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Differentially expressed plasma microRNAs in premature ovarian failure patients and the potential regulatory function of mir-23a in granulosa cell apoptosis

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Abstract

Recent studies implicate the regulatory function of microRNAs (miRNAs) in oocyte maturation and ovarian follicular development. Differentially expressed miRNAs are found in the plasma of premature ovarian failure (POF) patients and normal cycling women. In this study, miRNA-regulated signaling pathways and related genes were described using Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis. The effect of mir-23a on granulosa cell apoptosis was also studied by examining the protein expression of X-linked inhibitor of apoptosis protein (XIAP) and caspase-3, followed by subsequent counting of apoptotic cells after Hoechst 33258 staining. Both GO analysis and pathway analysis suggested that many signaling pathways, including the AKT signaling pathway, steroid hormone receptor signaling pathways, and others, were regulated by this group of differentially expressed miRNAs. A decrease in XIAP expression (mRNA and protein level) and caspase-3 protein levels and an increase in cleaved caspase-3 protein were observed in human ovarian granulosa cells transfected with pre-mir-23a, along with an increased occurrence of apoptosis. In conclusion, differentially expressed miRNAs in the plasma of POF patients may have regulatory effects on proliferation and apoptosis of granulosa cells by affecting different signaling pathways. Mir-23a may play important roles in regulating apoptosis via decreasing *XIAP* expression in human ovarian granulosa cells.

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Introduction

Premature ovarian failure (POF) is an ovarian disorder of multifactorial origin defined as the occurrence of amenorrhea, hypergonadotropism, and hypoestrogenism in women under the age of 40 years (Beck-Peccoz & Persani 2006). The incidence is one in 10 000 women at the age of 20, one in 1000 at the age of 30, and one in 100 by the age of 40. POF can be sporadic or familial (4–33%; Conway 1996, van Kasteren *et al*. 1999, Vegetti *et al*. 2000). Aside from its associated fertility problems, POF is a serious endocrine disorder and, if left untreated, can induce a twofold age-specific increase in mortality due to an increased incidence of cardiovascular disease, stroke, and osteoporosis (Snowdon *et al*. 1989). To better understand the pathogenesis of POF, it is necessary to remember that <500 (0.007%) of a woman's original seven million oocytes are released during her entire reproductive life, and the rest die during the process of folliculogenesis. Therefore, POF may result from either a reduced number of follicles formed during ovarian development or an increased rate of follicle loss. Studies have demonstrated that various factors, such as GNRH (Billig *et al*. 1994, Andreu *et al*. 1998), androgens (Billig *et al*. 1993), and Nodal (Wang *et al*. 2006), can induce apoptosis in ovarian granulosa cells and result in follicle loss.

MicroRNAs (miRNAs) are noncoding, single-stranded small RNAs of ∼22–24 nucleotides (nt) that constitute a novel class of gene regulators. The primary miRNA transcript, namely pri-miRNAs, is several kilobases long and undergoes substantial processing in the nucleus, resulting in the generation of a 70- to 90-nt stem–loop precursor miRNA (pre-miRNA). After subsequent processing in the cytoplasm by Dicer, a double-stranded miRNA duplex, which contains 2 nt-long 3′ overhangs that will unwind and form a single-stranded mature miRNA, is generated (Bernstein *et al*. 2001, Bernstein *et al*. 2003, Paroo *et al*. 2007). The mature miRNAs

repress translation or assist in mRNA degradation in a sequence-specific manner (Ambros 2004, Bartel 2004, Zamore & Haley 2005). In this way, miRNAs influence various cellular activities including cell proliferation, differentiation, and apoptosis under normal and diseased conditions.

Evidence generated in mouse studies suggests a regulatory function of miRNAs in oocyte maturation and ovarian follicular development (Murchison *et al.* 2007, Tang *et al.* 2007). Otsuka *et al.* (2008) found that *Dicer1* deficiency results in female infertility, which was caused by corpus luteum (CL) insufficiency and resulted at least in part from the impaired growth of new capillary vessels in the ovary. Furthermore, impaired CL angiogenesis in *Dicer1$^{d/d}$* mice was associated with a lack of miR17-5p and let-7b. Hong *et al.* (2008) showed that *Dicer1$^{fl/fl}$*, antiMüllerian hormone receptor *Amhr2$^{Cre/+}$* female mice, where *Dicer1* was selectively knocked out in Müllerian duct derivatives (i.e. the oviduct, uterus, and cervix) and in the granulosa cells of secondary and small antral follicles (Hong *et al.* 2008), showed a decreased ovulation rate and decreased ovary weights compared with wild-type controls (Hong *et al.* 2008). Choi *et al.* (2007) identified 177 miRNAs in the newborn mouse ovary and found that four miRNAs were downregulated approximately twofold in the mouse with a knockdown in an ovarian homeobox gene, a transcription factor necessary for oocyte differentiation. In 2006, Kim *et al.* (2006) identified 58 miRNAs in the pig genome and confirmed the expression of two of these miRNAs in the porcine ovary using northern blot analysis. Ro *et al.* (2007) identified 122 miRNAs in the ovaries of 2-week-old and adult mice. Fiedler *et al.* (2008) identified 13 differentially expressed miRNAs in mouse granulosa cells before and 4 h after human chorionic gonadotropin (hCG) treatment and their further investigation indicated that mir-21 can block apoptosis of mouse periovulatory granulosa cells.

In 2008, Chim *et al.* (2008) first reported the existence of miRNAs in maternal plasma. Since then, miRNAs have been detected in several kinds of body fluids including plasma, serum, and urine (Gilad *et al.* 2008). Plasma miRNAs have become promising potential biomarkers for a series of cancers and other diseases such as hepatocellular carcinoma (Yamamoto *et al.* 2009), gastric cancer (Tsujiura *et al.* 2010), prostate cancer (Mitchell *et al.* 2008), non-small-cell lung carcinoma (Chen *et al.* 2008), colorectal cancer (Chen *et al.* 2008), type 2 diabetes (Chen *et al.* 2008), ovarian cancer (Resnick *et al.* 2009), and drug-induced liver injury (Wang *et al.* 2009). In a previous study, we demonstrated differential miRNA expression profiles in the plasma of POF patients (Table 1) and normal cycling women by miRNA microarray analysis. The ten upregulated miRNAs were mir-202, mir-146a, mir-125b-2*, mir-139-3p, mir-654-5p, mir-27a, mir-765, mir-23a, mir-342-3p, and mir-126 and the two downregulated

Table 1 General characteristics of premature ovarian failure (POF) patients and normal cycling women.

	POF	Normal
Age	30.33±4.33	29.80±5.82
BMI (kg/m^2)	21.74±1.43	20.76±1.68
FSH (mIU/ml)	69.48±22.88	5.73±1.24

miRNAs were let-7c and mir-144 (Zhou *et al.* 2011). Nevertheless, the signaling pathways that regulate the expression of miRNAs during POF and the function of individual miRNAs in granulosa cell apoptosis remain unknown.

X-linked inhibitor of apoptosis protein (XIAP) exerts an antiapoptotic function through the direct inhibition of caspase-3 and modulating the mitochondrial death pathway by binding Smac/DIABLO (Asselin *et al.* 2001, Siegel *et al.* 2011). miRNAs, such as mir-23a, can target XIAP and regulate its function (Siegel *et al.* 2011). XIAP promotes the development of rat granulosa cells and ovarian follicles via its antiapoptotic function (Andreu *et al.* 1998, Li *et al.* 1998). However, the relationship between mir-23a and XIAP in granulosa cells during follicular development and atresia remains unclear.

In this study, we performed a bioinformatic analysis of miRNA-regulated signaling pathways and related genes on the basis of miRNA expression profiles. Additionally, the role of the differentially expressed mir-23a in granulosa cell apoptosis was also explored. Herein, we demonstrate that mir-23a, which we previously demonstrated to be differentially expressed in plasma of women with POF, induces apoptosis in cultured human granulosa cells. Furthermore, we provide evidence that this occurs via downregulation of XIAP at both the mRNA and protein levels, with a subsequent increase in caspase-3 cleavage. Taken together, our findings suggest a novel mechanism through which mir-23a affects granulosa cell apoptosis, which may help to explain the potential role of mir-23a in the pathogenesis of POF.

Results

Gene Ontology category

In our previous study, the differential miRNA profile in the plasma of POF patients and normal controls has been studied using miRNA microarray analysis (Zhou *et al.* 2011). Briefly, total RNA from the plasma of three POF patients was compared with three normal specimens using a customized miRNA microarray, which contained 821 human miRNAs from the miRNA Registry. Primary miRNA expression profiling with microarray identified 29 miRNAs based on the *P* value, 12 of which were differentially expressed between POF patients and normal women. The 12 upregulated miRNAs were

mir-202, mir-146a, mir-125b-2*, mir-139-3p, mir-654-5p, mir-27a, mir-765, mir-23a, mir-342-3p, and mir-126, and the two downregulated miRNAs were let-7c and mir-144. For validation of these findings, miRNAs were quantified using qRT-PCR analysis in the plasma of 39 POF patients and 20 normal women. Consistent with the microarray data, mir-146a, mir-27a, mir-23a, and mir-126 were highly expressed in the plasma from POF patients compared with the controls, with a fold change of 5.19, 2.98, 2.75, and 2.29 respectively.

The differentially expressed miRNAs were classified into different functional categories according to Gene Ontology (GO) analysis of biological process. The top six GO categories for upregulated genes were i) AKT signaling pathway, ii) regulation of mitochondrial membrane permeability, iii) steroid hormone receptor signaling pathway, iv) activation of mitogen-activated protein kinase kinase (MAPKK) activity, v) positive regulation of NF-κB transcription factor activity, and vi) induction of apoptosis by extracellular signals and apoptosis (Fig. 1A). The six primary GO categories for downregulated genes were i) AKT signaling pathway, ii) regulation of Wnt receptor signaling pathway, iii) regulation of growth, estrogen receptor signaling pathway, iv) induction of apoptosis by intracellular signals, v) androgen receptor signaling pathway, and vi) regulation of assembly reaction factor protein signal transduction (Fig. 1B).

Pathway analysis and the miRNA–mRNA regulatory networks

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for upregulated genes showed that the genes were largely involved in five pathways: i) ErbB signaling pathway, ii) p53 signaling pathway, iii) MAPK signaling pathway, iv) transforming growth factor β (TGFβ) signaling pathway, and v) apoptosis (Fig. 2A). The KEGG pathway analysis for downregulated genes showed that the genes were more related to the mammalian target of the following five pathways: i) rapamycin (mTOR) signaling pathway, ii) TGFβ signaling pathway, iii) MAPK signaling pathway, iv) p53 signaling pathway, and (v) vascular endothelial growth factor signaling pathway (Fig. 2B).

The miRNA–mRNA regulatory networks are shown in Fig. 3, which distinguished the putative target mRNAs between upregulated and downregulated miRNAs. Three overexpressed miRNAs (mir-27a, mir-23a, and mir-202) showed 44, 33, and 28 target mRNAs respectively. Possible mir-23a-regulated genes included the antiapoptotic gene *XIAP* and pro-apoptotic genes phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and caspase-7. These findings suggest that mir-23a may play a role in the regulation of granulosa cell apoptosis. The two downregulated miRNAs (mir-144 and let-7c) showed 27 and 41 target mRNAs, respectively, including p53, caspase-3, and PTEN.
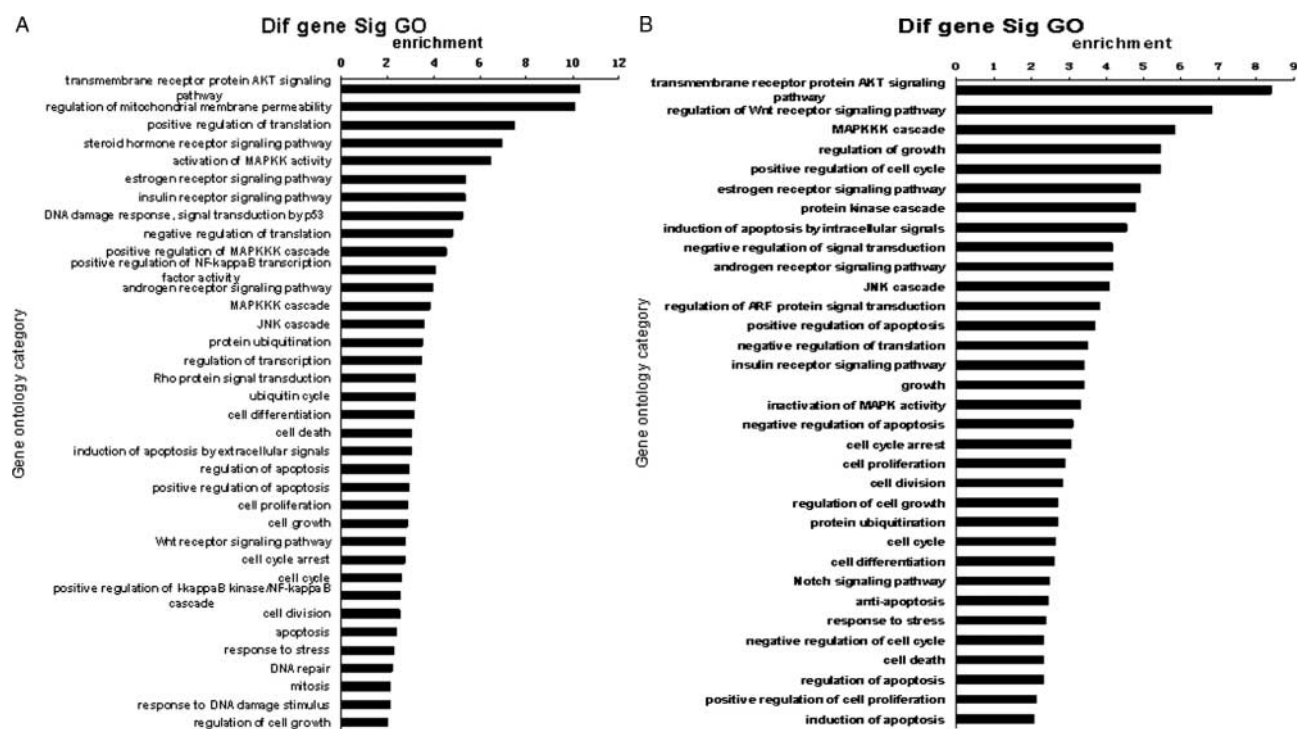

Figure 1 GOs targeted by miRNAs. (A and B) GOs targeted by the ten upregulated miRNAs and the two downregulated miRNAs respectively. All these GOs show an increased enrichment in each category by these miRNAs. The vertical axis is the GO category, and the horizontal axis is the enrichment of GO.
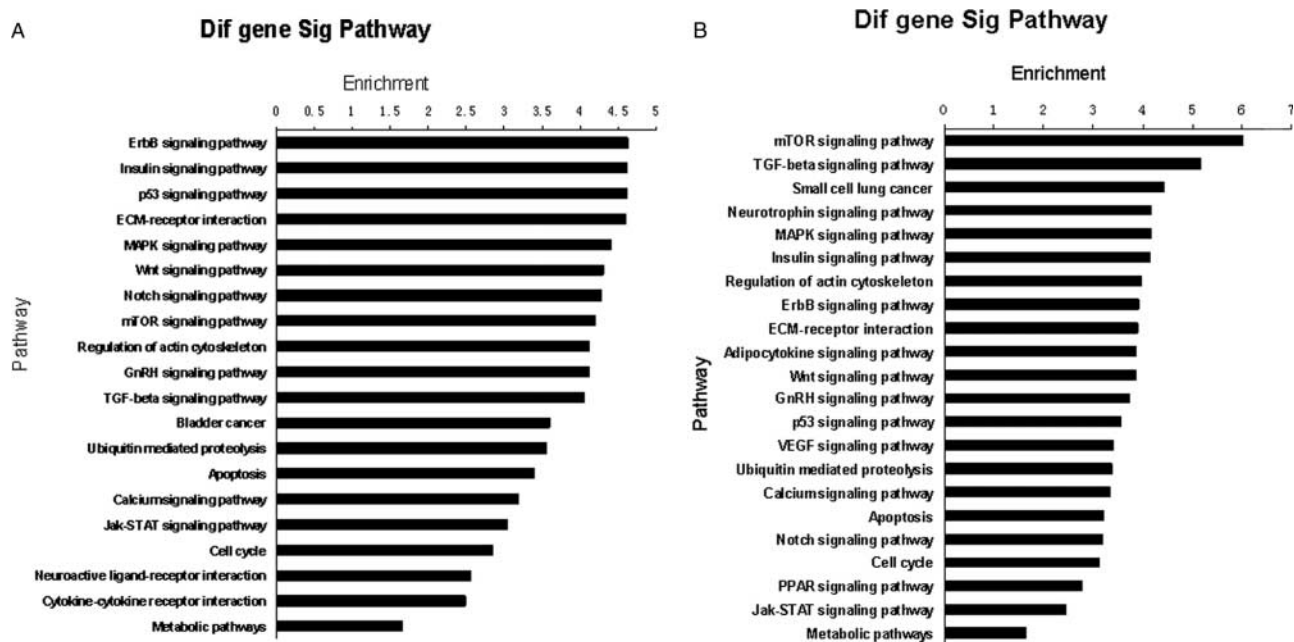
Figure 2 Pathway analysis based on miRNA-targeted genes. (A and B) Main pathways targeted by upregulated and downregulated miRNAs respectively. The vertical axis is the pathway category and the horizontal axis is the degree of enrichment of the pathway.

Mir-23a-induced apoptosis in human granulosa cells

To determine the effect of mir-23a on apoptosis in granulosa cells, the percentage of apoptotic cells in human granulosa cells transfected with pre-mir-23a or control pre-miRNA was assessed based on Hoechst 33258 staining. Mir-23a overexpression increased the rate of apoptosis in human granulosa cells. To further determine the role of mir-23a in apoptosis of granulosa cells, mir-23a inhibitor was transfected into granulosa cells. As shown in Fig. 4A, mir-23a inhibitor significantly decreased pre-mir-23a-induced apoptosis in granulosa cells ($P < 0.05$). These findings suggest that mir-23a induces apoptosis in human granulosa cells.

The effects of miR-23a on XIAP and caspase-3 expression in human granulosa cells

Recent studies have shown that mir-23a can induce caspase-dependent and -independent apoptosis in human embryonic kidney cells, both of which occur via the mitochondrial membrane disruption pathway (Chhabra *et al.* 2009). The KEGG pathway analysis demonstrated that mir-23a may regulate the antiapoptotic function of XIAP (Fig. 3). To investigate whether and how mir-23a is involved in granulosa cell apoptosis, the expression of XIAP and caspase-3 mRNA and protein in human granulosa cells after mir-23a overexpression was examined by RT-PCR and western blotting respectively. XIAP mRNA and protein contents were significantly downregulated in granulosa cells transfected with pre-mir-23a compared with the control pre-miRNA-transfected cells. Furthermore,

caspase-3 protein content was significantly decreased, with a corresponding increase in the cleaved caspase-3 level after transfection of pre-mir-23a, suggesting caspase-3 cleavage and activation. However, caspase-3 mRNA level was not altered after transfection of pre-mir-23a, suggesting that mir-23a regulates XIAP, not caspase-3, at both transcriptional and translational levels (Fig. 4B).

To confirm that mir-23a directly and specifically regulates *XIAP* expression, the mir-23a inhibitor or its control was transfected into granulosa cells. mir-23a inhibitor significantly increased XIAP mRNA and protein expression. In contrast, there was no change in caspase-3 protein and mRNA level (Fig. 5). These findings suggest that mir-23a induces apoptosis by decreasing *XIAP* expression at both transcriptional and translational levels, with a subsequent cleavage of caspase-3.

Discussion

Recruitment of growing follicles, atresia, ovulation, and luteal tissue formation and regression are dynamically regulated events that regenerate on a cyclical basis in the ovary (Carletti & Christenson 2009). These events involve dynamic changes in cellular growth, angiogenesis, steroidogenesis, cell cycle, and apoptosis and are accurately regulated at the endocrine and tissue levels (Carletti & Christenson 2009). Defects in the regulatory networks result in ovarian failure such as POF due to disruption of folliculogenesis, blockage of ovulation, and loss of oocytes via apoptosis. Understanding the molecular events during folliculogenesis and atresia
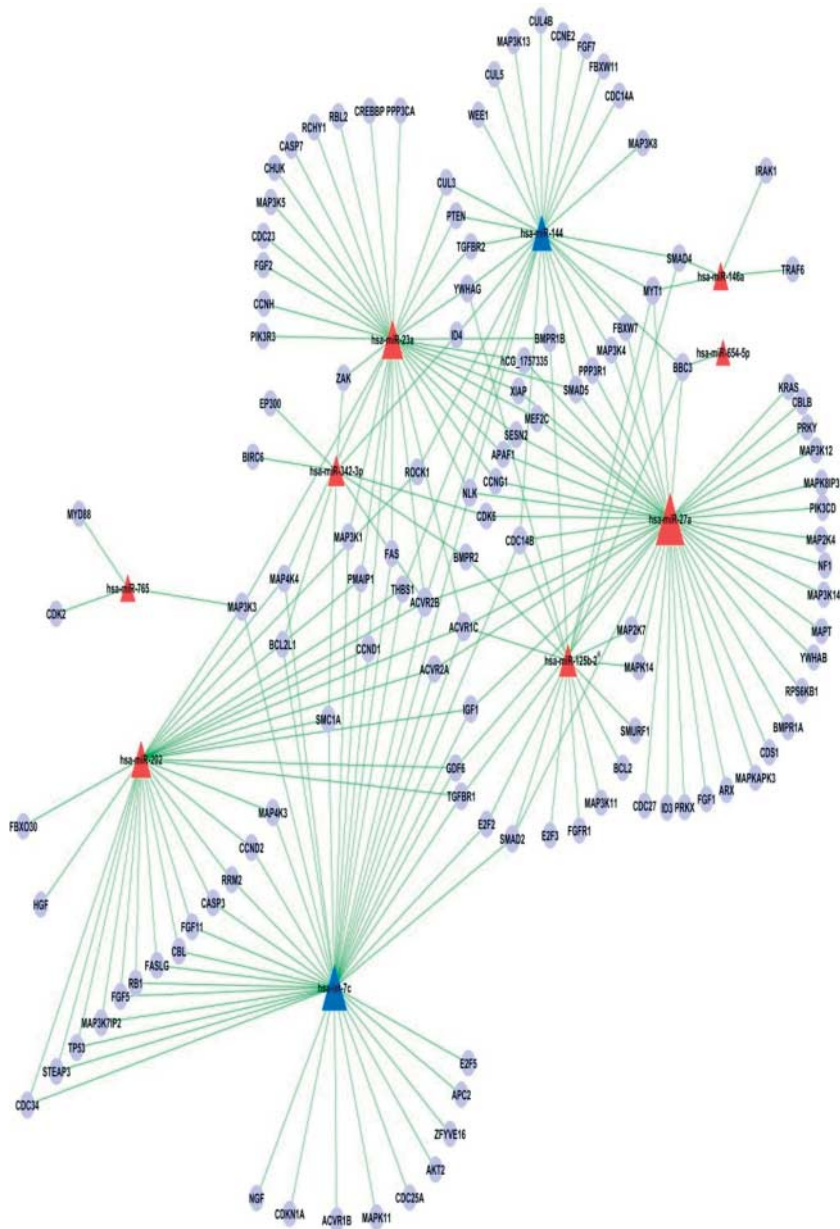
Figure 3 miRNA–mRNA network. Triangles represent miRNAs, and gray oval cycle nodes represent mRNAs. The green lines show the inhibitory effect of miRNAs on mRNAs. The red triangles represent upregulated miRNAs and blue triangles represent downregulated miRNAs.

will provide insights into enhancing reproductive efficiencies and alleviating deficiencies (Carletti & Christenson 2009).

The recent identification of miRNAs as an important posttranscriptional gene regulator has led to an explosion in our knowledge of the role of posttranscriptional gene regulation in reproductive organ such as the ovary. In addition, miRNAs have also been recognized to be involved in crucial cell processes, such as apoptosis, differentiation, and oncogenesis by regulating signal transduction pathways (Ambros 2004). In a previous study, we identified several differentially expressed miRNAs in the plasma of POF patients and normally cycling women, including ten upregulated miRNAs (mir-202, mir-146a, mir-125b-2*, mir-139-3p, mir-654-5p,

mir-27a, mir-765, mir-23a, mir-342-3p, and mir-126) and two downregulated miRNAs (let-7c and mir-144). We also verified four differentially expressed miRNAs (mir-146a, mir-27a, mir-23a, and mir-126) in POF and found that the results were consistent with miRNA microarray analysis (Zhou *et al.* 2011).

In order to further determine the function of the differentially expressed plasma miRNAs, GO analysis and KEGG pathway annotation were used to analyze their target gene pools. GO organized genes targeted by differential miRNAs into hierarchical categories based on biological processes and then outlined the roles of miRNAs. In this study, GO analysis illustrates that these miRNAs are most related to the AKT signaling pathway, regulation of mitochondrial membrane permeability,
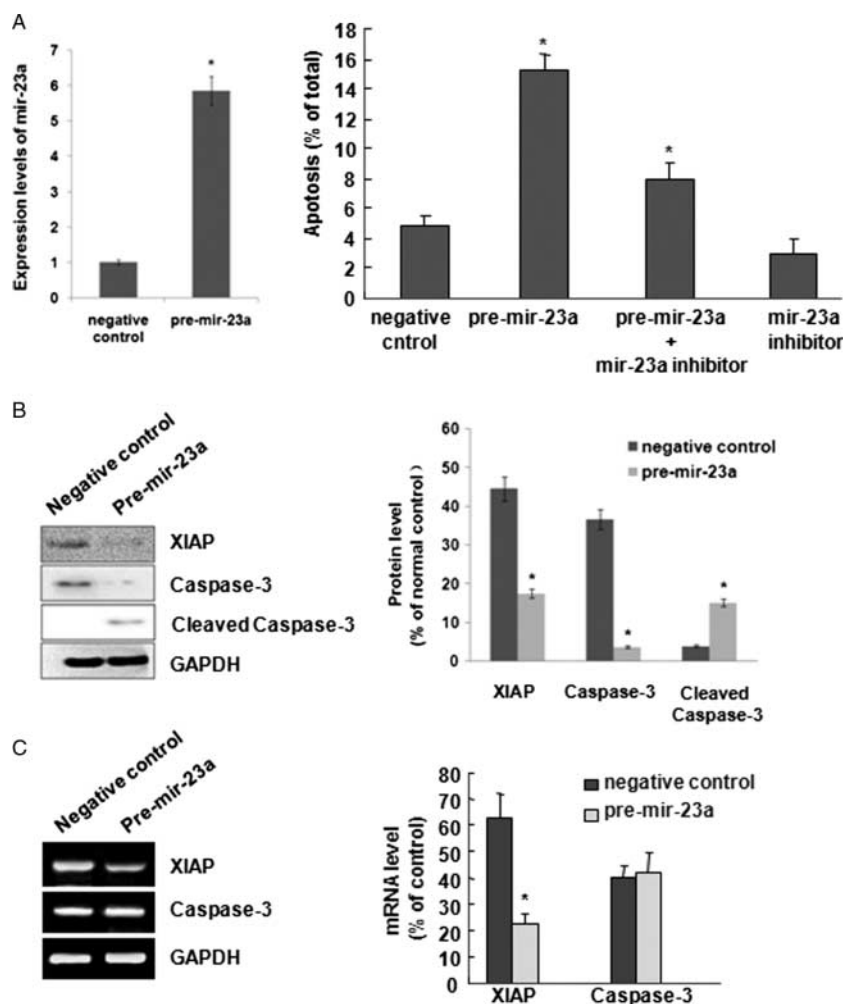
Figure 4 Mir-23a is pro-apoptotic in human granulosa cells. (A) Pre-mir-23a increased apoptosis and mir-23a inhibitor decreased apoptosis in human granulosa cells as assessed by Hoechst 33258 staining (*$P<0.05$, *t*-test, $n=3$). (B) Pre-mir-23a decreased XIAP and caspase-3 protein content and increased cleaved caspase-3 content in human granulosa cells (*$P<0.05$, *t*-test, $n=3$). GAPDH was included as a loading control. (C) *XIAP* mRNA level was decreased (*$P<0.05$, *t*-test, $n=3$) and there was no change in caspase-3 mRNA level ($P>0.05$, *t*-test, $n=3$) after transfection of pre-mir-23a.

steroid hormone receptor signaling pathway, activation of MAPKK activity, positive regulation of NF-κB transcription factor activity, induction of apoptosis by extracellular signals, apoptosis, and cell growth (Fig. 1). KEGG annotation showed that survival pathways (TGFβ and mTOR), importantly proliferative (ErbB, MAPK, Wnt, and cell cycle) and apoptotic (p53 signaling pathway and apoptosis) signaling pathways, were most abundant among the significantly enriched ones (Fig. 2), which was in accordance with the GO analysis. This functional identity revealed by different bioinformatic methods suggested that miRNAs may have regulatory effects on proliferation and apoptosis of granulosa cells by affecting the signaling pathways as mentioned earlier.

The miRNA–mRNA regulatory network analysis further integrated the bioinformatic observations and then outlined the main targets of miRNAs (Fig. 3). Mir-27a and mir-23a have 44 and 33 target mRNAs, respectively, that exhibit more target mRNAs compared with the other miRNAs. The mir-23a~27a~24-2 cluster has been shown to play important roles in several processes during normal and pathologic states and is tightly related to the cell cycle, proliferation,

differentiation, apoptosis, hematopoiesis, and cardiac hypertrophy (Huang *et al*. 2008, Chhabra *et al*. 2010). Studies have illustrated that the mir-23a~27a~24-2 cluster functions as a growth-promoting and antiapoptotic factor targeting the Smad pathway in hepatocellular carcinoma cells (Huang *et al*. 2008). However, a recent study demonstrated that the mir-23a~27a~24-2 cluster could induce caspase-dependent and -independent apoptosis in human embryonic kidney cells (Chhabra *et al*. 2009). The pro-apoptotic and antiapoptotic nature of the mir-23a~27a~24-2 cluster suggests that this cluster may play different roles under different physiological and pathological conditions. It is of great interest to identify the function of mir-23a in granulosa cells and follicular atresia.

In this investigation, several lines of evidence suggest that mir-23a promotes apoptosis of human granulosa cells. First, overexpression of mir-23a in granulosa cells can induce downregulation of antiapoptotic XIAP and increase the level of the pro-apoptotic cleaved form of caspase-3 as measured by western blotting analysis (Fig. 4A). Secondly, the percentage of apoptotic cells is significantly increased in mir-23a-treated granulosa cells
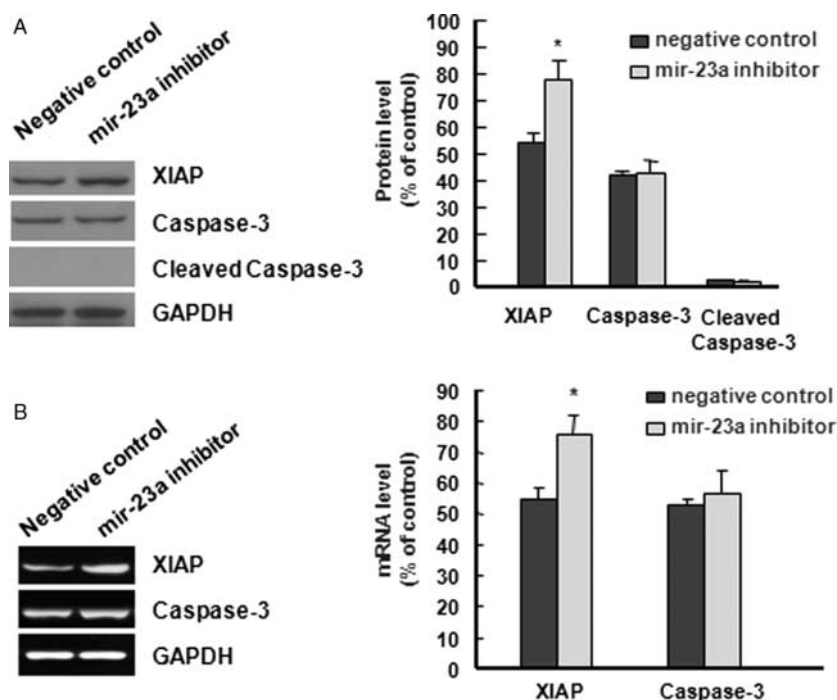
Figure 5 Mir-23a inhibitor increased XIAP protein and mRNA level. (A) XIAP protein content increased after transfection of mir-23a inhibitor, but there was no change in caspase-3 protein content (*$P<0.05$, t-test, $n=3$). (B) *XIAP* mRNA level increased after transfection of mir-23a inhibitor, but there was no change in caspase-3 mRNA level (*$P<0.05$, t-test, $n=3$).

compared with control miRNA-treated granulosa cells based on Hoechst 33258 staining (Fig. 4D). Furthermore, mir-23a inhibitor blocked mir-23a-induced apoptosis and decreased *XIAP* mRNA expression, with no effect on caspase-3 mRNA expression. XIAP is the endogenous inhibitor of caspase-3, and decreasing *XIAP* expression by mir-23a may contribute to enhancing caspase-3 activity (Siegel *et al.* 2011). These results provide clear evidence, for the first time, that mir-23a promotes apoptosis of granulosa cells via decreasing *XIAP* expression, which may contribute to the etiology of POF.

In conclusion, some plasma miRNAs are differentially expressed in POF patients and normal cycling women. Mir-23a, which is significantly upregulated in the plasma of POF patients, is essential for apoptosis induction in human granulosa cells by targeting XIAP and the caspase signaling cascade. In a word, these findings highlight the important roles of miRNAs in the nosogenesis of POF.

Materials and Methods

Study participants

All the samples were obtained following signed informed consent. All procedures for sample collection were approved by the Human Ethics Committees of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, and Institute of Zoology, Chinese Academy of Sciences.

Thirty-six infertile patients, <40 years of age who underwent their first IVF or ICSI–embryo transfer (ET) cycle, were included in this study (Table 2). The etiology of infertility was either due to tubal or male factors. The basal FSH, LH, estradiol (E_2), progesterone, PRL, and testosterone levels were measured on day 3 of the cycle before their stimulation cycle. The long

protocol of GNRH-a downregulation in the midluteal phase was used for the patients undergoing IVF or ICSI–ET. Recombinant FSH (150–225 IU) was administered on day 3 of the cycle. HCG (10 000 IU) was given when the leading follicle was ≥18 mm in diameter and there were at least two follicles ≥16 mm in diameter. Oocyte retrieval was arranged after 36 h. Samples of follicular fluid, for the isolation of ovarian granulosa cells, were collected from these patients undergoing stimulation cycles of IVF or ICSI–ET at the Department of Human Reproductive Medicine, Beijing Obstetrics and Gynecology Hospital.

Isolation of human granulosa cells

Isolation of human ovarian granulosa cells from follicular fluid was performed as described by Gillott *et al.* (2008). Briefly, all granulosa cells were disaggregated by incubating with 10% hyaluronidase for 15 min at 37 °C and separated from the red blood cells and lymphocytes by density gradient centrifugation over 50% Percoll (Sigma) for 15 min at 1000 ***g***. The granulosa cells at the interface were harvested. After being centrifuged at 500 ***g*** for 5 min, cells were cultured in six-well plates (∼10^6 cells/well) using Roswell Park Memorial Institute (RPMI)-1640 with glutamine and $NaHCO_3$ supplemented with 10% fetal calf plasma and 1% antibiotic–antimycotic (penicillin and streptomycin; Sigma) at 37 °C with 5% CO_2. Cultures were maintained for 18 h until the culture media were changed and any nonadherent cells were removed.

Transfection with miRNAs and miRNA inhibitor

Ovarian granulosa cells were transfected with 60 pM pre-mir-23a or pre-mir-negative control (Ambion Inc., Austin, TX, USA), as well as mir-23a inhibitor or its control (Exiqon, Vedbaek, Denmark), in six-well plates according to the

Table 2 General characteristics of the study population.

Age (years)	31.83 ± 3.63
Menstrual cycle length (days)	31.61 ± 3.73
Duration of infertility (years)	4.17 ± 2.50
Antral follicle number	11.11 ± 2.89
BMI (kg/m^2)	21.37 ± 1.73
Basal FSH (mIU/ml)	5.89 ± 1.03
Basal LH (mIU/ml)	4.77 ± 1.96
FSH/LH	1.47 ± 0.73
Basal E_2 (pg/ml)	26.41 ± 5.46
Basal progesterone (ng/ml)	0.83 ± 0.18
Basal PRL (ng/ml)	14.16 ± 7.08
Basal testosterone (ng/dl)	39.43 ± 8.50
Peak E_2	2776.949 ± 1156.806
Peak LH	2.44 ± 1.56
Peak P	1.62 ± 0.75
Retrieved oocytes (n)	10.83 ± 2.31
Metaphase II oocytes (n)	9.83 ± 1.82

manufacturer's instructions. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the granulosa cells in six-well plates reached 50–60% confluence. Cells were incubated with Opti-MEM (Invitrogen) for 6 h and then with fresh RPMI-1640 medium containing 10% fetal bovine serum for 24 h. Total RNAs and proteins were prepared 48 h after transfection and used for quantitative real-time-PCR or western blot analysis.

Western blotting analysis

Western blotting was done as described by Yang *et al*. (2006). Briefly, total protein extracts were prepared using whole-cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM sodium fluoride, 10% glycerol, and 1% Triton X-100) containing an inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate). Protein concentrations were determined using a standard Bradford assay, and 50 μg total protein were subjected to SDS–PAGE followed by electrotransfer onto nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies against human XIAP (R&D, Minneapolis, MN, USA), human caspase-3 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam) followed by incubation with secondary antibodies. Signals were developed using the enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Quantitative real time-PCR

Real-time PCR was performed using a standard SYBR Premix Ex Taq kit (Takara Bio Inc., Shiga, Japan) on an Applied Biosystems 7500 fast real-time PCR System (Applied Biosystems , Carlsbad, CA, USA). The 10 μl PCR reaction system included 5 μl SYBR Green PCR Master Mix, 1 μl cDNA, 0.2 μl ROX Reference Dye II, 0.5 μl specific primer (Table 1), and 3.3 μl RNase-free water. The reactions were performed in a 96-well plate at 95 °C for 30 s; followed by 45 cycles at 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 25 s; and finally 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. All reactions were run in triplicate. The threshold

cycle was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Negative control reactions without RT reaction and template were also performed.

RNA isolation and RT-PCR

Total RNA was isolated from human ovarian granulosa cells using TRIzol reagent according to the manufacturer's instructions. RNA (2 μg) was used for cDNA synthesis by RT as previously reported (Yang *et al*. 2004). The cDNAs obtained were amplified using specific primers (Asselin *et al*. 2001, Chen *et al*. 2009) as follows: XIAP (389 bp), 5′-GAA GAC CCT TGG GAA CAA CA-3′(sense) and 5′-CGC CTT AGC TGC TCT TCA GT-3′ (antisense); caspase-3 (445 bp), forward, 5′-CACAATAG-CACCCATCCG-3′ (sense) and 5′ -GGGACATCAGTCGCTTCA-3′ (antisense); and GADPH (230 bp), 5′-ACG GAT TTG GTC GTA TTG GG-3′ (sense) and 5′-TGA TTT TGG AGG GAT CTC GC-3′ (antisense).

To detect the mRNA of *XIAP* and caspase-3, PCR was performed at 94 °C for 15 s, 51 °C for 30 s, and 68 °C for 90 s for 30 cycles. *GAPDH* expression was used as an internal control.

Assessment of apoptosis

Cells were harvested and stained using Hoechst 33258 as previously reported (Wang *et al*. 2006). At least 200 cells in several selected areas were counted in each treatment group. Cells were counted with the counter 'blinded' to the sample identity to avoid experimental bias. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is stained by Hoechst 33258. This can take the form of crescents around the perimeter of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells can be visualized by fluorescence microscopy.

GO analysis

GO analysis was applied to analyze the main function of the differentially expressed genes according to the GO, which is the key functional classification of NCBI (Ashburner *et al*. 2000). Generally, Fisher's exact test and χ^2-test were used to classify the GO category. The false discovery rate (FDR; Dupuy *et al*. 2007) was calculated to correct the *P* value. The smaller the FDR, the smaller the error would be in judging the *P* value. The FDR was defined as $FDR = 1 - N_k/T$, where N_k refers to the number of Fisher's test *P* values less than χ^2-test *P* values and *T* refers to permutation test. *P* values were computed for the GOs of all differential genes. Enrichment provides a measure of the significance of the function: as the enrichment increases, the corresponding function is more specific, which helps to indicate GOs with more concrete function description in the experiment. Within the significant category, the enrichment Re was given by:

$$Re = \frac{(n_f/n)}{(N_f/N)}$$

with n_f being the number of differential genes within the particular category, n being the total number of genes within the same category, N_f being the number of differential genes in the entire microarray, and N being the total number of genes in the microarray.

MicroRNA–Gene Network

The relationship of the miRNA and genes were determined by their differential expression values, and according to the interactions of miRNA and genes in the Sanger miRNA database, the MicroRNA–Gene Network was built (Joung et al. 2007). In the MicroRNA–Gene Network, the circle represents gene and the shape of square represents miRNA, and their relationship was represented by one edge. The center of the network was represented by degree. Degree means the contribution of one miRNA to the genes around or the contribution of one gene to the miRNAs around. The key miRNA and the gene in the network always have the biggest degrees (Joung et al. 2007, Shalgi et al. 2007).

Pathway analysis

Pathway analysis was used to determine the significant pathways of the differentially expressed genes according to the KEGG and Biocarta and Reatome. Still, the Fisher's exact test and χ^2-test were used to select the significant pathways, and the threshold of significance was defined by P value and FDR. The enrichment Re was calculated as in the equation mentioned earlier (Kanehisa et al. 2004, Yi et al. 2006, Draghici et al. 2007).

Statistical analysis

Results are presented as mean±S.E.M. of at least three independent experiments. Quantitative data were compared with a student t-test between the two groups. $P<0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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