ORIGINAL ARTICLE: REPRODUCTIVE ENDOCRINOLOGY

Elevated circulating micro-ribonucleic acid (miRNA)-200b and miRNA-429 levels in anovulatory women

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Objective: To study the role of micro-RNA (miRNA)-200b and miRNA-429 in human ovulation and to measure their expression levels in ovulatory and anovulatory patients.
Design: Micro-RNA-200b and miRNA-429 expression analysis in human serum and granulosa cells at different phases of the ovulation cycle in normal cycling women and women undergoing assisted reproductive technology cycles.
Setting: University-affiliated hospital and academic research laboratory.
Patient(s): Forty women (7 normally ovulating, 15 normally ovulating with pure male infertility factor, and 18 with polycystic ovary syndrome) were included in this study.
Intervention(s): None.
Main Outcome Measure(s): The expression profile of circulating miRNAs and granulosa cells was assessed by means of real-time quantitative reverse transcription–polymerase chain reaction analysis.
Result(s): We identified miRNA-200b and miRNA-429 in the sera of all women tested. These miRNA expression levels were elevated during the early follicular phase of the cycle compared with serum levels during the early luteal phase. Anovulatory women, diagnosed with polycystic ovary syndrome, expressed significantly higher levels of miRNA-200b and miRNA-429 compared with spontaneously ovulating women. Ovulation induction with exogenous gonadotropins during an IVF cycle reduced these levels to the levels measured in normal ovulating women.
Conclusion(s): Our findings suggest an involvement of miRNA-200b and miRNA-429 in the pituitary regulation of human ovulation. Although it is unclear whether this altered miRNA expression profile is a cause or a result of anovulation, the levels of these molecules in the serum of anovulatory women may serve as serum biomarkers for the ovulation process. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: miRNA-200b, miRNA-429, ovulation, PCOS, pituitary, serum levels

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Ovulation of a mature and viable oocyte and the formation of a functional corpus luteum are essential for conception and the establishment of pregnancy. Numerous studies have shown that the LH surge initiates transcriptional up- and down-regulation of genes, including cytokines, transcription factors, and matrix-remodeling proteins within periovulatory granulosa cells (1). However, posttranscriptional gene regulation with respect to ovarian function, outside of oocyte development, has been largely overlooked.

Micro-RNAs (miRNAs) are endogenous small noncoding RNA molecules of approximately 23 nucleotides in length, which are known to form a novel class of regulatory determinants

Received August 2, 2016; revised September 20, 2016; accepted October 4, 2016.
I.E. has nothing to disclose. N.N. has nothing to disclose. M.N.P. has nothing to disclose. C.G. has nothing to disclose. D.G.-W. has nothing to disclose. A.H. has nothing to disclose. R.H.-K. has nothing to disclose. S.Y. has nothing to disclose. T.I. has nothing to disclose.
Supported by the Morasha program of the Israel Science Foundation (grant 1680/10) and by a grant from the 2015 joint research fund of the Hebrew University and Hadassah Medical Center (to T.I.).
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Fertility and Sterility® Vol. ■, No. ■, ■ 2016 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.10.003

of cellular signaling pathways, with a large set of target messenger RNAs (mRNAs) proposed for every single miRNA (2). It has been postulated that modulation of a single miRNA might be an effective way to target entire pathways for clinical benefit (3, 4).

The role of miRNA in follicular signaling and ovulation has been described in various systems, but the specific miRNAs involved and their mechanism of action are yet to be revealed (5–7). Carletti and Christenson (8) demonstrated that 212 known miRNAs are expressed in mouse mural granulosa cells, and of these, 13 were regulated by the LH surge. Additionally, disruption of miRNA processing seems to result in altered ovarian morphology and gene expression (9). A granulosa cell–specific Dicer knockout showed an accelerated early follicular recruitment, increased follicular degeneration, and alterations in genes involved in follicular development, such as *Amh1*, *Cyp17a1*, and *Cyp19a1*. These studies demonstrated the potential role for miRNAs in follicular development and function (9).

A recent murine study demonstrated that miRNA-200b and miRNA-429, members of the miRNA 200 family, strongly affect ovulation in female mice (10). Double knockout mice lacking miRNA-200b and miRNA -429 displayed a defective hypothalamic–pituitary–ovarian axis and failed to ovulate (11). The involvement of the miRNA 200 family in humans has been tied to tumorogenesis, particularly in the epithelial to mesenchymal transition (12, 13). This process is an important component of corpus luteum formation, especially during the intense angiogenesis occurring after the LH signal of ovulation is received (14). Interestingly, when miRNA-200b and miRNA-429 double knockout mice were super-ovulated by injecting pregnant mare serum gonadotropin and hCG, the miRNA-200b and miRNA-429 double knockout females ovulated, similar to control females. This finding suggests that impaired hormonal regulation prevented these mice from ovulating naturally and that ovulation can be activated by exogenous hormones. Among the putative target mRNAs listed, the 3′-untranslated regions of both mouse and human *ZEB1* and *ZEB2* have the highest number of loci that are complementary to the seed sequence of miR-200b and miR-429 (15). Specific knockout of these miRNAs in the mice pituitary increased *ZEB1* mRNA expression (11). In the mouse, ZEB1 protein is known to be a nuclear repressor of the LH gene, leading to inhibition of LH transcription and abolishment of the LH surge, which is vital for ovulation (11, 16). The miR-200b cluster is present in humans, and the upstream regions of human *LHB* gene also contain ZEB1-binding sites (11). These similarities imply that the roles of these miRNAs in maintaining normal ovulation in the mouse may also be applicable to human reproductive physiology.

To investigate the role of these two miRNAs in human ovulation, we analyzed their expression pattern during ovarian stimulation for IVF in anovulatory and ovulatory women, as well as in naturally ovulating women. Because miRNAs could not be measured directly in the human pituitary, we measured their serum levels during the different phases of the ovulatory cycle according to the known stability and resistance to degradation of miRNAs in body fluids (17, 18). We also measured the expression levels of miR-200b and miR-429 in the target organ of the reproductive axis, in freshly isolated granulosa lutein cells (GLCs).

MATERIALS AND METHODS

Subjects

The Hadassah Hebrew University Medical Center Institutional Review Board approved this study (HMO-0110-09), and all subjects gave written informed consent to participate in the study. All women were under 35 years of age. The first group was composed of normally cycling women, with a regular menstrual cycle of 27–30 days, without recent use of hormonal treatment, history of pelvic inflammatory disease, or any other general health problem. Group 2 included normally cycling women undergoing IVF treatment solely because of male factor infertility (MFI). The anovulatory women (group 3) were diagnosed with polycystic ovary syndrome (PCOS) according to the Rotterdam revised criteria (19). These women underwent IVF treatment after failure to conceive with at least four cycles of controlled ovarian stimulation and insemination.

Ovarian Stimulation Protocol

Women undergoing IVF were treated by the short GnRH antagonist protocol. In brief, controlled ovarian stimulation was initiated on day 2 or 3 of a spontaneous cycle. An initial dose of 150–225 IU recombinant FSH (Gonal-F [Merck-Serono] or Puregon [MSD]) or highly purified hMG (Menopur [Ferring Pharmaceuticals]) was administered. From day 6 onward, the gonadotropin dose was estimated according to serum E_2 levels and a transvaginal ultrasound scan. When a leading follicle reached 13–14 mm, a GnRH antagonist (Cetrotide [Merck-Serono] or Orgalutran [MSD]) was administered at 0.25 mg/d. Final oocyte maturation was triggered with the use of 250 μg recombinant hCG (Ovitrelle [Merck-Serono]) as soon as the mean diameters of two follicles were $\geq$18 mm. Oocyte retrieval was scheduled 36 hours after hCG injection. All accessible follicles were harvested, and oocytes were collected from follicular aspirates and subjected to fertilization. The residual follicular fluid aspirates containing GLCs were collected for further investigation.

Specimens

Blood samples were collected at two time points during the ovarian cycle, and serum was aliquoted and immediately stored at −80°C until used. The first sample was collected on day 3 of the cycle, before any gonadotropin treatment was administered, representing the early follicular phase. The second sample was collected at the early luteal phase, around day 14 in the first group, or during ovum pick up in the IVF patient groups. Ovulation was determined in the first group by measurement of serum levels of P and LH. Granulosa lutein cells originating from follicular fluid aspirates were