficiency of transfection, which was found to be higher than 90%.

RNA Interference

Granulosa cells were transfected with 100 nM SMAD5 siRNA (sc-38378; Santa Cruz Biotechnology, Dallas, TX) or control siRNA (sc-37007; Santa Cruz Biotechnology) using Lipofectamine 2000, according to the manufacturer's instructions.

RNA Extraction and RT-PCR

Total RNA was isolated from granulosa cells using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The cDNA synthesis reactions were performed using 2 μg of RNA. Based on previous studies, GAPDH and U6 are stably expressed in the human ovary, and their levels of expression are widely used as internal references [19, 20]. In this study, GAPDH was used as an internal control for determining the SMAD5 levels, whereas human U6 was used as an internal control for determining the miRNA levels. SuperScript II (Invitrogen) and the following primers (Invitrogen, Shanghai, China) were used for RT-PCR: hsa-miR-23a, 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG GAA AT-3′; hsa-miR-27a, 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG CGG AA-3′; and human U6, 5′-TAT GGA ACG CTT CAC GAA TTT G-3′. The cycle parameters used in this assay were as follows: 65°C for 5 min, 42°C for 3 min, 42°C for 50 min, and 72°C for 15 min.

Quantitative PCR

Quantitative RT-PCR (qRT-PCR) was performed using an SYBR Green kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Each 10-μl reaction mixture included 1 μl of cDNA, 5 μl of SYBR Green Mix, 0.5 μl of the specific primers, 0.2 μl of RoxReference Dye II, and 3.3 μl of RNase-free water. The sequences for the primers (Invitrogen, Shanghai, China) were as follows: hsa-miR-23a forward, 5′-CCG CGA TCA CAT TGC CAG GG-3′; hsa-miR-27a forward, 5′-GCC GCT TCA CAG TGG CTA AG-3′; miRNA Public reverse, 5′-GTG CAG GGT CCG AGG T-3′; human U6 forward, 5′-TCG CTT CGG CAG CAC ATA TAC-3′; human U6 reverse, 5′-TAT GGA ACG CTT CAC GAA TTT G-3′; SMAD5 forward, 5′-CCT CCA GTA TTA GTG CCT CGT-3′; SMAD5 reverse, 5′-TCA TTG TGG CTC AGG TTC C-3′; GAPDH forward, 5′-ACG GAT TTG GTC GTA TTG GG-3′; and GAPDH reverse: 5′-TGA TTT TGG AGG GAT CTC GC-3′. The PCR reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec, and 72°C for 15 sec. During each run, a ''no reverse transcriptase'' control was used to ensure that the samples were not contaminated with genomic DNA. All of the reactions were performed in triplicate.

Western Blotting Analysis

Western blotting was performed as previously described [21]. In brief, proteins were extracted from the cells using whole-cell lysis buffer (4 mM ethylene glycol tetraacetic acid; 3 mM ethylene diamine tetraacetic acid, pH 8.0; 0.5% Nonidet P-40; 12.5 mM HEPES; 1 mM dithiothreitol; 0.5 mM Na_3VO_4; 125 mM NaF; 2.5 mg/ml aprotinin; 25 mg/ml trypsin inhibitor; and 25 mM PMSF).

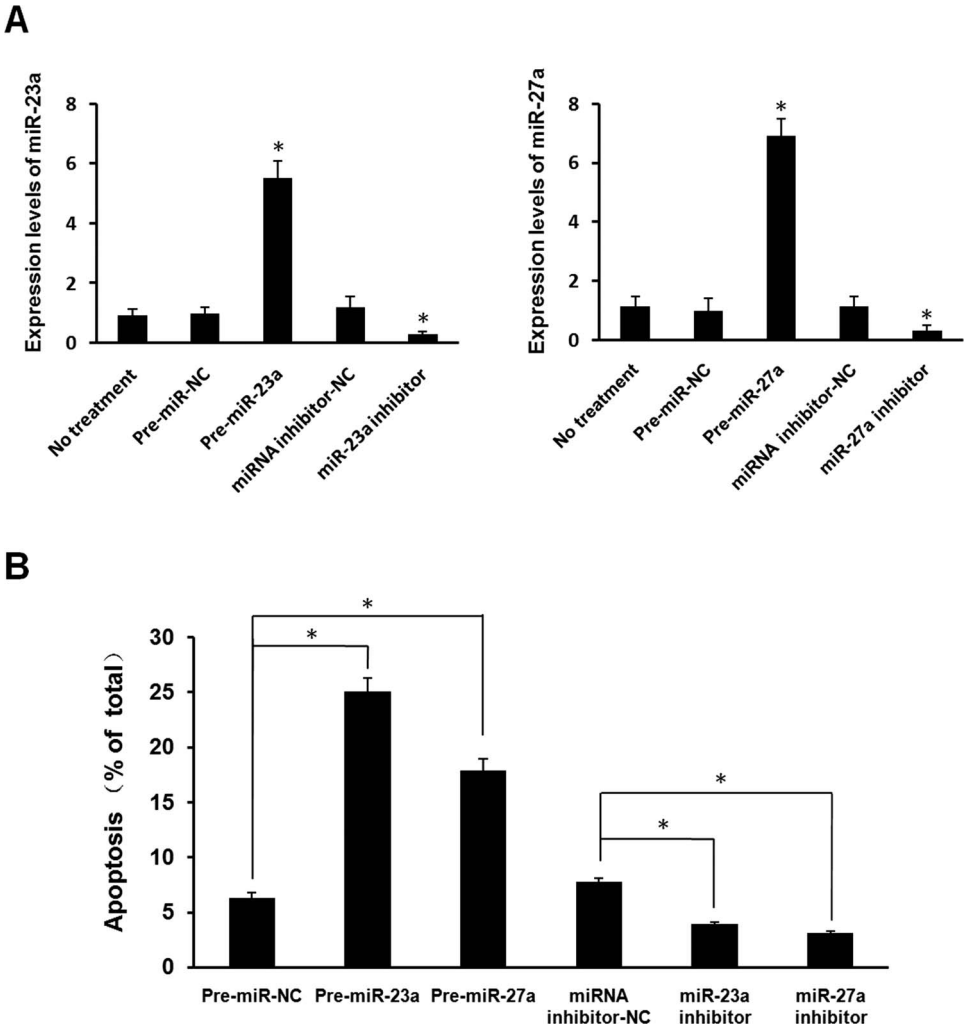

FIG. 1. miR-23a and miR-27a promote the apoptosis of granulosa cells. **A**) Quantitative RT-PCR was used to determine the transfection efficiency of pre-mir-23a, pre-mir-27a, the mir-23a inhibitor, and the mir-27a inhibitor. Human U6 was used for normalization. Data are mean ± SD (*$P < 0.05$). **B**) Effects of transfection using different constructs on the rate of apoptosis of granulosa cells were determined using Hoechst 33258 staining. The data were obtained from three independent experiments. Data are mean ± SD (*$P < 0.05$).

The protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). A total of 20 μg of total protein was subjected to SDS-PAGE and then was electrotransferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with the primary antibody (anti-SMAD5: no. 9517; 1:1000; Cell Signaling Technology, Beverly, MA; anti-Fas: sc-715; 1:1000; Santa Cruz Biotechnology; anti-Fasl: sc-834; 1:1000; Santa Cruz Biotechnology; anti-cleaved caspase-8: no. 9496; 1:1000; Cell Signaling Technology; anti-cleaved caspase-3: sc-22171-R; 1:1000; Santa Cruz Biotechnology; anti-SMAD2: ab53100; 1:1000, Abcam, Cambridge, U.K.; anti-SMAD4: ab40759; 1:1000; Abcam, Cambridge, U.K.; anti-GAPDH: ab22556; 1:1000; Abcam, Hong Kong, China). After incubation with the appropriate secondary antibodies (Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) at room temperature for 1 h, the reactive bands were visualized using an enhanced chemiluminescence kit (Pierce). The final results were analyzed using Scion Image software (http://scion-image.software.informer.com/).

Assessment of Apoptosis

Cells were stained using 10 μg/ml Hoechst 33258 (Sigma) at room temperature for 5 min in the dark. The apoptotic cells were identified as previously reported [22]. In each sample, at least 200 cells were counted in a random field to determine the percentage of apoptotic cells.

Luciferase Assay

A total of 100 ng of the indicated pGL3 firefly luciferase construct and 5 ng of a pGL3 *Renilla* luciferase normalization control were cotransfected into 293T cells using Lipofectamine 2000. At the same time, the indicated miR-23a or miR-27a expression plasmid or mimics (Viewsolid Biotech, Beijing, China) were transfected. The level of luciferase activity was determined at 48 h after transfection using the Dual-Glo luciferase assay system (Promega, Madison, WI). The level of *Renilla* luciferase activity was used as the internal control.

Statistical Analysis

The results were presented as the mean values ± SDs of three independent experiments. Student *t*-test was used for comparisons between two groups, and an ANOVA with a post hoc test was used for multiple comparisons. These statistical analyses were performed using SPSS software, version 16.0 (IBM Corp., Armonk, NY). The value indicating significant differences was set at $P < 0.05$.

RESULTS

miR-23a and miR-27a Increase the Apoptotic Rate of Human Granulosa Cells

We previously reported that the levels of miR-23a and miR-27a were upregulated in the serum of POF patients [15]. To explore the effects of miR-23a and miR-27a on the apoptosis of human granulosa cells, we transfected these cells with pre-miR-23a, pre-miR-27a, an miR-23a inhibitor, an miR-27a inhibitor, or their NCs at a final concentration of 100 nM. The expression levels of miR-23a and miR-27a were determined

using qRT-PCR. As shown in Figure 1A, the expression of miR-23a in cells transfected with pre-miR-23a was significantly increased compared with that of cells transfected with pre-miR-NC or cells given no treatment. In contrast, the expression level of miR-23a was significantly decreased after transfection with an miR-23a inhibitor compared with that of cells transfected with miRNA inhibitor-NC or cells given no treatment. Similar results were observed when the cells were transfected with pre-miR-27a or an miR-27a inhibitor.

The effects of miR-23a and miR-27a on granulosa cell apoptosis were determined using Hoechst staining. Overexpression of miR-23a or miR-27a dramatically increased the apoptotic rate, whereas the downregulation of miR-23a or miR-27a suppressed apoptosis (Fig. 1B). Taken together, these results indicated that miR-23a and miR-27a promoted apoptosis in cultured human granulosa cells.

miR-23a and miR-27a Are Involved in Fas-Induced Death Signals

The FasL-Fas system is one of the best characterized apoptotic signaling pathways of human granulosa cells [23]. FasL and Fas are expressed in granulosa cells and induce apoptosis [24]. Typically, the death receptor-initiated pathway is activated by caspase-8, followed by the activation of caspase-3 [25]. To explore how miR-23a and miR-27a regulate apoptosis in human granulosa cells, the levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3 in transfected cells were determined using Western blotting. As shown in Figure 2A, pre-miR-23a significantly increased the Fas, FasL, cleaved caspase-8, and cleaved caspase-3 levels. In contrast, the miR-23a inhibitor triggered a significant decrease in the levels of these proteins, suggesting that miR-23a is involved in the FasL-Fas pathway. Similar trends were observed when miR-27a was studied (Fig. 2B). Taken together, these findings indicate that mir-23a and mir-27a increase the rate of granulosa cell apoptosis.

SMAD5 *Is a Novel Target of miR-23a and miR-27a in Human Granulosa Cells*

Next, we identified target genes to investigate the mechanisms underlying the induction of apoptosis by pre-miR-23a and pre-miR-27a in granulosa cells. In our previous study, Kyoto Encyclopedia of Genes and Genomes pathway analysis suggested that miR-23a and miR-27a might coregulate the expression of SMAD5, which is involved in cell proliferation and apoptosis [15].

Using the TargetScan database, we predicted the potential binding sites of miR-23a and miR-27a in the SMAD5 3′ UTR (Fig. 3A). We constructed luciferase reporter vectors in which the miR-23a or miR-27a coding sequence was positioned upstream of the luciferase gene pGL3-SMAD5-3′ UTR. Cotransfection of the luciferase-reporter vector and the miR-23a or miR-27a mimic was performed using 293T cells. The luciferase activity was significantly decreased in the group cotransfected with pGL3-SMAD5-3′ UTR and the mimic of miR-23a or miR-27a compared with that of the miRNA mimic controls, indicating that miR-23a and miR-27a directly target *SMAD5* (Fig. 3B).

As determined using qRT-PCR, the SMAD5 mRNA level was significantly decreased after treatment with pre-miR-23a or pre-miR-27a and was dramatically increased after transfection with their inhibitors (Fig. 3, C and D). At the protein level, the SMAD5 content was reduced in cells transfected with pre-miR-23a or pre-miR-27a. However, miR-23a or the miR-27a inhibitor increased the level of SMAD5 protein compared with that of the control (Fig. 3, E and F).

In order to systematically investigate the target genes of both miR-23a and miR-27a, we also searched for other potential targets of miR-23a and miR-27a among the other SMAD (SMAD1/2/3/4/6/7/9) genes. With the help of bioinformatics analysis, we found that the *SMAD2* and *SMAD4* genes may be cotargeted by miR-23a and miR-27a because they contain several possible miRNA-binding sites based on prediction analysis. However, as shown in Supplemental Figure S1 (available online at www.biolreprod.org), we did not find any alterations in the SMAD2 and SMAD4 protein levels when the expression of these miRNAs were changed, indicating that *SMAD2* and *SMAD4* are not direct targets of miR-23a/miR-27a in human granulosa cells.

Taken together, these data suggest that miR-23a and miR-27a act upon the 3′ UTR of *SMAD5* and that there is a significant inverse correlation between the miR-23a/miR-27a levels and the SMAD5 expression level. Thus, these data strongly suggest that *SMAD5* is a direct target of miR-23a and miR-27a.

miR-23a and miR-27a Regulate the Fas Signaling Pathway by Targeting SMAD5

To further assess the role of SMAD5 in granulosa cells, SMAD5 siRNA was transfected into granulosa cells. As shown in Figure 4A, the level of SMAD5 protein significantly decreased after transfection with SMAD5 siRNA. Furthermore, cells treated with SMAD5 siRNA exhibited significant increases in the levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3 expression (Fig. 4B), strongly indicating that the downregulation SMAD5 activates the FasL-Fas pathway.

An increase in the rate of granulosa cell apoptosis was observed after SMAD5 downregulation, suggesting that SMAD5 works as an apoptosis inhibitor in these cells (Fig. 4C). Interestingly, the decrease in the apoptotic rate induced by the miR-23a/miR-27a inhibitor was reversed by introducing SMAD5 siRNA, supporting the hypothesis that SMAD5 is involved in the apoptosis-promoting effects of both miR-23a and miR-27a (Fig. 5, A and B).

To determine whether SMAD5 was associated with the miR-23a-induced and miR-27a-induced Fas apoptosis pathway, we determined the protein levels of several members of the FasL-Fas pathway after cotransfection of miR-23a or the miR-27a inhibitor and SMAD5 siRNA. As mentioned above, the miR-23a/miR-27a inhibitor decreased the Fas, FasL, cleaved caspase-8, and cleaved caspase-3 levels, whereas additional transfection of SMAD5 siRNA reversed the effects of these miRNA inhibitors. These results confirm that SMAD5 is a functional mediator of miR-23a-induced or miR-27a-induced FasL-Fas pathway activation (Fig. 5, C and D).

In conclusion, miR-23a and miR-27a positively regulate the FasL-Fas pathway by targeting *SMAD5* in granulosa cells.

DISCUSSION

Folliculogenesis is a highly regulated event, forming the basis of physiological ovarian function. Malfunction of this process results in ovarian and endocrine disorders, such as POF. Folliculogenesis is regulated by a large number of intraovarian and endocrine factors, including hormones, cytokines, and growth factors, and the fate of a follicle is determined by a delicate balance among these regulators. Recent studies have shown that miRNAs are involved in the regulation of numerous physiological and pathological pro-

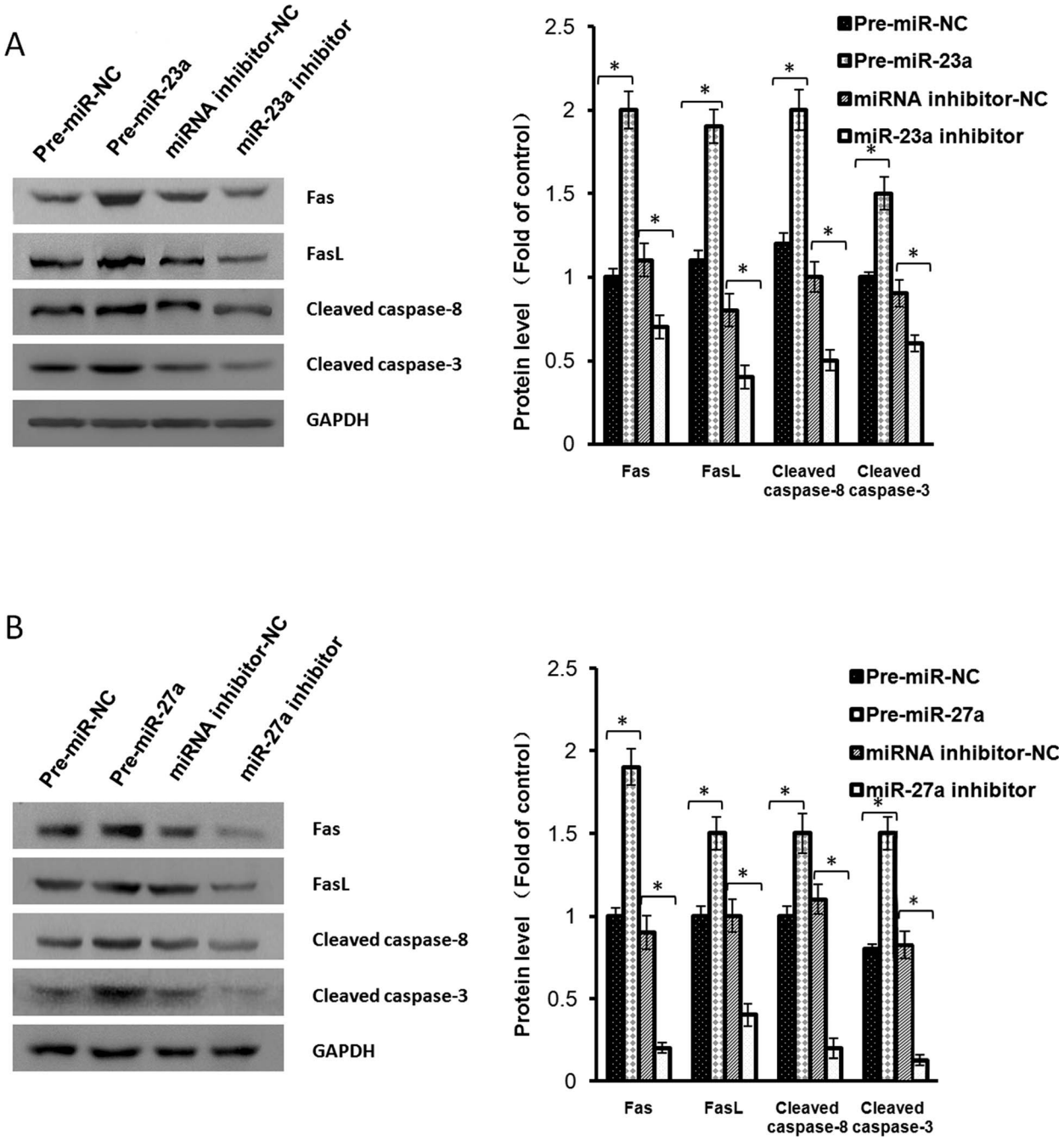

FIG. 2. miR-23a and miR-27a induce Fas-mediated apoptosis. **A**) Western blot analysis of the levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3 protein after transfection with pre-mir-23a or the mir-23a inhibitor. The protein expression levels were normalized to those of GAPDH. Values are the means ± SDs of three independent experiments. Statistical analysis was used to evaluate the significance of the differences (*$P < 0.05$). **B**) Western blot analysis of the levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3protein after transfection with pre-mir-27a or the mir-27a inhibitor (*$P < 0.05$).

cesses, including folliculogenesis [26]. Jiang et al. [27] found that the level of miR-93 is increased in PCOS granulosa cells and that it targets Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A) to promote proliferation and cell cycle progression [27]. Recently, miR-30a was found to function as an oncogene during ovarian development. In cultured COV434 cells, miR-30a overexpression upregulated the levels of BCL2A1, immediate early response 3 (IER3), and cyclin D2 expression by inhibiting the expression of the forkhead transcription factor gene 2 (FOXL2); furthermore, downregulation of the FOXL2 level was associated with ovarian granulosa cell tumorigenesis [28]. miR-23a and miR-27a act as apoptosis regulators in many systems. For example, miR-23a represses the apoptosis of pancreatic cancer cells by directly targeting apoptotic protease-activating factor-1 (APAF1) [29]. In addition, miR-23a and miR-27a contribute to neuronal cell death after experimental traumatic brain injury by upregulating the expression of proapoptotic Bcl-2 family members [30]. In our previous study, several differentially expressed miRNAs were identified in the plasma when comparing POF patients to normally cycling women. Among these differentially expressed miRNAs, miR-23a and miR-27a were found to be consistently

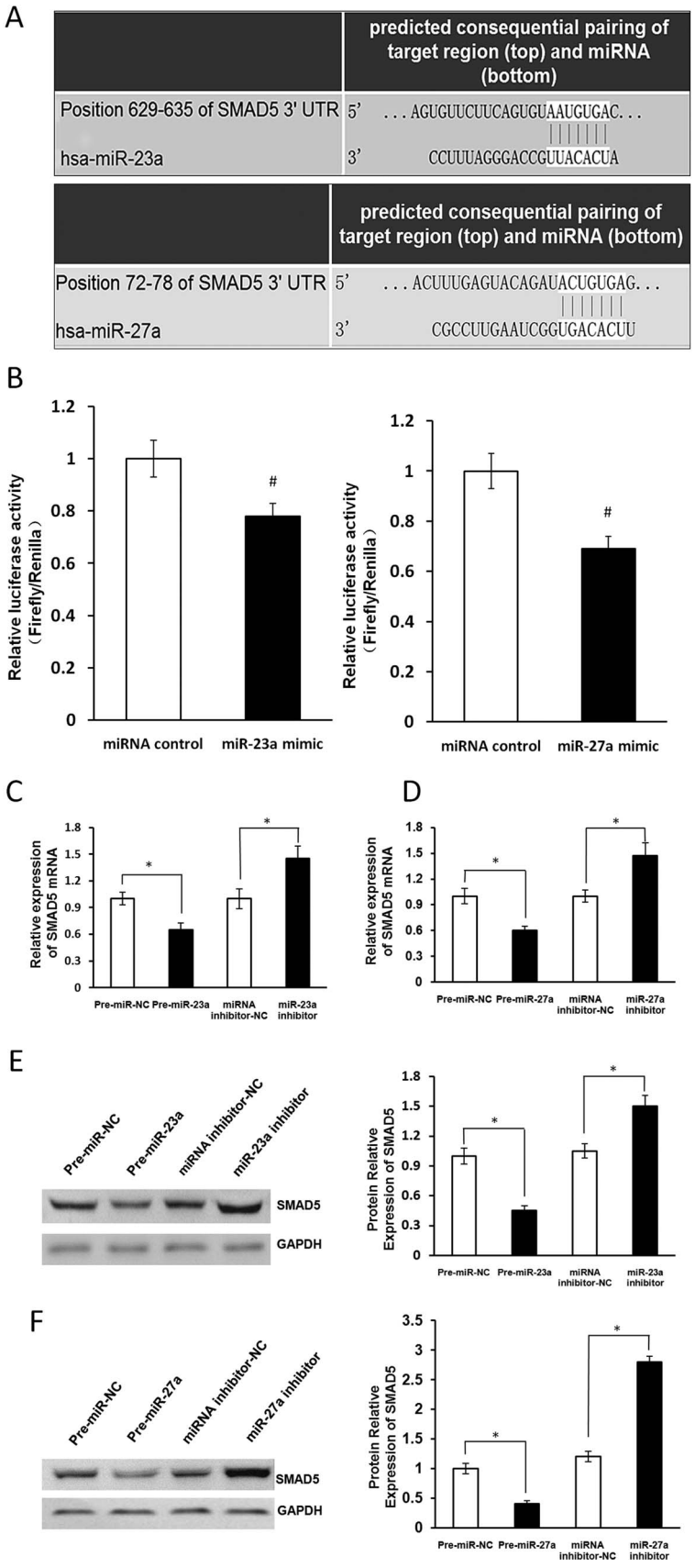

FIG. 3. miR-23a and miR-27a directly target *SMAD5* in granulosa cells. **A**) Consequential pairing of the *SMAD5* region and miR-23a/miR-27a, as predicted using the TargetScan program. **B**) *SMAD5* is a target gene of miR-23a and miR-27a. Results are from luciferase reporter assays of 293T cells cotransfected with pGL3-SMAD5-3′UTR and miR-23a/miR-27a mimics or NCs (#$P < 0.01$) are shown. **C**) The mRNA expression of SMAD5 in granulosa cells transfected with pre-mir-23a, the miR-23a inhibitor, or their NCs. Data are the mean values ± SD of n = 3 experiments (*$P < 0.05$). **D**) The mRNA expression of SMAD5 in granulosa cells transfected with pre-mir-27a, the miR-27a inhibitor, or their NCs. Data are the mean values ± SD of n = 3

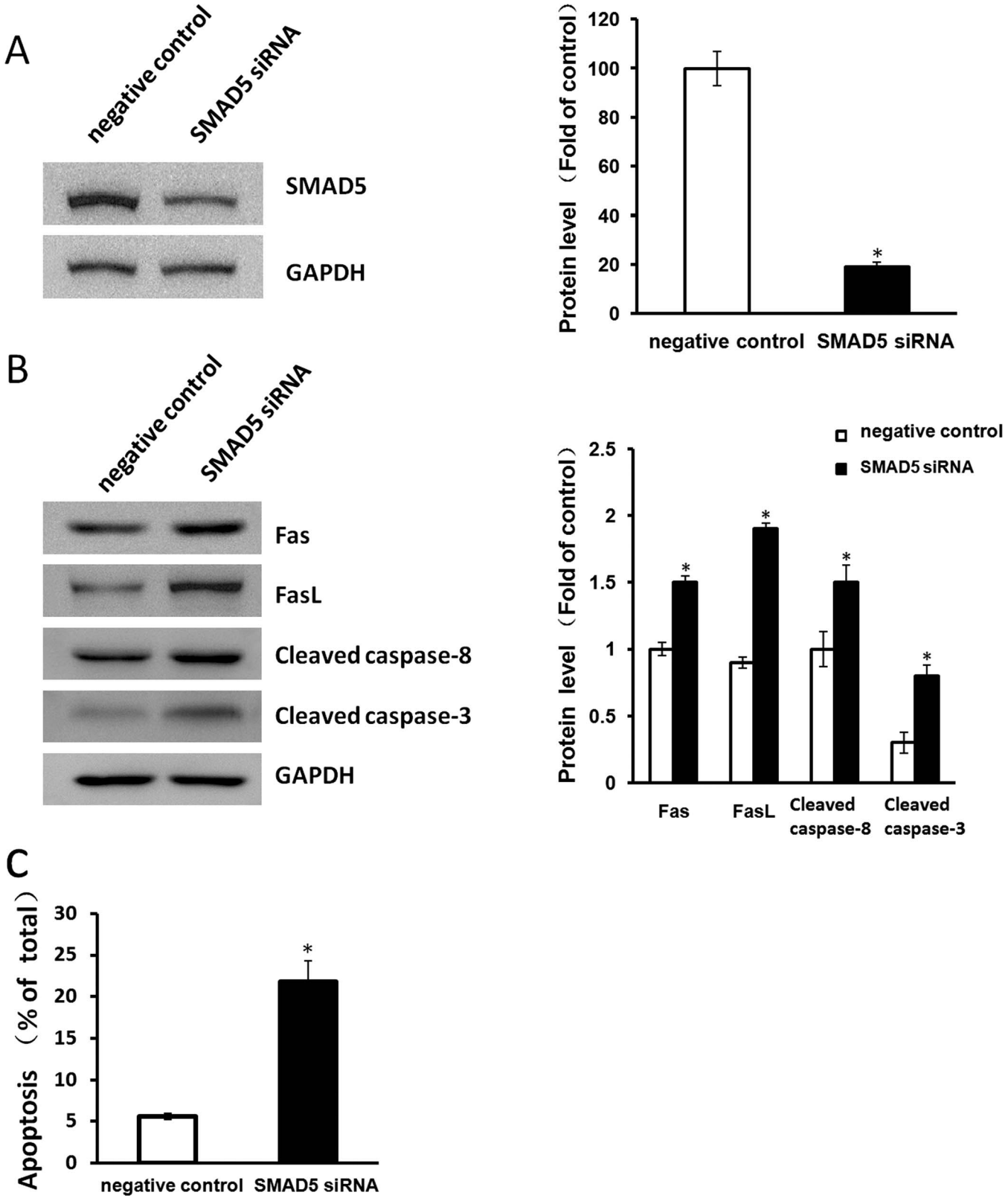

FIG. 4. Apoptosis of granulosa cells is promoted by SMAD5 siRNA. **A**) SMAD5 protein level in granulosa cells transfected with SMAD5 siRNA or control siRNA. GAPDH was used as the loading control. Data are mean ± SD (*$P < 0.05$). **B**) Levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3 proteins in granulosa cells transfected with SMAD5 siRNA or control siRNA. GAPDH was used as the loading control. Data are mean ± SD (*$P < 0.05$). **C**) Hoechst 33258 staining was performed to measure the rate of apoptosis of granulosa cells transfected with SMAD5 siRNA or control siRNA. Data are mean ± SD (*$P < 0.05$).

experiments (*$P < 0.05$). **E**) SMAD5 protein expression in granulosa cells transfected with pre-mir-23a, the miR-23a inhibitor, or their negative controls. Data are the mean values ± SD of n = 3 experiments (*$P < 0.05$). **F**) SMAD5 protein expression in granulosa cells transfected with pre-mir-27a, the miR-27a inhibitor, or their NCs. Data are the mean values ± SD of n = 3 experiments (*$P < 0.05$).

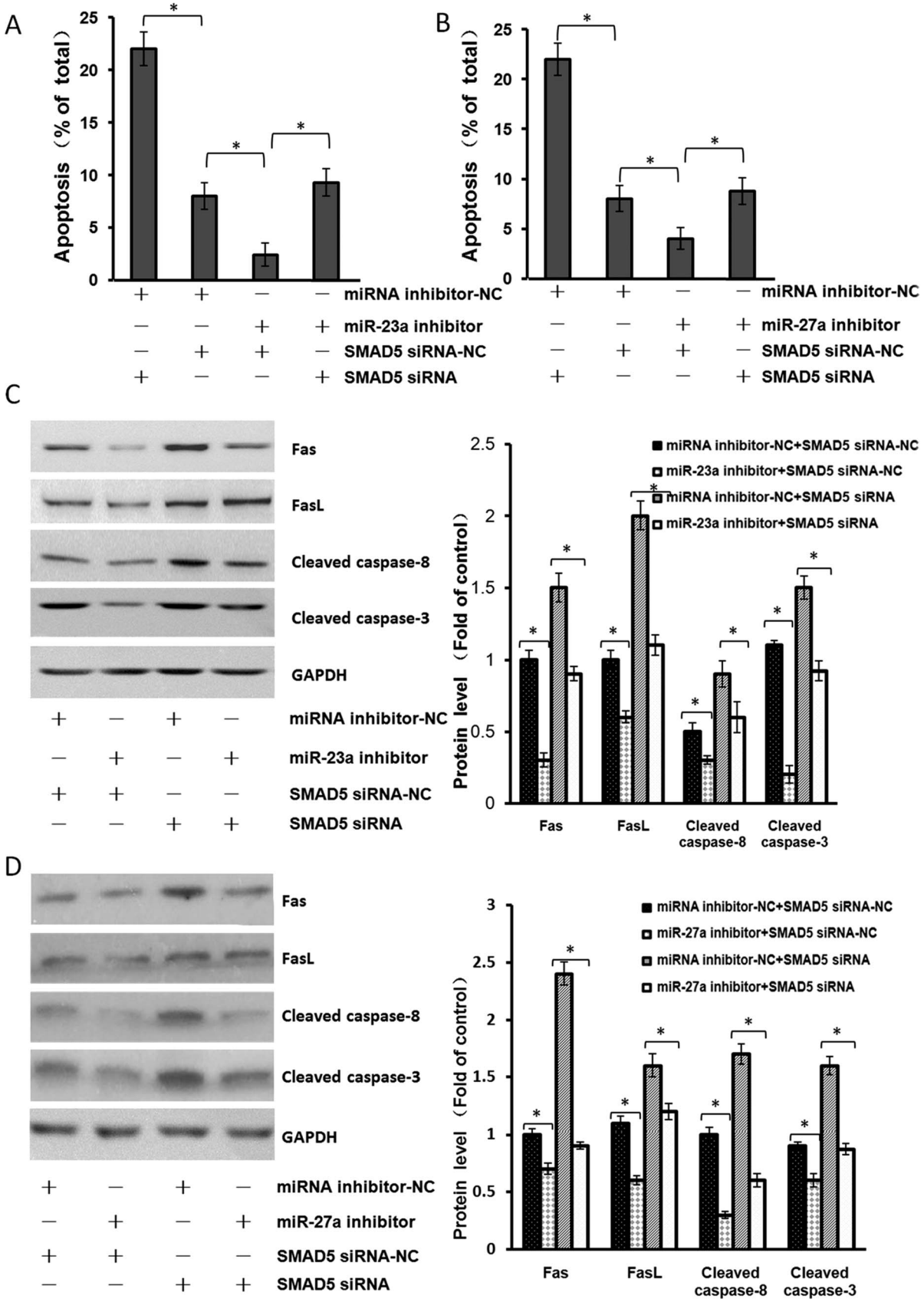

FIG. 5. SMAD5 siRNA prevents the antiapoptotic effects of the miR-23a and miR-27a inhibitors in granulosa cells. **A** and **B**) SMAD5 siRNA prevented the inhibitory effects on apoptosis induced by the inhibitor of miR-23a or miR-27a. Data are mean ± SD (*$P < 0.05$). **C**) Transfection with SMAD5 siRNA and the miR-23a inhibitor abolished the reduction in the Fas, FasL, cleaved caspase-8, and cleaved caspase-3 expression levels induced by the miR-23a inhibitor. The level of endogenous GAPDH was used for normalization. Data are mean ± SD (*$P < 0.05$). **D**) SMAD5 expression prevented the effects of miR-27a on granulosa cells. The miR-27a inhibitor mediated the suppression of FasL, cleaved caspase-8, and cleaved caspase-3 expression, which was abrogated upon cotransfection with SMAD5 siRNA and the miR-27a inhibitor. Data are mean ± SD (*$P < 0.05$).

expressed in previous microarray analyses. Many target genes of mir-23a and mir-27a have been identified. For example, miR-23a and miR-27a have been reported to be closely associated with apoptosis via repressing the expression of the Apaf-1 and bcl-2 proapoptotic proteins, including Noxa, Puma, and Bax, in neuronal cells [30]. Furthermore, miR-27a regulates apoptosis in nucleus pulposus cells by targeting PI3K [31]. Additionally, a very recent study has provided strong support for our data by showing that miR-23a and miR-27a protect differentiating embryonic stem cells from Bone Morphogenetic Protein 4 (BMP-4)-induced apoptosis by targeting *SMAD5* [32]. In the present study, we found that two differentially expressed miRNAs, miR-23a and miR-27a, promote apoptosis in human granulosa cells via the FasL-Fas pathway, partially by directly targeting *SMAD*5.

Based on our previous data, we performed the present study to elucidate the effects of miR-23a and miR-27a on the survival of ovarian granulosa cells. Our results showed that pre-mir-23a or pre-mir-27a enhanced the apoptosis rate of granulosa cells, whereas knocking down the miR-23a or miR-27a level had the opposite effect. These findings clearly suggest a proapoptotic function for miR-23a or miR-27a, consistent with the results obtained using an in vitro mouse model of ovarian follicle development, in which an miR-27a mimic decreased the oocyte maturation rate compared with that of the control group [16]. However, previous reports have also established miR-23a and miR-27a as oncogenes in multiple cancers [33–35]. For example, in one study, an miR-27a inhibitor enhanced the paclitaxel sensitivity and increased the rate of paclitaxel-induced apoptosis of ovarian cancer cells [36]. Therefore, miR-23a and miR-27a have different functions in the regulation of apoptosis, which might be explained by the large number of their target genes in different cell types.

The FasL-Fas signaling pathway is involved in the regulation of granulosa cell apoptosis [24]. In this study, the overexpression of miR-23a and miR-27a significantly increased the levels of Fas and FasL protein and caused the appearance of specific small cleavage fragments of caspase-8 and caspase-3 in ovarian granulosa cells. These findings are supported by FasL being more highly expressed in the granulosa cells of atretic follicles than in those of the dominant follicles and by the finding that the overexpression of FasL induces granulosa cell apoptosis via the caspase-8-mediated and caspase-3-mediated apoptotic pathway [37]. Thus, these results provide evidence that miR-23a and miR-27a activate the Fas-FasL signaling pathway and induce apoptosis in human ovarian granulosa cells.

MicroRNAs repress target-gene expression at the posttranscriptional level, which inspired us to search for the direct target mRNAs of miR-23a and miR-27a. In our previous study, *SMAD5* was shown to be a target gene that was coregulated by miR-23a and miR-27a based on microarray analyses and bioinformatic predictions [15]. SMAD5 is a receptor-regulated SMAD (R-SMAD) protein that is phosphorylated and activated by BMP signaling pathways [38]. Several BMPs play roles in oocyte and follicular development. BMP-4 and BMP-7 have been identified as prosurvival factors in ovarian granulosa cells that act through various signaling pathways [39]. As a mediator of signal transduction through the BMP pathway, SMAD5 could directly affect granulosa cell function. Tripurani et al. [40] demonstrated that the conditional deletion of *SMAD1* and *SMAD5* in ovarian granulosa cells resulted in the upregulation of platelet-derived growth factor alpha (PDGFA) expression and led to the development of metastatic granulosa cell tumors, suggesting that SMAD5 is an important regulator of multiple aspects of granulosa cell development. Further detailed analysis showed that there was a potential binding site for miR-23a and miR-27a in the SMAD5 mRNA 3′ UTR, suggesting that *SMAD5* might be a novel target gene for miR-23a and miR-27a. We verified this prediction by demonstrating that miR-23a and miR-27a directly bound to the 3′ UTR of SMAD5 mRNA using a luciferase assay and that the mRNA and protein levels of SMAD5 were significantly decreased after miR-23a and miR-27a transfection of granulosa cells.

BMP signaling plays many roles in apoptosis. Bmpr1a-mutant mice have defective nasal cartilage and higher levels of phosphorylated SMAD1 and SMAD5 in their nasal tissue, accompanied by increased levels of p53, Bax, and caspase-3 protein compared with those of control mice [41]. As a member of the BMP signaling pathway, SMAD5 plays critical roles in the regulation of female fertility because double *SMAD1*/*SMAD5* and triple *SMAD1*/*SMAD5*/*SMAD8* conditional knockout mice become infertile and develop metastatic granulosa cell tumors [42]. To examine the role of SMAD5 in ovarian granulosa cell apoptosis, we performed SMAD5 siRNA knockdown experiments. In the current study, the apoptosis rate of granulosa cells was enhanced after SMAD5 siRNA transfection because of the increased protein levels of Fas, FasL, cleaved caspase-8, and cleaved caspase-3. These results indicate that the Fas-mediated apoptosis signaling pathway is partially regulated by SMAD5 in granulosa cells (Fig. 4).

In addition, we knocked down the expression of SMAD5 in cells transfected with an inhibitor of miR-23a or miR-27a to explore the role of SMAD5 in miR-23a-mediated and miR-27a-mediated apoptosis. As expected, inhibiting the expression of SMAD5 reversed the miR-23a/miR-27a inhibitor-mediated suppression of apoptosis and abolished the inactivation of the Fas pathway, suggesting that miR-23a and miR-27a activate FasL-Fas signaling and promote granulosa cell apoptosis by targeting *SMAD*5. As previously reported, miR-23a induced apoptosis in human granulosa cells by decreasing the level of XIAP and increasing the rate of caspase-3 cleavage [15]. Apoptosis is regulated by various pathways. XIAP gene expression and function have been shown to be positively regulated by TGFβ signaling in a SMAD-dependent manner in uterine cancer cells [43]. The interactions among the various pathways that regulate the apoptotic process will be evaluated in a future study.

In conclusion, miR-23a and miR-27a induce granulosa cell apoptosis in vitro, and these two miRNAs activate the SMAD5-mediated FasL-Fas signaling pathway. These findings provide a novel perspective regarding the role of miRNAs in the regulation of human granulosa cell apoptosis in vitro. Studies performed using follicles and further in vivo experiments are required to confirm the regulatory roles of miR-23a and miR-27a in follicular development and follicular atresia.

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