

# Poor ovarian response in women undergoing in vitro fertilization is associated with altered microRNA expression in cumulus cells

Cengiz Karakaya, Ph.D.,<sup>a,b</sup> Ozlem Guzeloglu-Kayisli, Ph.D.,<sup>a</sup> Asli Uyar, Ph.D.,<sup>a,c</sup> Amanda N. Kallen, M.D.,<sup>a</sup> Elnur Babayev, M.D.,<sup>a</sup> Nuray Bozkurt, M.D.,<sup>b</sup> Evrim Unsal, Ph.D.,<sup>d</sup> Onur Karabacak, M.D.,<sup>b</sup> and Emre Seli, M.D.<sup>a</sup>

<sup>a</sup> Division of Reproductive Endocrinology and Infertility, Department of Obstetrics, Gynecology, and Reproductive Sciences, Yale School of Medicine, New Haven, Connecticut; <sup>b</sup> Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Gazi University School of Medicine, Ankara, Turkey; <sup>c</sup> Department of Computer Engineering, Okan University, Istanbul, Turkey; and <sup>d</sup> Department of Histology and Embryology, Istanbul Bilim University School of Medicine, Istanbul, Turkey

**Objective:** To analyze the association of micro-ribonucleic acid (miRNA) expression with the number of oocytes retrieved, in women undergoing in vitro fertilization (IVF).

**Design:** Experimental study.

**Setting:** Academic medical center.

**Patient(s):** A total of 189 women undergoing IVF–intracytoplasmic sperm injection (ICSI).

**Intervention(s):** Pooled cumulus cells were collected.

**Main Outcome Measure(s):** Poor responders were identified as patients who produced fewer oocytes than the 25th percentile of their respective age group. MicroRNAs were extracted from cumulus cells, and an miRNA microarray was performed, comparing poor responders ( $n = 3$ ) to non-poor responders ( $n = 3$ ). Expression of miR-21-5p (active strand of miR-21) and miR-21-3p was tested in poor responders ( $n = 21$ ) and non-poor responders ( $n = 29$ ), using reverse transcription real-time polymerase chain reaction (qRT-PCR). Regulation of miR-21-5p and miR-21-3p, in human granulosa-like tumor (KGN) cells, by estradiol ( $E_2$ ), was tested in vitro.

**Result(s):** MicroRNA microarray analysis showed up-regulation of 16 miRNAs and down-regulation of 88 miRNAs in poor responders. Notably, miR-21 was significantly up-regulated 5-fold in poor-responder samples. Analysis using qRT-PCR confirmed that miR-21-5p expression was significantly up-regulated in poor responders, whereas miR-21-3p expression was significantly lower, suggesting that elevated miR-21-5p expression in cumulus cells is not regulated at the pre-miR-21 level in poor responders. Both miR-21-5p and miR-21-3p were increased in KGN cells in response to higher doses of  $E_2$ ; their expression was not affected at lower  $E_2$  concentrations.

**Conclusion(s):** We found that poor response to IVF is associated with altered miRNA expression in cumulus cells, specifically with elevated expression of miR-21-5p, and that this elevated expression is independent of lower serum  $E_2$  levels in poor responders. (Fertil Steril® 2015;103:1469–76. ©2015 by American Society for Reproductive Medicine.)

**Key Words:** IVF, miRNA, miR-21, poor ovarian response, cumulus cells

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Current affiliation for O.G.-K.: Department of Obstetrics and Gynecology, University of South Florida, Tampa, Florida. Current affiliation for A.U.: The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut.

C.K. and O.G.-K. should be considered similar in author order.

Reprint requests: Emre Seli, M.D., 310 Cedar St, L50G 304B, New Haven, Connecticut 06520-8063 (E-mail: [emre.seli@yale.edu](mailto:emre.seli@yale.edu)).

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The process of oocyte maturation and ovulation is a tightly regulated event requiring the rapid, yet highly ordered, phenotypic transformation of multiple cell types within the developing follicle. A thorough understanding of this process is particularly important for the successful treatment of infertility, which may require controlled ovarian hyperstimulation (COH) and in vitro fertilization

(IVF). The goal of COH is the recruitment of multiple follicles that may yield fertilizable oocytes of good quality, thus allowing for optimal early embryo development and successful embryo transfer (1). Individual ovarian response to COH varies widely, ranging from a poor to a strong response to gonadotropins (2); a poor response to ovarian stimulation typically results in fewer retrieved oocytes (3).

Opinions differ widely as to the exact definition of a poor ovarian response (POR). In a consensus statement from the European Society of Human Reproduction and Embryology, 24 previous definitions were cited, with the authors concluding that  $\geq 2$  of the following 3 features must be present: (1) advanced maternal age ( $\geq 40$  years) or any other risk factor for POR; (2) a previous POR (defined as  $\leq 3$  oocytes, using a conventional stimulation protocol); or (3) an abnormal ovarian reserve test (i.e., antral follicle count  $< 5$ – $7$  follicles or antimüllerian hormone  $< 0.5$ – $1.1$  ng/ml). Alternatively, 2 episodes of POR after maximal stimulation were considered sufficient to define a patient as having POR in the absence of advanced maternal age or abnormal ovarian reserve testing (3). Another classification defines poor responders as those patients who produce fewer oocytes than 25% of women in their respective age group (2).

In any case, the molecular mechanisms responsible for a so-called “poor response” to COH remain largely unknown. Several germline-specific transcriptional changes that occur during the process of oocyte maturation have been identified (4, 5), and we are just beginning to understand the mechanisms by which posttranscriptional alterations in gene expression can facilitate oocyte development. One such process is via changes in micro ribonucleic acid (miRNA) expression, and gonadal-selective miRNAs may play important roles in ovarian development and female fertility.

MicroRNAs are a large family of short, noncoding RNAs that repress expression of target genes via degradation of target messenger RNAs (mRNAs) or inhibition of their translation by interactions with the 3'-untranslated regions (3'-UTRs). MicroRNAs regulate essential cellular processes, including growth, differentiation, and apoptosis. They are therefore required for normal mammalian development (6), and their function is conserved across evolution, from yeast to mammals (7–11).

MicroRNAs are processed from precursor molecules (which correspond to either transcripts of independent miRNA genes or the introns of protein-coding mRNAs) and are subsequently matured in a 2-step process. First, in the nucleus, Drosha catalyzes the processing of primary miRNAs to approximately 70-nucleotide pre-miRNAs; then, these pre-miRNAs are transported into the cytoplasm, where they are cleaved by the ribonuclease III endonuclease Dicer. Afterward, 1 strand of the resulting approximately 21-base pair miRNA duplex is preferentially incorporated into the RNA-induced silencing complex. These single-stranded miRNAs harbor a 7-nucleotide seed sequence that mediates suppression of target transcripts by binding to partially complementary sequences within the 3'-UTR (6).

MicroRNAs seem to play a role in granulosa cell (GC) function and thus may be important for follicular signaling

and oocyte development. Carletti et al. (12) and Fiedler et al. (13) demonstrated that 212 known miRNAs are expressed in mouse mural GCs. Of these miRNAs, 13 were regulated by the luteinizing hormone (LH) surge, with 3 in particular (miR-21, miR-132, and miR-212) being highly up-regulated by the LH surge. Additionally, miR-21 increases in vivo in response to human chorionic gonadotropin (hCG) and has been implicated as an antiapoptotic factor in mouse GCs (12).

In addition, disruption of miRNA processing seems to result in altered ovarian morphology and gene expression. Lei et al. (14) generated a GC-specific Dicer knockout and observed accelerated early follicular recruitment and increased degeneration, as well as alterations in genes involved in follicular development, such as *Amh1*, *Cyp17a1*, and *Cyp19a1*. These studies lend weight to the potential role for miRNAs in follicular development and function.

As miRNAs seem to be important mediators of differentiation, proliferation, and apoptotic events in GCs, we hypothesized that an altered response to gonadotropin stimulation in women with infertility may be associated with altered miRNA expression in somatic follicular cells. We therefore sought to determine whether expression of miRNAs in cumulus cells is altered in women with infertility who demonstrate poor response to COH-IVF.

## MATERIALS AND METHODS

### Stimulation Protocols and Collection of Cumulus Cells

Pooled cumulus cells and clinical data were collected from a total of 189 consecutive cycles in women undergoing infertility treatment with IVF and intracytoplasmic sperm injection (ICSI) at Gazi University School of Medicine IVF Center (Ankara, Turkey) between January 22, 2010 and October 31, 2011. Only 1 cycle from each patient was included in the study. Causes of infertility included diminished ovarian reserve, male factor, tubal factor, anovulation, endometriosis, and unexplained infertility, or a combination of these factors. This study was approved by the Gazi University Institutional Review Board committee.

For patients undergoing agonist cycles, treatment was initiated by pituitary suppression with gonadotropin releasing hormone (GnRH)-agonists during the luteal phase of the preceding cycle. Stimulation with gonadotropins was initiated only after down-regulation had been achieved (estradiol [ $E_2$ ] level  $< 50$  pg/ml in the absence of ovarian cysts on transvaginal sonography). For patients undergoing antagonist cycles, treatment with gonadotropins was initiated on cycle-day 3 when serum progesterone was  $< 1$  ng/ml and transvaginal sonography confirmed absence of ovarian cysts. A GnRH-antagonist was added for pituitary suppression after 5 days of gonadotropin stimulation.

Stimulation protocols included 150–300 international units (IU) per day of gonadotropins, either recombinant (Gonal-f, Merck Serono) or in combination with human menopausal gonadotropin (hMG; Menopur, Ferring Pharmaceuticals, Inc). The use of an agonist or antagonist protocol for controlled ovarian stimulation (COS) was decided based on physician

preference. Both protocols were used in women predicted to be non-poor or poor responders. Patients received hCG (250 ug; Ovidrel, Merck Serono) when  $\geq 2$  follicles  $> 18$  mm in diameter were present with an adequate  $E_2$  response. Oocytes were collected 36 hours after hCG injection.

Percentiles of the number of retrieved oocytes were determined separately for 4 age groups within the study population ( $< 35$  years, 35–37 years, 38–40 years, and  $> 40$  years). The number of oocytes included in the first quartile, which corresponds to percentile values  $< 25\%$ , was considered an indication of poor response to COS.

Retrieved cumulus-oocyte complexes were placed in culture medium (G-MOPS, VitroLife), and cumulus cells were dissected from the oocyte mechanically in the absence of hyaluronidase. Cumulus cells from each patient were pooled in a single Eppendorf tube (Denville Scientific Inc.). Samples were washed twice with 0.5 ml of 1X phosphate-buffered saline (PBS) and centrifuged at  $1,000 \times g$  for 1 minute, with the supernatant removed after each wash. After the final wash, the cell pellet was resuspended in 50  $\mu$ l of SideStep Lysis and Stabilization Buffer (Stratagene). Samples were stored at  $-80^\circ\text{C}$  until analysis.

### MicroRNA Extraction and Microarray Analysis

To purify miRNAs from cumulus or human granulosa-like tumor (KGN) cells, the Qiagen miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit were used according to the manufacturer instructions. MicroRNAs extracted from pooled cumulus-cell samples of 3 poor-responder and 3 non-poor-responder patients were sent to Keck Biotechnology Resource Laboratory at Yale University for microarray analysis using Affymetrix GeneChip miRNA 2.0 Arrays. Sample labeling and hybridization were performed per manufacturer instructions.

The probe intensities were extracted from microarray scan images, and raw data were uploaded to the MATLAB programming environment (R2011b; MathWorks) in .CEL format for background correction, normalization, and probe-set summarization, according to the Robust Multichip Average algorithm (15). The relative expressions of miRNAs in cumulus cells were compared across poor and non-poor responders. Differential expression was evaluated in terms of both statistical significance and biological significance. Probe sets with a fold change of  $> |1.5|$  and a  $P$  value of  $< .05$  were considered differentially expressed.

### Reverse Transcription and Real-Time Polymerase Chain Reaction

To synthesize cDNA (complementary deoxyribonucleic acid), reverse transcription (RT) master mix was prepared using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer protocol. In brief, 5  $\mu$ l of isolated total small RNAs was combined with 7  $\mu$ l of RT master mix, including RT buffer, deoxynucleotide triphosphates (dNTPs), MultiScribe RT (Life Technologies), and RNase inhibitor. Reverse transcription primer specific for each miRNA was added to a total volume of 15  $\mu$ l. The reaction was incubated on ice for 5 minutes, followed by RT in the

C1000 Touch (Bio-Rad) thermal cycler with the following parameters:  $16^\circ\text{C}$  for 30 minutes;  $42^\circ\text{C}$  for 30 minutes;  $85^\circ\text{C}$  for 5 minutes.

Real-time polymerase chain reaction (qPCR) was performed using the TaqMan MicroRNA Assay Kits for miR-21-3p, miR-21-5p, let7-f, miR150, and RNU-43 (Applied Biosystems) according to manufacturer protocol. In brief, 10  $\mu$ l of TaqMan 2x Universal PCR master mix was combined with 7.67  $\mu$ l of nuclease-free water, 1  $\mu$ l of 20x TaqMan MicroRNA Assay Mix, and 1.33  $\mu$ l of RT reaction in the PCR reaction tube. Amplification was carried out using 40 cycles of PCR in CFX96 Touch Real-Time PCR Detection System (Bio-Rad), with the following program: initial denaturation at  $95^\circ\text{C}$  for 10 minutes, followed by 40 cycles at  $95^\circ\text{C}$  for 15 seconds, and  $60^\circ\text{C}$  for 60 seconds. All reactions were run in triplicate. Expression of the target microRNAs was normalized to RNU-43 levels to ensure the testing of an equal amount of miRNAs from the various samples and groups, and the  $2^{-\Delta\Delta\text{Ct}}$  method was used to calculate relative expression levels. Results were reported as a fold change in gene expression between groups.

### In Vitro Studies

To determine the effect of  $E_2$  on miRNA expression, we performed in vitro experiments using the KGN cell line. These cells were obtained from Maria Lalioti, Ph.D., and cultured in  $\alpha$ MEM (1:1 v/v; Sigma-Aldrich) containing 10% fetal bovine serum (10% v/v; Gibco/BRL); penicillin (100 U/ml); and streptomycin (200  $\mu$ g/ml), in a standard 95% air: 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ . When they reached confluence, cells were passaged with Trypsin-EDTA (0.25%; Sigma-Aldrich) and incubated in serum-free, phenol red-free media (Sigma-Aldrich) for 24 hours before treatment with vehicle ( $E_2$ , at  $10^{-8}$  M or  $10^{-7}$  M; Sigma-Aldrich), for 6 hours. After treatments, media were removed, and plates were rinsed in PBS and stored at  $-80^\circ\text{C}$  until analysis. To measure expression of miRNAs, cells were lysed with QIAzol Lysis Reagent (Qiagen), and miRNA extraction and qRT-PCR were performed, as described in the preceding section.

### Statistical Analysis

Statistical analyses were performed using MATLAB (R2011b; MathWorks), GraphPad Prism version 6.00 for Windows (GraphPad Software), and SigmaStat version 3.0 (Systat Software, Inc). For clinical parameters or qRT-PCR results, parametric tests ( $t$  test or 1-way ANOVA), followed by Dunnett's multiple comparisons test, were used, as appropriate. When values did not follow a Gaussian distribution, owing to small sample size, logarithmic transformation of data was performed to approach normality before statistical analysis.

## RESULTS

### Clinical Characteristics

The clinical characteristics of the study population are represented in Table 1, by age group and response levels in terms of number of cycles, number of oocytes retrieved, amount of

TABLE 1

Clinical characteristics of study population represented by age groups and ovarian response levels.

Characteristic	< 35 (y)		35–37 (y)		38–40 (y)		> 40 (y)	
	Poor	Non-poor	Poor	Non-poor	Poor	Non-poor	Poor	Non-poor
Response level								
Cycles, n <sup>a</sup>	37	98	7	17	5	14	2	9
Retrieved oocytes <sup>b</sup>	5.8 ± 0.2	16.8 ± 0.5	4.4 ± 0.4	13.9 ± 0.9	2.6 ± 0.3	11.1 ± 1.2	1.5 ± 0.2	9.0 ± 1.6
Total FSH administered (IU)	2,984 ± 128	2,694 ± 97	3,439 ± 391	2,827 ± 194	3,660 ± 501	3,130 ± 278	4,000 ± 0	3,094 ± 425
E2 value on the day of hCG (pg/ml)	1,598 ± 161 <sup>c</sup>	2,205 ± 122 <sup>c</sup>	1,017 ± 176	2,222 ± 533	1,076 ± 170	1,643 ± 355	750 ± 249	1,735 ± 608

Note: Values are presented as mean ± SEM, unless specified otherwise.

<sup>a</sup> Only one cycle from each patient was included in the study.

<sup>b</sup> The number of retrieved oocytes is significantly different among the poor and non-poor responder patients within the <35 y, 35–37 y, and >40 y age groups. The difference between the oocyte numbers was not significant in >40 y age group ( $P = .063$ ).

<sup>c</sup> Significantly different from each other ( $P < .01$ ).

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administered follicle-stimulating hormone (FSH), and the E<sub>2</sub> value on the day of hCG injection (Table 1). A cutoff value for the number of retrieved oocytes was calculated for each age group, to define POR. These values correspond to first-quartile points and were determined as 8, 6, 4, and 3 for age groups of <35 years, 35–37 years, 38–40 years, and >40 years, respectively. The patients who had fewer oocytes than the cutoff values were classified as poor responders.

No statistical difference was found in the amount of total gonadotropin used for COH for poor responders, compared to non-poor responders in any of the age groups, although there was a trend toward higher doses for poor responders (Table 1). Serum E<sub>2</sub> levels on the day of hCG injection were lower in poor responders; however, the difference was only statistically significant in the group with those aged <35 years (Table 1). As the response levels were defined according to oocyte numbers, the number of retrieved oocytes was significantly different between the poor and non-poor responders within the age groups of <35 years, 35–37 years, and 38–40 years. Surprisingly, the difference was not significant in the >40 years age group, likely owing to a low number of samples with a high variation of oocyte numbers in poor and non-poor responders.

### Poor Ovarian Response Is Associated With Differential MicroRNA Expression in Cumulus Cells

We first determined whether poor responders differ from non-poor responders in cumulus-cell miRNA expression. MicroRNAs were extracted from cumulus-cell samples of 6 individual patients, including 3 poor and 3 non-poor responders, and analyzed using Affymetrix GeneChip miRNA 2.0 Arrays. Supplemental Table 1 (available online) represents clinical characteristics of the patients whose cumulus-cell samples were used for microarray analysis.

We applied principal component analysis (PCA) to the expression values obtained from microarray analysis of cumulus cells. Samples from both poor and non-poor-responder groups are illustrated in Supplemental Figure 1 (available online) in a 3-dimensional plot across the first 3 principal components, accounting for 88.2% of the total variance in the data. The PCA plot shows a separation between the miRNA expression profiles of poor and non-poor-responder

groups and high within-group variations (Supplemental Fig. 1). The variation of overall miRNA expressions between poor-responder samples is higher, compared with the non-poor-responder samples.

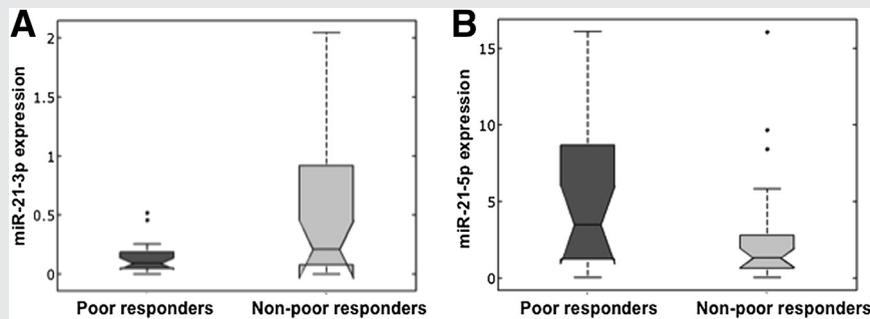
Differential expression analysis revealed up-regulation of 16 miRNAs (Supplemental Table 2, available online) and down-regulation of 88 miRNAs (Supplemental Table 3, available online) in poor responders, compared with non-poor responders. The statistical and biological significance of the differences of expression values between groups are additionally shown as a volcano plot (Supplemental Fig. 2, available online). Notably, miR-21 was significantly up-regulated five-fold in poor-responder samples ( $P = .03$ ), with the highest fold-change among all the up-regulated miRNAs (Supplemental Table 2).

### The MicroRNA miR-21 5p Is Elevated in the Cumulus Cells of Poor Responders

Microarray analysis identified miR-21 as an miRNA that is significantly elevated in cumulus cells of women with poor response. As miR-21 was previously implicated as a regulator of GC function in mouse, we further investigated miR-21 in human cells. First, we tested cumulus-cell samples from poor ( $n = 21$ ) and non-poor ( $n = 29$ ) responders for miR-21-5p (active strand of miR-21) expression, using qRT-PCR. Although the causes of infertility for patients involved in the study included diminished ovarian reserve, male factor, tubal factor, anovulation, endometriosis, and unexplained infertility, validation studies were performed in non-poor age-matched responders who had tubal factor or male factor infertility and poor response. We found miR-21-5p expression to be significantly up-regulated in poor responders ( $P < .05$ ) (Fig. 1).

The stem-loop precursor of miR-21 (pre-miR-21) contains both miR-21-5p (the active form implicated for most biological functions, with the sequence 5'-uagcuuaucaugacugauguuga-3'), and miR-21-3p (the complementary sequence in the closed loop configuration with the sequence 5'-aacacagucgagggcugu-3') (16). We therefore asked whether the altered miR-21 expression affects the pre-miR-21 with resulting parallel changes in cumulus-cell miR-21-5p and miR-21-3p levels, or whether the effect is specific for

FIGURE 1



The distributions of miR-21 expression according to ovarian response are shown as box plots. (A) miR-21-3p expression is significantly lower in the poor-responder group ( $P < .05$ ). (B) miR-21-5p expression is significantly higher in the poor-responder group ( $P < .01$ ). The shaded area and the error bars represent 75% and 95%, respectively.

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miR-21-5p. Using qRT-PCR in the same samples, we found miR-21-3p expression to be significantly lower in poor responders ( $P = .003$ ) (Fig. 1), suggesting that elevated miR-21-5p expression in cumulus cells is not regulated at the pre-miR-21 level in poor responders.

To validate our microarray results, we tested 2 additional miRNAs: let7-f and miR-150. Consistent with microarray findings, miR-150 was significantly down-regulated in poor responders ( $P < .05$ ), and let7-f was significantly up-regulated ( $P < .05$ ) (Fig. 2).

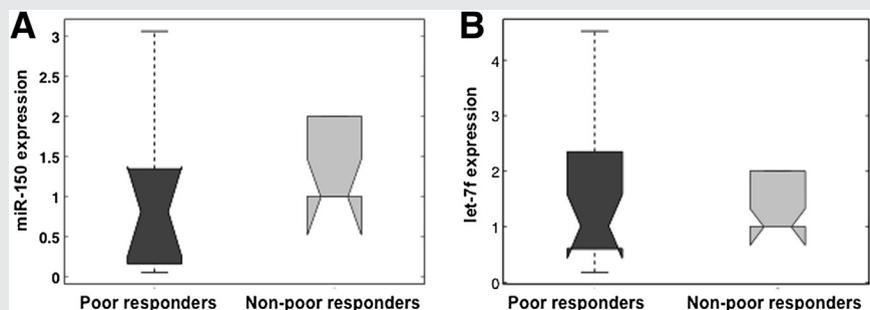
### Altered miR-21 Expression in Poor Responders Is Not Associated With Maternal Age in the Assessed Patient Population

Clinical characteristics of the patients used for qPCR validation of miR-21 expression are given in Supplemental Table 4 (available online). No significant difference was found in the age, total FSH administered, or  $E_2$  value on the day of hCG injection, for poor vs. non-poor responders.

The only difference observed was in number of retrieved oocytes, as the separation of poor and non-poor responders was based on the oocyte numbers in the entire study population.

In the PCA graph (Supplemental Fig. 1), 1 of each of the poor-responder and non-poor -responder samples seems slightly different from the other 2 samples from the same group. The samples that are located relatively apart in the PC space belong to a 24-year-old poor responder and a 25-year-old non-poor responder (Supplemental Table 1). As both patients are the youngest of the 3 patients in their respective groups, we investigated the association between miR-21 expression and maternal age. The entire qPCR cohort was divided into 2 age groups: patient age  $< 35$  years ( $n = 30$ ) and patients age  $\geq 35$  years ( $n = 20$ ). Expression of miR-21-5p and miR-2-3p were compared among the age groups, and no significant difference was found. This result indicates a lack of relationship between age and miR-21 expression in the study population (Supplemental Fig. 3, available online).

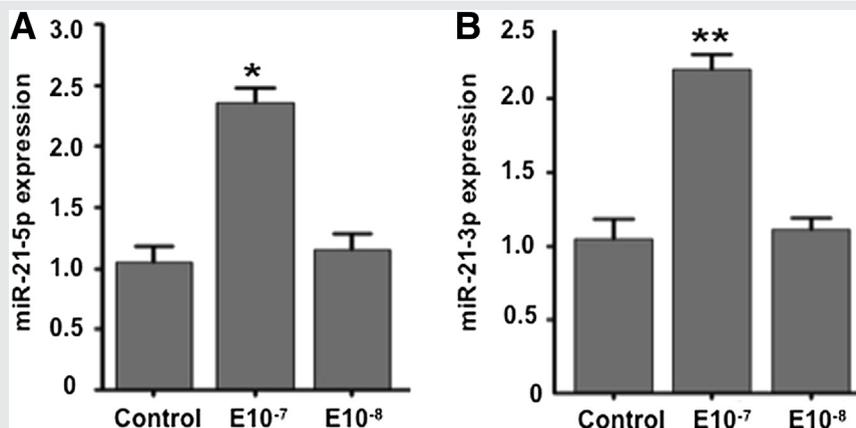
FIGURE 2



The distributions of miR-150 and let7-f expression, according to ovarian response, are shown as box plots. (A) Expression of miR-150 is significantly lower in the poor-responder group ( $P < .05$ ). (B) Expression of let7-f is significantly higher in the poor-responder group ( $P < .05$ ). The shaded area and the error bars represent 75%, and 95%, respectively.

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FIGURE 3



Expression of miR-21 in KGN cells in response to E<sub>2</sub> treatment. The KGN cells were treated with 10<sup>-7</sup> and 10<sup>-8</sup> M of E<sub>2</sub>, respectively. Raw threshold cycle (Ct) values were normalized to RNU43 housekeeping snoRNA (small nucleolar RNA), and relative expression was determined using the 2<sup>-ΔΔCt</sup> method. The fold-change, compared with controls treated with media alone, was determined. One-way ANOVA was used to determine statistical differences between groups. Expression of both miR-21-5p (A) and miR-21-3p (B) increased significantly in response to treatment with 10<sup>-7</sup> M of E<sub>2</sub>. The results represent mean ± SD; \*P<.05; \*\*P<.001.

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### Elevated miR-21 Expression in Poor Responders Is Not Likely to Be a Result of Decreased Serum Estradiol Levels in These Women

As women with poor response had lower serum E<sub>2</sub> levels, and miR-21 expression has previously been shown to be down-regulated by E<sub>2</sub> in MCF-7 cells, we tested whether E<sub>2</sub> affects miR-21-5p or miR-21-3p expression in KGN cells in culture. We found that both miR-21-5p and miR-21-3p are increased in KGN cells in response to higher doses of E<sub>2</sub> (P<.05), whereas their expression is not affected at lower E<sub>2</sub> concentrations (Fig. 3). Although KGN cells and cumulus cells cannot conclusively be said to respond similarly to E<sub>2</sub>, the cell line provides a useful mechanism for studying patterns of miRNA regulation, and these findings suggest that the elevated miR-21-5p expression in cumulus cells of poor responders may be independent of lower serum E<sub>2</sub> levels in these women.

### DISCUSSION

Our understanding of the regulation of follicular development and oocyte maturation by miRNAs is rapidly evolving. We now know, for example, that miRNAs are differentially expressed in MII oocytes and cumulus cells, including those predicted to regulate genes that are important for cumulus-oocyte communication (17). Additionally, miRNA expression profiles in GCs are associated with the maturity of adjacent oocytes, and modulation of these profiles may regulate oocyte maturation (18). Data are sparse, however, on miRNA expression patterns in “poor responder” women, who demonstrate a subpar response to COH. Such knowledge has the potential to further the development of therapeutic interventions in fertility treatment.

In this study, we demonstrate a significant difference between women with poor and normal responses to COH in

terms of miRNA expression. In women with a poor response to COH-IVF, 88 miRNAs are down-regulated, and 16 miRNAs are up-regulated. Specifically, we found that the expression of miR-21, which is highly up-regulated by the LH surge (12), is increased in poor responders. We confirmed our findings in a large number of samples, using qRT-PCR, and determined that the active form of miR-21—miR-21-5p—is specifically elevated in cumulus cells of women with poor response, and that this increase is not likely to be mediated by the lower serum E<sub>2</sub> levels observed in poor responders.

A thorough understanding of the genetic and molecular basis of POR is imperative for designing successful therapies. Many autosomal genes identified in animal and human models have been implicated as possible contributors to premature ovarian failure (which is, in turn, associated with the early onset of POR). These genes include *Fshr*, *Fmr1*, *Foxl2*, *Foxo3a*, *Gdf9*, *Nobox*, and *Oct4*, among others (19). With respect to POR in particular, the most well-studied potential biomarker seems to be the FSH receptor. Variants in the receptor itself, as well as abnormal signaling pathways, have been implicated in several forms of infertility (2, 19). Other polymorphisms under investigation include *Esr1* and *Esr2* (20). However, although progress has been made, much remains to be learned regarding the molecular pathogenesis of POR.

Our findings, which demonstrate differential expression of miR-21 in poor vs. non-poor responders, add to a growing body of literature exploring the role of miRNAs in reproductive function. Hong et al. (21) generated mice with a targeted deletion of *Dicer* (which is required for miRNA and small interfering RNA synthesis) in GCs. These mice demonstrated decreased ovarian weight, fewer large antral follicles, and decreased ovulation rates (22).

Conversely, Nagaraja et al. (6) demonstrated normal folliculogenesis with increased follicular atresia in female mice, and ultimate sterility, after targeted Dicer deletion in somatic cells of the female reproductive tract. In another mouse model with 80% reduction in Dicer expression, female mice had normal ovulation rates, but corpora lutea function was reduced (as evidenced by decreased progesterone production), and the mice were unable to sustain pregnancies (23). In any case, disruption of proper miRNA synthesis is likely to lead to dysfunctional reproductive outcomes. In addition, DICER1 mutations have been identified in ovarian Sertoli-Leydig cell tumors in women (24), and double conditional knockout of *Dicer* and *Pten* (a key negative regulator of the PI3K pathway) resulted in the development of high-grade serous ovarian cancer in a mouse model (25).

MicroRNA-21 is a particularly interesting target of study because it is highly up-regulated by the hCG and LH surge, and may function as an antiapoptotic factor in GCs (12). Mir-21 is encoded by the Mir21 gene; it was one of the first mammalian miRNAs identified, and the mature sequence is strongly conserved throughout evolution. The human miRNA-21 gene is located on the plus strand of chromosome 17q23.2 within coding gene *TMEM49* (also known as vacuole membrane protein).

Despite its location within intronic regions of a coding gene in the direction of transcription, the gene has its own promoter regions (MiRBase). Within the approximately 3,433-nucleotide-long, independently transcribed primary miR-21 transcript (pri-miR-21) resides a miR-21 RNA stem-loop precursor (pre-miR-21) (16). This precursor, found between nucleotides 2,445 and 2,516, contains both miR21-5p (the active form, derived from the 5' arm of the precursor miRNA, which is implicated in most biological functions) and miR21-3p (the complimentary sequence in the closed loop configuration, derived from the 3' arm) (26). In addition to its roles in reproductive function, aberrant miR-21 expression has been implicated in multiple other disease states, including cancer (expression of miR-21 has been found to be deregulated in nearly all cancer types) and cardiovascular disease (22).

In our study, miR-21-5p was elevated in cumulus cells of poor responders, whereas miR-21-3p was, in fact, significantly decreased, suggesting that the increase in miR-21-5p expression is not regulated at the pre-mir-21 level in these poor-responder patients. Additionally, we found that both miR-21-5p and miR-21-3p are increased in a GC line in response to higher doses of E<sub>2</sub> (while expression was not affected at lower E<sub>2</sub> concentrations), suggesting that altered miR-21 expression in poor responders is not a result of decreased serum E<sub>2</sub> levels in these women. The use of the KGN GC line in these experiments provided a readily accessible system for performing mechanistic experiments to better characterize miR-21 regulation.

Our findings on the effect of E<sub>2</sub> on miR-21 expression differ from those of Wickramasinghe et al. (27), who showed that E<sub>2</sub> repressed the expression of miR-21 by activating the estrogen receptor in MCF-7 cells (27). However, these differences could be explained by cell type-specific effects of E<sub>2</sub>

on miR-21. Perhaps E<sub>2</sub> has both agonist and antagonist effects on miR-21 expression and exerts these effects via both estrogen receptor-dependent and -independent mechanisms.

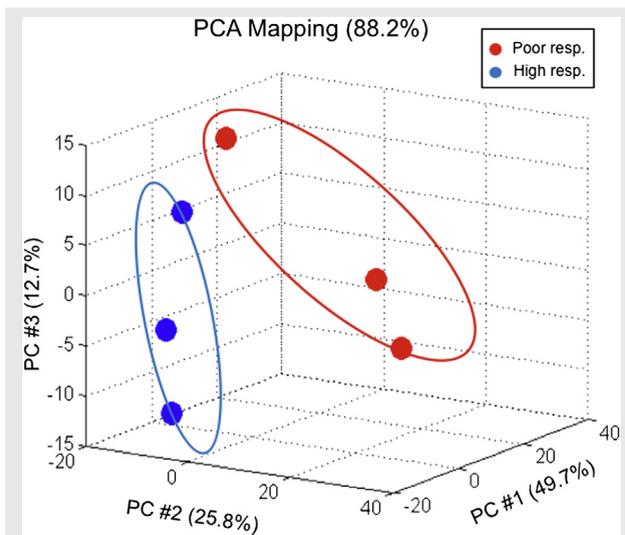
In this study, we identified a list of miRNAs with altered expression in the cumulus cells of women with a poor response to COH-IVF. In addition, we identified miR-21-5p as a specific miRNA that is altered in poor responders. Our findings suggest a role for miRNAs in human cumulus-cell function and potentially in the pathogenesis of POR in women undergoing infertility treatment with COH-IVF. Potential target mRNAs that might be regulated by miR-21 in human cumulus cells and GCs remain to be identified. In addition, other miRNAs identified as differentially expressed in women with poor response to IVF remain to be validated and characterized.

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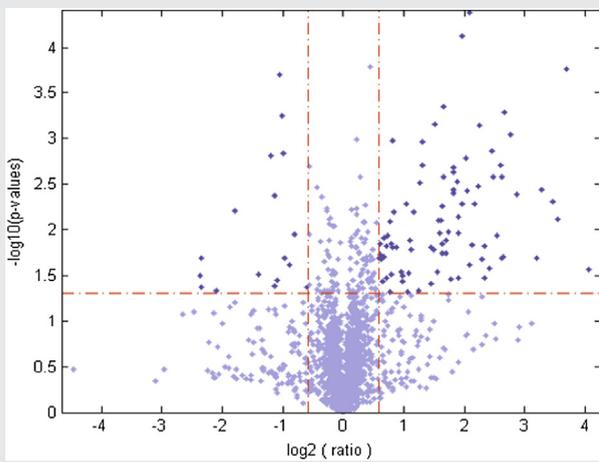
## SUPPLEMENTAL FIGURE 1



Principal component analysis (PCA) of miRNA expression in cumulus cells of poor-responder (red circles) and non-poor-responder samples (blue circles) is shown. The percentages of variability represented by the first 3 principal components are displayed across PC #1, #2, and #3 on the x, y, and z axes, respectively. resp. = responder.

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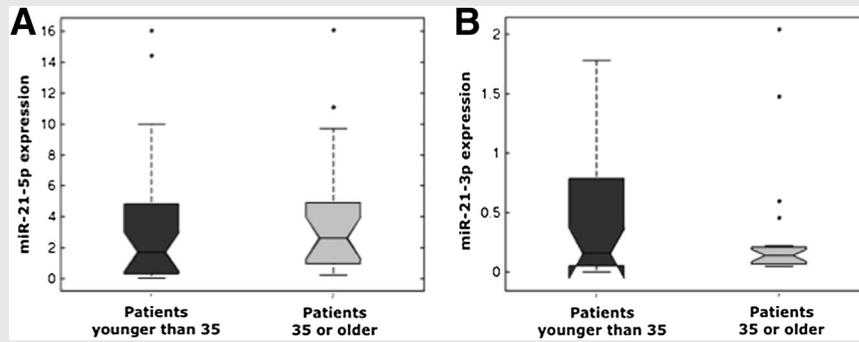
## SUPPLEMENTAL FIGURE 2



A volcano plot of microarray results highlights differentially expressed miRNAs. The horizontal axis represents the biological significance as  $\log_2$  (ratio), with ratio being the expression fold-change between sample groups. The vertical axis,  $(-\log_{10}[\text{p-values}])$ , denotes statistical significance. Significantly up- and down-regulated miRNAs are shown in the upper-right and upper-left regions of the plot, respectively. Overall, 16 miRNAs (including miR-21) are up-regulated, and 88 miRNAs are down-regulated in poor responders, with respect to non-poor responders.

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## SUPPLEMENTAL FIGURE 3



The box plot representation of miR-21 expression levels investigated in 2 groups, by maternal age (given in years): patient age <35 years, and patient age  $\geq$ 35 years. (A) Expression of miR-21-5p shows quite similar distributions in the younger and older age groups; no statistically significant difference was found between the groups ( $P=.639$ ). (B) The difference between miR-21-3p expressions for the 2 groups was not significant ( $P=.543$ ).

Karakaya. Poor ovarian response and microRNA. *Fertil Steril* 2015.