

## miR-106a Increases Granulosa Cell Viability and Is Downregulated in Women With Diminished Ovarian Reserve

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**Context:** Women with diminished ovarian reserve (DOR) have reduced fertility, cardiovascular events, and osteoporosis. Although differential microRNA (miRNA) expression has been described in several ovarian disorders, little is known about the role of miRNAs in the pathogenesis of DOR.

**Objective:** Identify differentially expressed miRNAs in DOR and explore the role of miR-106a in human granulosa cell proliferation.

**Design:** miRNA microarray (n = 3) and quantitative reverse transcription polymerase chain reaction (n = 30) were used to examine miRNA expression in serum and granulosa cells from normal-cycling and women with DOR. Primary human granulosa cells were treated alone or in combination with miR-106a mimic, miR-106a inhibitor, apoptosis signal-regulating kinase 1 (ASK1) small interfering RNA (siRNA), or p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580) before assessment of cell viability and apoptosis. Western blot was used to measure ASK1 protein and phosphorylation/activation of p38 MAPK. Binding of miR-106a to ASK1 mRNA was examined by 3' untranslated region (3'UTR) luciferase analysis.

**Results:** Fifteen miRNAs were differentially expressed (n = 30), and miR-106a was downregulated in serum and granulosa cells of women with DOR. miR-106a mimic increased cell viability and attenuated apoptosis, whereas the converse occurred following treatment with miR-106a inhibitor. miR-106a suppressed ASK1 expression by directly targeting its 3'UTR. miR-106a inhibitor increased p38 MAPK phosphorylation/activation, and this effect was abolished by treatment with ASK1 siRNA. Whereas knockdown of ASK1 abolished the effects of miR-106a inhibitor on cell viability/apoptosis, pretreatment with SB203580 did not significantly alter the effects of miR-106a inhibitor.

**Conclusions:** Downregulation of miR-106a may contribute to the pathogenesis of DOR by reducing granulosa cell viability and promoting apoptosis via enhanced ASK1 signaling. (*J Clin Endocrinol Metab* 103: 2157–2166, 2018)

**D**iminished ovarian reserve (DOR) is characterized by women of reproductive age who have regular menses but reduced ovarian response to ovarian

stimulation or fecundity compared with healthy women of comparable age (1). The prevalence of DOR increased from 19% to 26% between 2004 and 2011 and

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Abbreviations: 3'UTR, 3' untranslated region; ASK1, apoptosis signal-regulating kinase 1; DMEM, Dulbecco's modified Eagle medium; DOR, diminished ovarian reserve; FSH, follicle-stimulating hormone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GO, gene ontology; IVF, *in vitro* fertilization; MAPK, mitogen-activated protein kinase; miRNA, microRNA; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; POF, premature ovarian failure; siRNA, small interfering RNA.

represents a major challenge in reproductive medicine (2). The primary pathological features of DOR are premature depletion of primordial follicles, accelerated follicle atresia, and inhibition of follicle growth (3, 4). Although familial, idiopathic, iatrogenic, autoimmune, and genetic factors can account for most cases (5), the underlying molecular etiology of DOR is still poorly understood.

MicroRNAs (miRNAs) are small (20 to 25 nt) non-coding RNA molecules that regulate gene expression by directly binding to the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs), leading to their degradation or the inhibition of translation (6). miRNAs exert pleiotropic effects under physiological and pathological conditions by regulating diverse cellular processes, such as cell proliferation, differentiation, and apoptosis (7). Studies suggest that miRNAs are involved throughout the process of ovarian follicle development, including follicle growth, atresia, and ovulation (8–11). Consequently, miRNAs could play vital roles in the pathogenesis of DOR, including aberrant gonadotropin levels and apoptotic phenomena associated with oocytes and the surrounding granulosa cells (12). Recent studies have shown that miRNAs are differentially expressed in women with polycystic ovary syndrome and premature ovarian failure (POF) (9–11). For example, we have shown that plasma miR-23a levels are elevated in women with POF and that treatment with pre-miR-23a induces apoptosis of cultured human granulosa cells *in vitro* (9).

Apoptosis signal-regulating kinase 1 (ASK1; also termed MAP3K5) is a mitogen-activated protein kinase (MAPK) kinase family member that serves as an upstream activator of p38 MAPK signaling. ASK1/p38 MAPK signaling affects multiple cellular functions, and upregulation of ASK1 promotes apoptosis via activation of caspase-9 and caspase-3 (13, 14). Recent studies have shown that ASK1 may contribute to drug-induced ovarian cancer cell and small preantral follicle apoptosis/atresia (15–17). Several miRNAs have been shown to regulate ASK1 levels in extraovarian cell types (18–20), but potential relationships between miRNAs, ASK1, and the pathogenesis of DOR are unknown.

In the current study, we identify differentially expressed miRNAs in serum and granulosa cells from women with or without DOR. In particular, miR-106a is the only miRNA with reduced levels in both serum and granulosa cells from women with DOR compared with normal-cycling women. miR-106a is one of four miRNAs previously shown to be downregulated in several models of cell and organismal aging (21), and thus we investigated the role of miR-106a in human granulosa cell apoptosis. We demonstrate that downregulation of miR-106a enhances human granulosa cell apoptosis and increases the levels of ASK1 and phosphorylated

p38 MAPK. These findings contribute to a better understanding of the molecular mechanisms underlying DOR.

## Materials and Methods

### Study population

This study was approved by the Human Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University and performed from February 2012 to May 2016. All women were fully counseled, and informed consent was obtained in writing from all participants. Serum and ovarian granulosa cells were obtained from women with DOR (n = 30) and normal-cycling infertile women (control group; n = 30). All women were undergoing their first *in vitro* fertilization (IVF) or intracytoplasmic sperm injection-embryo transfer cycle due to tubal and/or male factors (Table 1). The inclusion criteria for women with DOR included (1) age <40 years, (2) subfertility >1 year, (3) basal follicle-stimulating hormone (FSH) level of 12 to 25 mIU/mL, and (4) antral follicle count <5 on days 2 to 3 of a spontaneous cycle. Women were excluded if they had (1) a history of ovarian cystectomy or oophorectomy, (2) received cytotoxic chemotherapy, and (3) received pelvic irradiation.

### IVF treatment and specimen collection

Controlled ovarian stimulation was performed with recombinant FSH and gonadotropin-releasing hormone antagonist adjuvant therapy. Recombinant FSH (150-225IU; Merck Serono, Inc., Geneva, Switzerland) was administered on days 2 to 3 of the cycle. Gonadotropin-releasing hormone antagonist (Cetrotide 0.25 mg/d; STAMedica, Amsterdam, Netherlands) was initially administered when the leading follicle reached 13 to 14 mm in diameter and maintained until human chorionic gonadotropin injection. A total of 250 µg recombinant human chorionic gonadotropin (Merck Serono, Inc.) was given when the leading follicle was ≥18 mm in diameter or there were at least two follicles ≥17 mm in diameter, and oocyte retrieval was performed after 36 hours. Ovarian granulosa cells were isolated from follicular fluid aspirates. Serum samples were collected on days 2 to 3 of the menstrual cycle before the start of the IVF treatment cycle.

**Table 1. General Characteristics of the Study Population**

Characteristics	DOR Group	Control Group	P Value
Age, y	33.32 ± 3.98	29.4 ± 3.37	0.977
BMI, kg/m <sup>2</sup>	21.46 ± 2.28	20.56 ± 1.85	0.129
Menstrual cycle length, d	29.56 ± 1.87	29.52 ± 1.76	0.938
Basal FSH, mIU/mL	14.35 ± 2.17	5.29 ± 1.09	<0.001
Basal LH, mIU/mL	4.06 ± 1.55	4.50 ± 1.02	0.335
FSH/LH	3.94 ± 1.57	1.21 ± 0.32	<0.001
Antral follicle number	3.37 ± 0.64	11.72 ± 3.16	<0.001
Basal E <sub>2</sub> , pg/mL	24.87 ± 20.40	18.87 ± 9.64	0.189

Values are presented as mean ± standard deviation.

Abbreviations: BMI, body mass index; LH, luteinizing hormone.

## Preparation of primary human granulosa cells

Primary human granulosa cells were isolated from follicular fluid aspirates obtained at oocyte retrieval as described by Shi *et al.* (22). Aspirates were taken from follicles with a diameter  $\geq 14$  mm, and the number of retrieved oocytes varied from 6 to 15 in normal-cycling women and 2 to 4 in women with DOR. Briefly, follicular fluid from each patient was centrifuged at  $400 \times g$  for 10 minutes, and the layers of granulosa cells with the red blood cell pellet were resuspended. After shaking at 200 rpm for 20 minutes at  $37^\circ\text{C}$ , the cell suspensions were layered on 8.0 mL Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA) and centrifuged at  $600 \times g$  for 20 minutes. Granulosa cells at the interface were harvested and washed three times with 10 mL Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F-12 Ham (DMEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin sulfate, and  $1 \times$  GlutaMAX (Invitrogen, Shanghai, China). After a final centrifugation for 5 minutes at  $600 \times g$ , the cells were resuspended and cultured in six-well plates ( $\sim 10^6$  cells/well) with DMEM/F-12 at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

## miRNA microarrays

Total RNA was extracted from granulosa cells using TRIzol reagent (Invitrogen, Shanghai, China), whereas serum RNA was isolated with TRIzol LS according to the manufacturer's instructions. RNA samples were quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Beijing, China), labeled with the miRCURY Hy3/Hy5 Power labeling kit, and hybridized to a miRCURY LNA Array (v.16.0) containing 1891 capture probes, covering all human, mouse, and rat miRNAs annotated in miRBase 16.0 (Kangcheng Biotech Company, Shanghai, China). After washing, the slides were scanned using the Axon GenePix 4000B microarray scanner and images were imported into GenePix Pro 6.0 software (Molecular Devices, Beijing, China) for grid alignment and data extraction. Signals from four replicate spots for each probe on the same slide were averaged, and normalization was carried out by the median normalization method (23). Normalized data (foreground minus background divided by median) were used for the identification of significantly differentially expressed miRNAs using volcano plot filtering and R statistical software. miRNAs were defined as being significantly differentially expressed if the *P* value was  $< 0.05$  and the fold change  $> 1.5$ . miRNA microarray data have been deposited in Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under the reference series accession number GSE111774.

## Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using TRIzol reagent, and 2  $\mu\text{g}$  was used for complementary DNA synthesis by reverse transcription with specific stem-loop reverse transcription primers for miRNAs (Supplemental Table 1) or oligo(dT) for mRNAs. Quantitative real-time polymerase chain reaction (PCR) was performed using a standard SYBR Premix Ex Taq kit protocol in 96-well plates. Each 10- $\mu\text{L}$  PCR reaction consisted of 5  $\mu\text{L}$  SYBR Green PCR Master Mix, 1  $\mu\text{L}$  complementary DNA, 0.2  $\mu\text{L}$  ROX Reference Dye II, 1  $\mu\text{L}$  specific primers, and 2.8  $\mu\text{L}$  RNase-free water. Primers used for miRNA amplification are listed in Supplemental Table 1 and U6 small nuclear RNA was used for normalization. Primers used amplification of ASK1 and

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were as follows: ASK1 forward primer, CTG CAT TTT GGG AAA CTC GAC T; ASK1 reverse primer, AAG GTG GTA AAA CAA GGA CGG; GAPDH forward primer, TGT TGC CAT CAA TGA CCC CTT; and GAPDH reverse primer, CTC CAC GAC GTA CTC AGC G. The cycling parameters were  $95^\circ\text{C}$  for 30 seconds, 40 cycles of  $95^\circ\text{C}$  for 5 seconds,  $60^\circ\text{C}$  for 20 seconds and  $72^\circ\text{C}$  for 15 seconds, and  $95^\circ\text{C}$  for 15 seconds, as well as a final melting step with slow heating from  $60^\circ\text{C}$  to  $95^\circ\text{C}$ . All reactions were run in triplicate, and negative control reactions without reverse transcription reaction and template were also performed. Relative gene expression was calculated using the comparative Ct method and the equation  $2^{-\Delta\Delta\text{Ct}}$  (24).

## miRNA transfection

Granulosa cells were seeded in six-well plates and transfected the next day at 80% confluence. Cells were transfected with 100 nM miR-106a mimic or miR-106a inhibitor as well as their respective negative controls (RiboBio, Guangzhou, China) using Lipofectamine RNAiMAX (Invitrogen). A fluorescein isothiocyanate-labeled inhibitor control was included to demonstrate the efficiency of transfection, which was found to be higher than 90%.

## Small interfering RNA transfection

Granulosa cells were transfected with 100 nM ASK1 small interfering RNA (siRNA) (sc-29748; Santa Cruz Biotechnology, Dallas, TX) or control siRNA (sc-37007; Santa Cruz Biotechnology) using Lipofectamine RNAiMAX according to the manufacturer's instructions.

## Cell viability assay

Assessment of cell viability was performed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported (25). Briefly, granulosa cells were cultured in 24-well plates and transfected at 30% confluence with miR-106a mimic or inhibitor, ASK1 siRNA, or relevant controls for 6 hours. At each time point (0, 24, 48, and 72 hours), the culture medium was changed to 500  $\mu\text{L}$  DMEM/F-12 medium and 50  $\mu\text{L}$  MTT reagent (5 mg/mL) and incubated for 4 hours before being replaced with 500  $\mu\text{L}$  dimethyl sulfoxide for 10 minutes. Samples were transferred to 96-well plates for analysis of optical density at 570 nm. The experiments were performed in triplicate.

## Assessment of apoptosis

Granulosa cells were transfected for 48 hours, stained with Hoechst 33258 as described by Wang *et al.* (26), and morphologically assessed by fluorescence microscopy. At least 200 cells in a selected area were counted in each treatment group, with individuals counting blinded to sample identity. Normal cells show round and intact nuclei, whereas apoptotic cells exhibit morphological changes with karyopyknosis or fragmentation.

## Western blot analysis

Western blot was performed as described by Yang *et al.* (9). Briefly, cells were washed with cold phosphate-buffered saline and lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 1.5 mM  $\text{MgCl}_2$ , 100 mM sodium fluoride, 10% glycerol, and 1%

Triton X-100) containing protease inhibitor cocktail (Sigma-Aldrich, Shanghai, China). Then, 50  $\mu\text{g}$  protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose membranes. After being blocked with 5% skim milk for 1 hour, the membranes were incubated overnight at 4°C with primary antibodies against proliferating cell nuclear antigen (PCNA, 1:1000; #13110; Cell Signaling Technology, Shanghai, China), ASK1 (1:1000; #8662; Cell Signaling Technology), p38 MAPK (1:1000; #9212; Cell Signaling Technology), phospho-p38 MAPK (1:1000; #4511; Cell Signaling Technology), or GAPDH (1:2000; #ab22556; Abcam, Shanghai, China). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 1 hour at room temperature, and signals were developed using the enhanced chemiluminescence system (Pierce, Rockford, IL). Densitometric quantification was performed using Scion Image software (Scion Corp., Frederick, MD).

### Gene ontology analysis

Gene ontology (GO) analysis was applied to analyze the main function of the differentially expressed miRNAs and was performed as described by Yang *et al.* (9). Briefly, Fisher exact test and  $\chi^2$  test were used to classify the GO categories, and a false discovery rate was calculated to correct the *P* value computed for the GOs of all differentially expressed genes. The measure of the significance of the function was provided by enrichment: as the enrichment increased, the corresponding function was more specific, which helped to indicate GOs with more defined functions.

### Pathway analysis

Pathway analysis was a functional analysis mapping genes to KEGG pathways as previously described by Yang *et al.* (9). Fisher exact test and  $\chi^2$  test were used to select the significant pathways, and the threshold of significance was defined by *P* value and false discovery rate (the lower the *P* value, the more significant the pathway).

### 3'UTR luciferase reporter assay

ASK1 3'UTR luciferase reporter analysis was performed with 293T cells seeded in 24-well tissue culture plates ( $1 \times 10^5$  cells per well, antibiotic-free medium) and transfected at 80% confluence. The predicted ASK1 3' UTR (205 bp) was inserted in the downstream region of the Firefly luciferase dual-luciferase GV306 vector (Genechem, Shanghai, China) and was termed *GV306-ASK1*. As a negative control, a mutant ASK1 3'UTR sequence was also inserted into the GV306 vector and was termed *GV306-mASK1*. The miR-106a precursor was cloned into the GV268 vector (Genechem) and was

termed *GV268-miR-106a*. Cells were cotransfected with 1.2  $\mu\text{g}$  *GV306-ASK1*, 1.2  $\mu\text{g}$  *GV306-mASK1*, and 0.4  $\mu\text{g}$  *GV268-miR-106a* using Lipofectamine 2000. *Renilla* luciferase from the GV306 vector was used as an internal control. After transfection for 48 hours, the cells were harvested and Firefly luciferase activity was analyzed relative to *Renilla* luciferase activity in the same sample using the Dual-Luciferase Reporter Assay System (Promega, Beijing, China). Three independent experiments were performed and assayed in quadruplicate per group.

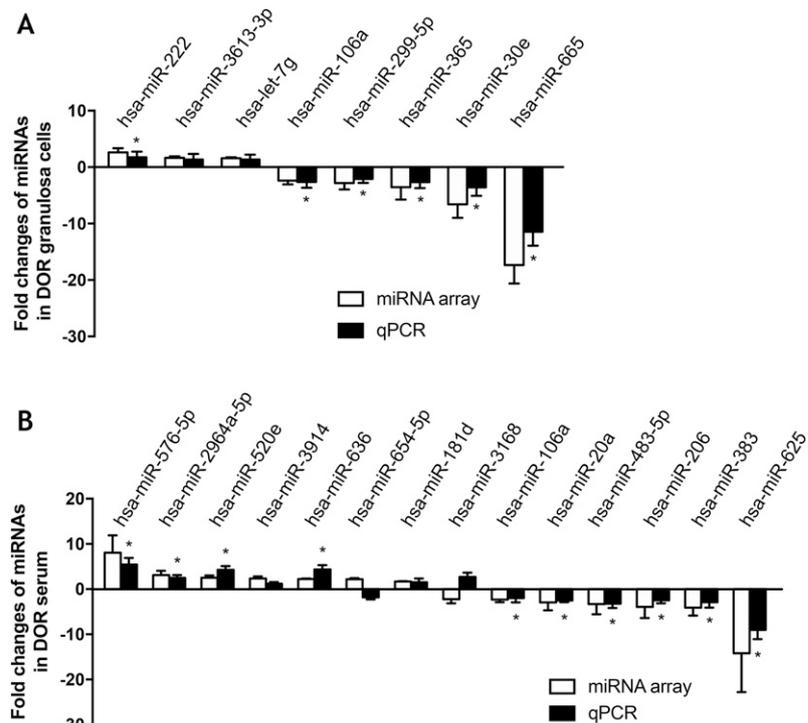
### Statistical analysis

Results are presented as the mean  $\pm$  standard deviation of at least three independent experiments. Student *t* test was used for comparisons between two groups, and Student-Newman-Keuls *q* test was used for multiple comparisons. Statistical analyses were performed using SPSS software, version 16.0 (IBM Corp., Armonk, NY). *P* < 0.05 was considered statistically significant.

## Results

### Downregulation of miR-106a in granulosa cells and serum from women with DOR

We used miRNA microarray analysis to study the differential expression of miRNAs in granulosa cells and serum from three women with DOR and three



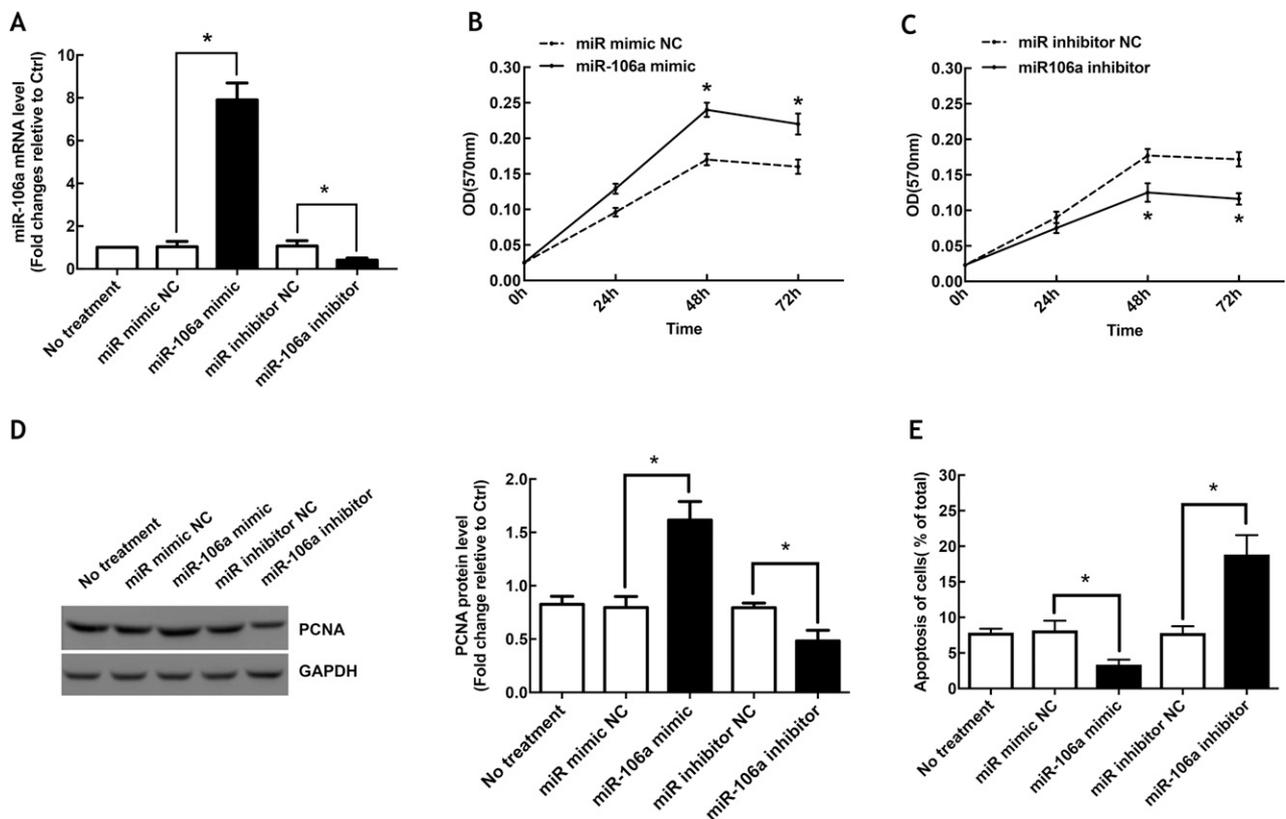
**Figure 1.** Differentially expressed miRNAs in (A) granulosa cells and (B) serum from women with DOR. Expression of miRNAs was profiled by microarray, and fold changes were calculated with respect to normal-cycling controls (white bars). Quantitative reverse transcription PCR (qRT-PCR) was used to validate differential expression of miRNAs in granulosa cells and serum from an expanded cohort of 30 women with DOR and 30 normal-cycling women (black bars). Data are presented as the mean  $\pm$  standard deviation. \*Significant differences in qRT-PCR analyses (*P* < 0.05).

normal-cycling women. We found 8 differentially expressed miRNAs in granulosa cells (3 up and 5 down; Fig. 1A) and 14 in serum (7 up and 7 down; Fig. 1B) from women with DOR compared with normal-cycling women. Next, we used stem-loop quantitative reverse transcription PCR to validate the differential expression of these 21 miRNAs in granulosa cells and serum from an expanded cohort of 30 women with DOR and 30 normal-cycling women. We found that granulosa cells from women with DOR had upregulation of one miRNA (miR-222) and downregulation of five miRNAs (miR-106a, miR-299-5p, miR-365, miR-30e, and miR-665) compared with granulosa cells from normal-cycling women (Fig. 1A). In serum from women with DOR, four miRNAs were upregulated (miR-576-5p, miR-2964a-5p, miR-520e, and miR-636) and six were downregulated (miR-106a, miR-20a, miR-483-5p, miR-206, miR-383, and miR-625) compared with serum from normal-cycling women (Fig. 1B). Only miR-106a was downregulated in both granulosa cells and serum from women with DOR. GO and KEGG pathway analyses were performed with the 22 miRNAs that were differentially expressed in

granulosa cells and serum from women with DOR (Supplemental Table 2).

### miR-106a increases cell viability and inhibits apoptosis in human granulosa cells

To explore the effects of miR-106a on primary human granulosa cell viability, we transfected cells with miR-106a mimic, miR-106a inhibitor, or their respective controls at a final concentration of 100 nM. Quantitative reverse transcription PCR results confirmed significant increases or decreases in miR-106a levels in cells transfected with miR-106a mimic or inhibitor, respectively, compared with cells transfected with the relevant negative control or receiving no treatment (Fig. 2A). Next, we used the MTT assay to examine the effects of miR-106a mimic or inhibitor on the viability of primary human granulosa cells. As shown in Fig. 2B, treatment with miR-106a mimic significantly increased granulosa cell viability at both 48 and 72 hours. In contrast, cell viability was reduced following treatment with miR-106a inhibitor for 48 or 72 hours (Fig. 2C).



**Figure 2.** miR-106a increases cell viability and suppresses apoptosis in primary human granulosa cells. (A) Quantitative reverse transcription PCR was used to confirm transfection with miR-106a mimic and miR-106a inhibitor (normalized to human U6). Granulosa cells were transfected with (B) miR-106a mimic or (C) miR-106a inhibitor for 24, 48, or 72 hours, and cell viability was determined by the MTT assay. (D) Western blot analysis of PCNA protein levels (normalized to GAPDH) after transfection with miR-106a mimic or inhibitor for 48 hours. (E) Granulosa cells were transfected with miR-106a mimic or inhibitor for 48 hours, and the proportion of apoptotic cells was measured by Hoechst 33258 staining. Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. \*Significant differences ( $P < 0.05$ ). NC, negative control.

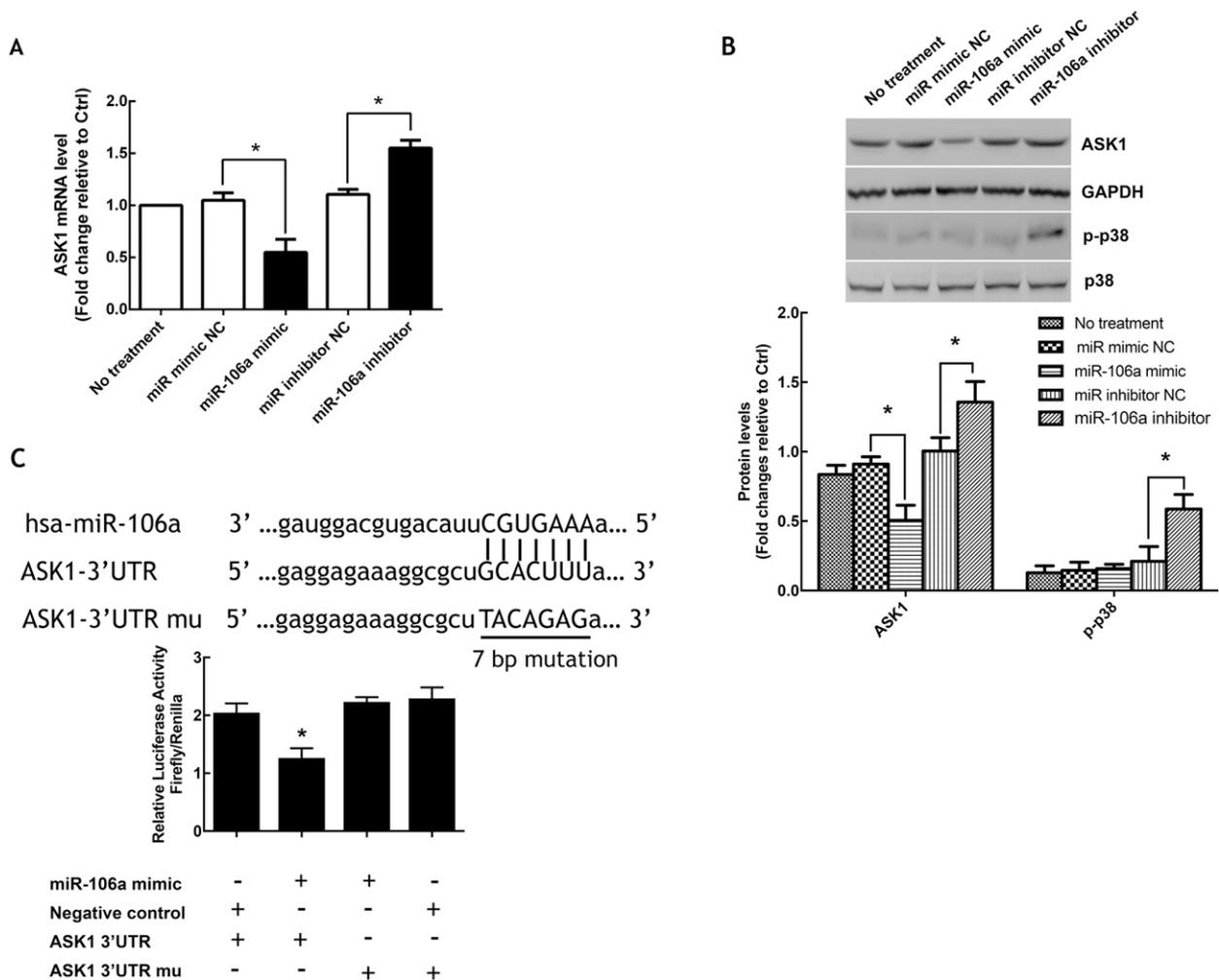
To further confirm the role of miR-106a on human granulosa cell viability, we used Western blot analysis to examine its effects on the expression of PCNA. As shown in Fig. 2D, PCNA levels were significantly increased after transfection with miR-106a mimic, whereas they were significantly decreased following transfection with miR-106a inhibitor.

Next, we examined the effects of treatment with miR-106a mimic or inhibitor on granulosa cell apoptosis by morphological assessment using Hoechst 33258 staining and fluorescence microscopy. Transfection of primary human granulosa cells with miR-106a mimic significantly reduced the percentage of apoptotic cells, whereas transfection miR-106a inhibitor increased the percentage of apoptotic cells (Fig. 2E). Together, these findings suggest that miR-106a

enhances viability and inhibits apoptosis of human granulosa cells.

**miR-106a negatively regulates ASK1 levels in human granulosa cells**

ASK1 has been shown to activate p38 MAPK signaling and induce apoptosis in other cell types (13, 14). Our KEGG pathway analysis suggested regulation of MAPK signaling, and TargetScan/miRBase database analyses predicted potential binding sites for miR-106a in the ASK1 3'UTR. To investigate the regulation of ASK1 by miR-106a, we first examined the effects of treatment with miR-106a mimic or inhibitor on ASK1 levels. As shown in Fig. 3A, transfection of primary human granulosa cells with miR-106a mimic significantly downregulated ASK1 mRNA levels,



**Figure 3.** miR-106a negatively regulates ASK1 levels in primary human granulosa cells. (A) Granulosa cells were transfected with miR-106a mimic or miR-106a inhibitor for 24 hours before measuring ASK1 mRNA levels by Quantitative reverse transcription PCR (normalized to GAPDH). (B) Western blot analysis of the levels of ASK1, phosphorylated p38 MAPK (p-p38), and p38 MAPK following transfection with miR-106a mimic or inhibitor for 48 hours. Quantified ASK1 protein levels were normalized to GAPDH, whereas those of phosphorylated p38 MAPK were normalized to total p38 MAPK. (C) TargetScan predicted alignments of miR-106a with wild-type and mutant binding sites in the 3'UTR of ASK1. 293T cells were treated with or without miR-106a mimic, and 3'UTR luciferase analysis was performed with wild-type ASK1-3'UTR or mutant ASK1-3'UTR. Data are presented as the mean ± standard deviation of at least three independent experiments. \*Significant differences ( $P < 0.05$ ). NC, negative control.

whereas transfection with miR-106a inhibitor increased the levels of ASK1 mRNA. Western blot analysis revealed similar decreases or increases in ASK1 protein levels following treatment with miR-106a mimic or inhibitor, respectively (Fig. 3B).

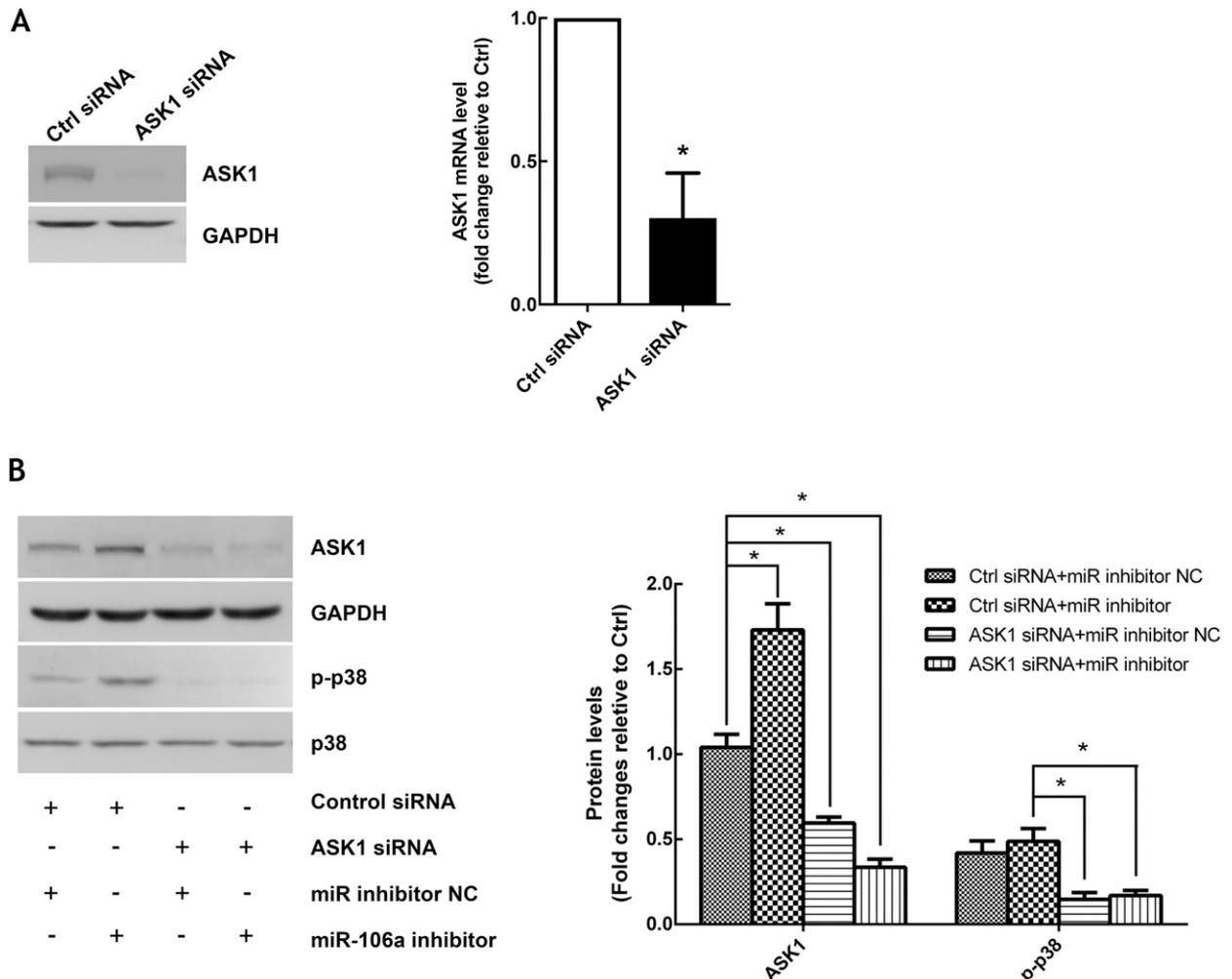
To examine the effects of miR-106a on p38 MAPK signaling/activation, primary human granulosa cells were treated with miR-106a mimic or inhibitor, and Western blot was used to measure the levels of phosphorylated p38 MAPK in relation to its total levels. As shown in Fig. 3B, treatment with miR-106a inhibitor significantly increased the levels of phosphorylated p38 MAPK, but basal phosphorylated p38 MAPK levels were not further reduced by treatment with miR-106a mimic.

To confirm that ASK1 is a direct target of miR-106a, we performed 3'UTR luciferase analysis with miR-106a mimic and reporter vectors containing wild-type ASK1

3'UTR and mutant ASK1 3'UTR. In cells transfected with the ASK1 3'UTR luciferase reporter, miR-106a treatment significantly reduced reporter luciferase activity compared with treatment with miRNA negative control (Fig. 3C). In contrast, treatment with miR-106a mimic failed to suppress mutant ASK1 3'UTR reporter luciferase activity (Fig. 3C). These results suggest that miR-106a directly targets ASK1 by binding to its 3'UTR.

### ASK1 mediates the effects of miR-106a on p38 MAPK signaling

To investigate the role of ASK1 in mediating the effects of miR-106a on p38 MAPK phosphorylation/activation, siRNA was used to knock down ASK1 in primary human granulosa cells. As shown in Fig. 4A, pretreatment with ASK1 siRNA significantly reduced ASK1 mRNA and protein levels. Western blot analysis



**Figure 4.** ASK1 mediates the effects of miR-106a on p38 MAPK signaling. (A) Western blot and Quantitative reverse transcription PCR were used to measure levels of ASK1 protein (*left*) and mRNA (*right*, normalized to GAPDH) in primary human granulosa cells transfected for 24 hours with ASK1 siRNA or control siRNA. (B) Granulosa cells were transfected for 24 hours with control or ASK1 siRNA before treatment of 48 hours with miR-106a inhibitor. Western blot analysis was used to measure the levels of ASK1, phosphorylated p38 MAPK (p-p38), and p38 MAPK. Quantified ASK1 protein levels were normalized to GAPDH, whereas those of phosphorylated p38 MAPK were normalized to total p38 MAPK. Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. \*Significant differences ( $P < 0.05$ ). Ctrl, control; NC, negative control.

showed that p38 MAPK phosphorylation was significantly increased after transfection with miR-106a inhibitor, and this effect was abolished by pretreatment with ASK1 siRNA (Fig. 4B). These findings suggest that miR-106a suppresses ASK1/p38 MAPK signaling in human granulosa cells.

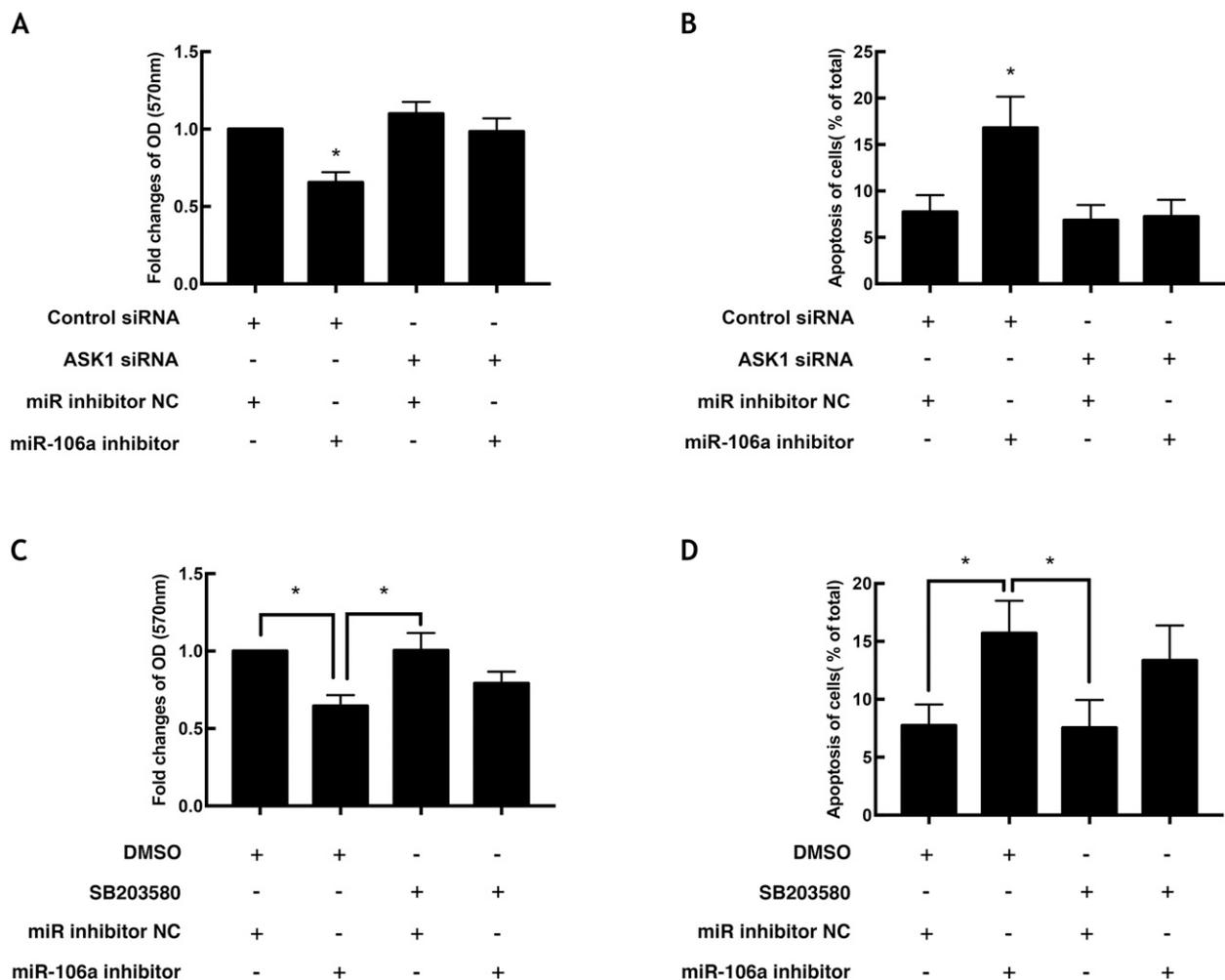
### ASK1 mediates the effects of miR-106a on cell viability and apoptosis

Next we investigated the involvement of ASK1 and p38 MAPK signaling in miR-106a-regulated granulosa cell viability. To study the role of ASK1, primary human granulosa cell viability and apoptosis were examined after knockdown of ASK1 and treatment with or without miR-106a inhibitor. As shown in Fig. 5A and 5B, treatment with miR-106a inhibitor reduced granulosa cell viability and increased apoptosis, and these effects were abolished

by pretreatment with ASK1 siRNA. To investigate the involvement of p38 MAPK signaling, granulosa cell viability and apoptosis were examined following treatment with p38 MAPK inhibitor SB203580 in the presence or absence of miR-106a inhibitor. Interestingly, pretreatment with SB203580 did not significantly alter the effects of miR-106a inhibitor on granulosa cell viability and apoptosis (Fig. 5C and 5D). These findings suggest that miR-106a enhances human granulosa cell viability by suppressing ASK1.

### Discussion

During follicle development, precise cross-talk between the oocyte and surrounding granulosa cells is essential for generating a fertilizable oocyte and regulating ovarian functions (27). Thus, the decline of oocyte competence in women with DOR may arise from abnormal regulation



**Figure 5.** ASK1 but not p38 MAPK signaling mediates the effects of miR-106a on primary human granulosa cell viability. (A, B) Primary human granulosa cells were transfected for 24 hours with control or ASK1 siRNA before treatment of 48 hours with miR-106a inhibitor. (A) Cell viability was determined by the MTT assay, and (B) apoptosis was measured by Hoechst 33258 staining. (C, D) Granulosa cells were treated with miR-106a inhibitor in the presence or absence of 1  $\mu$ M SB203580 for 48 hours, and (C) cell viability and (D) apoptosis were determined by the MTT assay and Hoechst 33258 staining, respectively. Data are presented as the mean  $\pm$  standard deviation of at least three independent experiments. \*Significant differences ( $P < 0.05$ ). DMSO, dimethyl sulfoxide; NC, negative control.

by granulosa cells. It is well established that miRNAs are capable of regulating many cellular processes, including cell proliferation, apoptosis, and differentiation. Indeed, increasing evidence suggests that miRNAs play critical roles in the regulation of granulosa cell functions. Differential expression and dysregulation of miRNAs have been associated with several ovarian disorders. For example, miR-22-3p has been implicated in apoptotic processes, and its levels are reduced in women with POF (28). Moreover, our previous studies demonstrated that miR-23a is upregulated in women with POF and increases human granulosa cell apoptosis by suppressing the X-linked inhibitor of apoptosis (9). In the current study, we identified several differentially expressed miRNAs in granulosa cells and serum from women with DOR. Among these differentially expressed miRNAs, miR-106a was uniquely downregulated in both granulosa cells and serum from women with DOR. miR-106a is a member of the miR-17 family, and recent studies have shown that its downregulation is associated with aging in humans (21).

GO and KEGG pathway analyses with differentially expressed miRNAs identified by microarray suggested involvement in apoptotic and proliferative processes. Interestingly, studies have shown that miR-106a participates in the regulation of cell senescence (29) and is reported to be upregulated in gastric cancer (30) and colorectal cancer (31). Considering the relevance of granulosa cell apoptosis to oocyte viability and overall folliculogenesis (32), we investigated the effects of miR-106a on granulosa cell viability. We found that miR-106a increases the viability of primary human granulosa cells, likely by promoting cell proliferation as well as suppressing cell apoptosis. Thus, downregulation of miR-106a in the granulosa cells of women with DOR may reduce their viability, which could negatively affect oocyte viability and follicle development. One limitation of our study is that it involved women undergoing ovarian stimulation for IVF, and thus the role of miR-106a in normal folliculogenesis (*i.e.*, unstimulated cycles) requires further verification. Moreover, we cannot rule out the influence that differences in follicle growth between healthy women and women with DOR (*i.e.*, number of oocytes retrieved) might have on the differential expression of miRNAs in general and miR-106a in particular. Thus, future studies examining the effects of miR-106a downregulation on granulosa cells and follicle development *in vivo* will be of great interest.

To investigate how miR-106a might contribute to granulosa cell apoptosis, we used TargetScan to identify potential target genes and then focused on ASK1 because it is an upstream activator of p38 MAPK signaling that has previously been implicated in cell

apoptosis (13, 14). Studies suggest that ASK1 is activated in response to various types of cellular stress and is involved in several pathological conditions (15, 33). For instance, elevated expression of miR-106a inhibited apoptosis of thyroid cancer cells by targeting retinoic acid receptor  $\beta$  to regulate ASK1/p38 MAPK signaling (34). Similarly, ASK1 has been shown to participate in lipopolysaccharide-induced apoptosis of endothelial cells (19). We have demonstrated that miR-106a negatively regulates ASK1 mRNA and protein levels. Moreover, we used 3'UTR luciferase analysis to show that miR-106a directly targets the 3'UTR of ASK1. Our knockdown studies with ASK1 siRNA demonstrated that ASK1 mediates the effects of miR-106a on p38 MAPK signaling and human granulosa cell viability and apoptosis. Interestingly, our p38 MAPK inhibitor studies do not support the involvement of p38 MAPK signaling in the effects of miR-106a on human granulosa cell viability and apoptosis. Future studies are required to determine how miR-106a-regulated p38 MAPK signaling contributes to granulosa cell and/or oocyte function.

Our results suggest that the downregulation of miR-106a may contribute to the pathogenesis of DOR by promoting granulosa cell apoptosis via the upregulation of ASK1. This study provides insight into the molecular mechanisms contributing to DOR and may be relevant to ovarian aging or other ovarian disorders.

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