Abstract

Background: Recent animal studies demonstrated that regulating the microRNA (miRNA) in granulosa cells (GCs) modulates the meiotic competence of oocytes. However, the difference in expression profiles of miRNAs in human GCs according to the maturity of the oocyte still remains to be elucidated.

Objective: This observational study investigated whether the miRNA profile of human GCs differs according to the maturity of the retrieved oocyte after controlled ovarian stimulation for in vitro fertilization (IVF).

Methods: Ten women who underwent ovarian stimulation cycles with GnRH agonist long protocols were recruited. The follicular fluid (FF) from the FF and miRNA was analyzed using real-time PCR.

Results: Mean number of MII oocytes in the mature group was 1.6 ± 0.9 with none in the immature group (p = 0.008). The total number of retrieved oocytes was 8.8 ± 1.9 in the mature group and 2.0 ± 1.2 in the immature group (p = 0.008). The GCs of the mature group showed a significantly lower expression of hsa-let-7b compared to the GCs of the immature group (p < 0.001).

Conclusion: Taken together, the miRNA expression profiles of human GCs obtained from dominant follicles are associated with maturity of the adjacent oocyte and may be useful as a prognosticator of IVF outcome.
Introduction

Although outcomes after in vitro maturation (IVM) have improved in recent years and are now clinically acceptable, obtaining meiotically competent oocytes (metaphase II, MII) is an essential precondition for optimal in vitro fertilization (IVF). The meiotic competence of oocytes depends on the potential of the respective follicles which support the oocytes’ maturation [1,2]. During the process of oocyte development, ovulation and fertilization, each oocyte is usually surrounded by several layers of granulosa cells (GCs). GCs play an important role as mediators of follicular potential by communicating with oocytes via paracrine signals [3,4]. During ovarian stimulation in IVF cycles, many genes expressed on GCs have been described as being controlled by both maternal and oocyte-derived signals, and it has been suggested that GCs may provide information about the maturation competence of their associated oocytes [5–7].

MicroRNAs (miRNAs) are endogenously produced non-coding RNA molecules that post-transcriptionally regulate gene expression by inhibiting the translation or cleaving of complementary target messenger RNAs (mRNAs) by binding to 3′ untranslated regions (UTR) [8,9]. Since the discovery of miRNA (lin-4) in Caenorhabditis elegans [10], miRNAs have been shown to play an important role in cellular signaling, apoptosis, metabolism, development and organogenesis [11–15]. Our recent study demonstrated that regulating miRNA in GCs can modulate the meiotic competence of oocytes during in vitro maturation in a mouse follicles model [16]. The results implied that oocyte maturation could be influenced by the miRNA profile of adjacent GCs. However, differences in the expression profiles of miRNAs in human GCs according to the maturity of their associated oocytes still remain to be elucidated.

This study aimed to evaluate the miRNA expression profiles of human GCs and to detect differences between the miRNA expression of GCs of human MII oocytes and that of MI oocytes using human GCs and to detect differences between the miRNA expression profiles of GCs of human MII and MI oocytes. How- ever, differences in the expression profiles of miRNAs in human GCs according to the maturity of their associated oocytes still remain to be elucidated.

This study aimed to evaluate the miRNA expression profiles of human GCs and to detect differences between the miRNA expression of GCs of human MII oocytes and that of MI oocytes using follicular fluid (FF) obtained during oocyte retrieval in IVF cycles.

Materials and Methods

The subjects

Ten women who underwent ovarian stimulation cycles with GnRH agonist long protocols were recruited. All subjects presented with unexplained infertility. As a first step, pituitary down-regulation was initiated by the injection of triptorelin (decapet- tyl depot, 1.88 mg; Ferring, Sweden) from the mid-luteal phase of the previous cycle. For controlled ovarian stimulation, administration of 150–300 IU of FSH (Gonal-F; Serono, Geneva, Switzerland), depending on the expected response, was started on day 3 of the menstrual cycle and continued until the leading follicle had a mean diameter of 17 mm. Recombinant human chorionic gonadotropin (hCG) (Ovidrel, Serono, Geneva, Switzerland) was injected subcutaneously 36 hours before transvaginal oocyte retrieval. No serious complications were reported during ovarian stimulation or after oocyte retrieval.

FF was individually aspirated in each patient during oocyte retrieval from the first accessible dominant follicle. The maturation of the retrieved oocytes was recorded for each FF sample. After centrifugation of FF, human GCs were collected from five patients who had more than one MII oocyte; they were classified as the “mature group”. GCs were also collected from five patients who did not have MII oocytes; they were classified as the “immature group”. Immature oocytes were fertilized after in vitro maturation (IVM). This study was approved by the institutional review board.

Collecting granulosa cells from aspirated follicular fluid

After cumulus-oocyte complexes were collected for IVF, 20–50 mL of the remaining FF was centrifuged at 950 g for 15 minutes at room temperature. To remove most of the red blood cells, the pellet was centrifuged at 400 g for 20 min on a single-layer discontinuous Percoll gradient (50% Ham’s F12 medium, Gibco-BRL; Life Technologies, Cergy-Pontoise, France). The 50% fraction was collected and washed twice with 20 mL of phosphate buffered saline to remove the remaining red blood cells. Following centrifugation, the pellet was washed with fresh medium (Ham’s F12). The final pellet of GCs was frozen at −80°C until RNA extraction.

RNA extraction and real-time PCR for microRNA

TRizol® (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from the individual frozen pellets of GCs. Poly(A) tailing and complementary DNA (cDNA) synthesis for total miRNAs were performed with 100 pg of total RNA using a commercial kit (NCode Vilo miRNA cDNA Synthesis Kit, Invitrogen), according to the manufacturer’s protocols (http://www.invitrogen.com) and as carried out in our previous study [16]. Quantification of miRNAs by RT-PCR was done with EXPRESS SYBR GreenER miRNA qRT-PCR Kit, Invitrogen). A two microliter reverse transcription product was used as the template, and miRNA levels were assessed using miRNA-specific PCR primers (1 µL), universal miRNA reverse primers (EXPRESS SYBR GreenER miRNA qRT-PCR Kit; 1 µL), and commercial reagents (EXPRESS SYBR GreenER miRNA qRT-PCR Kit, Invitrogen; 10 µL). The total volume of the mixture for a single reaction was set to 20 µL with DEPC-treated water. According to the web database (http://www.microrna.org), the miRNAs analyzed in this study are expressed in the human ova- ry. All of the human miRNA-specific PCR primers used in this study are listed in Table 1.

Real-time (RT) PCR was done using a Rotor-Gene instrument (Corbett Life Science, Sydney, Australia). Quantitative RT-PCR was carried out with incubation of the 20-µL reaction solution (Roche, Madison, WI, USA) at 95°C for 15 minutes, followed by up to 62 cycles at 95°C for 20 seconds and at 60°C for 40 seconds. Expression levels of microRNAs were normalized to human U6 and the relative expression levels of miRNAs calculated as (cycle threshold value [Ct] of miRNA)/(ct of control RNA) [17].

Statistics
Means and standard deviations were compared using Mann-Whitney U-test for IVF outcomes and Student’s t-test for miRNA expression. Differences were considered statistically significant when the p-value was less than 0.05. All data were analyzed using the Statistical Package for the Social Sciences for Windows software (version 12.0, SPSS Inc., Chicago, IL, USA).

Results

IVF outcomes
The oocyte maturity profile of subjects is shown in Table 2. Mean age was 35.4 ± 1.7 years for women in the mature group and 36.6 ± 2.1 years for women in the immature group. Mean number of MII oocytes in the mature group was 1.6 ± 0.9; no MII were retrieved in the immature group (p = 0.008). Mean number of MI oocytes was 5.6 ± 2.1 in the mature group and 1.0 ± 0.0 in the immature group (p = 0.008). The total number of retrieved oocytes was 8.8 ± 1.9 in the mature group and 2.0 ± 1.2 in the immature group (p = 0.008). The mean number of two pronucleate (2PN) oocytes was 5.8 ± 1.6 in the mature group and 0.6 ± 0.5 in the immature group (p = 0.001). All women in the mature group became pregnant while none of the women in the immature group became pregnant (Table 2).

Comparison of miRNAs from mature and immature oocyte groups
The relative expression of miRNAs normalized to U6 expression in human GCs according to oocyte maturity is shown in Fig. 1. GCs of MII oocytes had significantly lower expression levels of hsa-let-7b compared to GCs of MI oocytes, based on RT-PCR (5.7-fold lower, p < 0.001). Other tested miRNAs showed no significant differences between the two groups (Fig. 1).

Discussion
Recently we reported that the regulation of miRNAs in GCs modulates the meiotic competence of oocytes during in vitro maturation in a mouse follicles model [16]. In this context, the current study sought to investigate whether the expression profiles of miRNAs in human GCs differed according to the maturity of oocytes retrieved during IVF cycles. Our data showed that the GCs of MII oocytes showed lower expression levels of hsa-let-7b compared to GCs of MI oocytes, based on RT-PCR (5.7-fold lower, p < 0.001). Other tested miRNAs showed no significant differences between the two groups (Fig. 1).

FR: fertilization rate

Table 1 List of primers used for real-time PCR of human microRNAs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>qRT-PCR forward</th>
<th>qRT-PCR reverse</th>
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</thead>
<tbody>
<tr>
<td>human U6</td>
<td>CTCGCTTCGGCACGACAA</td>
<td>AACGCTTTCAAGCATATTCCG</td>
</tr>
<tr>
<td>hsa-let-7a</td>
<td>GCGGTTAGGATATGGTTGTATAGT</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-let-7b</td>
<td>CTCGCTTCGGTGTGTTGTGTTG</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-miR-16</td>
<td>TAGCAGCAGCTATAAATTGGCG</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-miR-26a</td>
<td>GATTTCAGCTATCCGATCGCC</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-miR-99a</td>
<td>ACCCGTAGCTCCAGCTCTG</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-miR-125b</td>
<td>TCCTGAGAGCTAAGCTGGA</td>
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</tr>
<tr>
<td>hsa-miR-126</td>
<td>TCGACGACCTGTACATGG</td>
<td>universal reverse primer</td>
</tr>
<tr>
<td>hsa-miR-143</td>
<td>CCTGAGATGAGCGACTCTG</td>
<td>universal reverse primer</td>
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</table>

Table 2 Oocyte maturity profile of subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Number of retrieved oocytes</th>
<th>2PN</th>
<th>FR</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MII</td>
<td>MI</td>
<td>GV</td>
<td>Total</td>
</tr>
<tr>
<td>Mature 1</td>
<td>35</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Mature 2</td>
<td>35</td>
<td>2</td>
<td>6</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td>Mature 3</td>
<td>37</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Mature 4</td>
<td>37</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Mature 5</td>
<td>33</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Immature 1</td>
<td>39</td>
<td>–</td>
<td>1</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Immature 2</td>
<td>37</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Immature 3</td>
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<td>34</td>
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<td>Immature 5</td>
<td>38</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

FR: fertilization rate
oocytes is related to lower ovarian response and whether this effect is the culmination of an insufficient expression of certain miRNAs. Some studies have suggested that the maturation of retrieved oocytes reflects the ovarian response to stimulation [18, 19].

In our results, the expression of miRNA in GCs differed according to the maturity of the oocytes. Let-7b has been reported to play an important role in the oocyte maturation process [20–22]. In previous mouse [16] and human [23] studies, the expression level of let-7b was reported to differ in GCs of MI oocytes compared to GCs of MII oocytes, which is in line with the findings of this study. Although miRNAs were previously shown to influence the meiotic competence of oocytes, further functional studies into the specific target genes of miRNAs are necessary before this strategy can be applied clinically.

Our study has some limitations. Firstly, the number of analyzed miRNAs in this study was small. We selected nine of the most highly expressed miRNAs in the human ovary based on the web database. Our data cannot rule out a possible impact of minor miRNAs expressed in the human ovary on oocyte maturation. Secondly, our data did not include a functional study of target genes of miRNAs such as gain- and loss-of-function. However, investigations which use small amounts of human GCs from aspirated FF have a fundamental limitation with respect to mass evaluations of candidate target genes.

In conclusion, miRNA expression profiles of human GCs from dominant FF are associated with maturity of the adjacent oocyte and can be considered a potential prognosticator of outcome after IVF cycles.

**Funding**

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**Conflict of Interest**

The authors declare they have no existing or potential conflicts of interest.

**References**


**Fig. 1** Relative expression of miRNAs in human GCs normalized to U6 according to oocyte maturity (cycle threshold of miRNA/cycle threshold of U6). The expression of hsa-let-7b in the GCs of MII oocytes was significantly lower compared to that of MI oocytes (27.6 × 10^-2 and 4.8 × 10^-3, respectively; p < 0.001).