miR-320a-3p Levels in Human Granulosa Cells: A Promising Biomarker of Good Quality Embryo and Clinical Pregnancy after IVF/ICSI.

Yu Liu
Institute of Reproductive Health and Center for Reproductive Medicine

Qiaojuan Mei
Institute of Reproductive Health and Center for Reproductive Medicine

Qiuzi Shen
Institute of Reproductive Health and Center for Reproductive Medicine

Jiahao Yang
Institute of Reproductive Health and Center for Reproductive Medicine

Min Zou
Institute of Reproductive Health and Center for Reproductive Medicine

Jiao Li
Institute of Reproductive Health and Center for Reproductive Medicine

Huaibiao Li
Institute of Reproductive Health and Center for Reproductive Medicine

Ling Zhang
Institute of Reproductive Health and Center for Reproductive Medicine

Wenpei Xiang (✉️ wpxiang2010@gmail.com)
Huazhong University of Science and Technology Tongji Medical College  https://orcid.org/0000-0001-9510-116X

Research

Keywords: miR-320a-3p, miR-483-5p, human granulosa cells, good quality embryo rate, clinical pregnancy rate

DOI: https://doi.org/10.21203/rs.3.rs-577307/v1

License: ☺️ ⚅️ This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background

miRNAs in body fluids are considered potential biomarkers of diseases. This study investigated whether miR-320a-3p and miR-483-5p levels in human granulosa cells from follicular fluids were associated with embryo developmental competence.

Methods

We collected 195 patients’ granulosa cells samples undergoing in vitro fertilization (n = 147) or intracytoplasmic sperm injection (n = 48) cycles, and gathered information about the outcomes of the treatment. miR-320a-3p and miR-483-5p levels were measured using qRT-PCR.

Results

The miR-320a-3p levels in human granulosa cells across different patient groups were significantly different in the good quality embryo rates, with lowest levels in the Q4 intervals (P<0.05). The relative expression levels of miR-320a-3p were negatively associated with clinical pregnancy rate (P<0.05) and positively correlated with the patient age (P=0.0033). Moreover, both the basal FSH (P=0.0003) and ovarian stimulation protocol (P=0.006 and P=0.004) significantly and positively affected miR-320a-3p levels. The days of stimulation was negatively correlated with the relative expression of miR-320a-3p (P=0.0466). The relative expression levels of miR-483-5p were significantly positively correlated with AMH (P=0.0047). Neither miR-320a-3p nor miR-483-5p levels in granulosa cells were associated with normal fertilized rate, blastulation rate and abortion rate.

Conclusions

The miR-320a-3p levels in human granulosa cells were negatively correlated with the good quality embryo rate and clinical pregnancy rate and positively correlated with the patient age, indicating that miR-320a-3p can be used as a potential indicator to predict embryo development ability and clinical pregnancy.

Background

MicroRNAs (miRNAs) are highly conserved, single stranded, small non-coding functional RNAs of 19–25 nucleotides, which contribute to post-transcriptional levels by binding the 3′-untranslated region of messenger RNAs (mRNAs), with destabilization or translation repression (1). Moreover, miRNAs are widely expressed in biological systems. Although many miRNAs are commonly expressed, specific expression of miRNAs are common in tissues, suggesting that different tissues have unique requirements for miRNAs and that these miRNAs have specific functional roles in different tissues. Owing to tissue-specific miRNAs expression, miRNAs are considered potential biomarkers (2). MiRNAs are very stable in biological fluids (3) and resistant to a wide range of storage conditions making them biomarkers in some states (4), such as retinoblastoma (5), Parkinson's disease (6), and pregnancy (7).

In reproduction, several studies have identified miRNAs are not only expressed in ovarian follicles cells, also found in the biological fluids, such as follicular fluid (8-10). The miRNAs in follicular fluid are involved in regulating various biological processes, include ovarian cell proliferation and apoptosis (11, 12), and oocyte quality and maturation (12, 13). Recent studies have reported that the miRNAs expression in the follicular fluid can lead to downstream events that will affect fertilization and day 3 embryo morphology (14) and are significantly negatively related to viable blastocyst formation (15). Moreover, miRNAs might be represented as promising biomarkers during in vitro fertilization (IVF) (16), and polycystic ovarian syndrome (PCOS) (17). In human embryos culture media, some of miRNAs are differentially expressed according to the fertilization method, chromosomal status, and pregnancy outcome, which makes them potential biomarkers for predicting IVF success (18). These studies suggest that miRNAs play an important role in the oocyte development and fertilization. Our previous study found that the expression of miR-320a-3p and miR-483-5p levels were decreased in the follicular fluid exosomes of elderly women. However, to date, no studies have reported the relationship of the two miRNAs expression profile in the human mural granulosa cells and ART outcomes during IVF/ICSI.

Therefore, the aim of this study was to investigated the relationship between miRNAs (miR-320a-3p and miR-483-5p) in human granulosa cells expression profile and oocyte developmental competence and explored the effect of patient clinical characteristics on miRNAs (miR-320a-3p and miR-483-5p) expression profile in human granulosa cells.
Methods

Patients’ characteristics

This study recruited 195 women enrolled in IVF (n = 147) or intracytoplasmic sperm injection (ICSI) (n = 48) cycles at the Center for Reproductive Medicine of Tongji Medical College at the Huazhong University of Science and Technology from December 2019 to January 2021. Participants were required to meet the following eligibility requirements: conventional controlled ovulation induction schemes were used. Patients were excluded if they were diagnosed with infectious disease, malignant tumors, premature ovarian failure, polycystic ovary syndrome, systemic diseases and hereditary diseases. The women’s ages ranged from 21-46 years (mean ± SD: 34.39 ± 5.19 years) and their body mass index (BMI) ranged from 8.93 kg/m² to 32.40 kg/m² (mean ± SD: 22.53 ± 3.39 kg/m²). Baseline hormonal levels including follicle-stimulating hormone [FSH], luteinizing hormone [LH], and 17β-estradiol [E2] and anti-Müllerian hormone (AMH) were measured on the third day of menstruation. The number of days of stimulation ranged from 5 to 22 days (mean ± SD: 9.97 ± 2.48 days), and the total dose of gonadotropins received ranged from 900 to 6450 IU (mean ± SD: 2344.27 ± 842.52 IU).

The controlled ovulation induction schemes used included ultra-long protocol, long protocol, antagonist protocol, progestin-primed ovarian stimulation (PPOS), mild stimulation protocol, and luteal phase stimulation. FSH stimulation was monitored by measuring serum E2 levels and follicular size. Human chorionic gonadotrophin (hCG) (Livzon, Zhuhai, China) was injected when at least three follicles are 18 mm or larger in diameter by ultrasound. After hCG injection 36 h, oocytes were extracted by transvaginal ultrasound-guided puncture.

Human granulosa cell collection and identification

Granulosa cells were collected from the follicular fluid of 195 patients as described (19). After collection, granulosa cells were seeded and cultured on coverslips for 48 h. Then the granulosa cells were fixed in 4% (v/v) paraformaldehyde for 20 min for immunofluorescence as before (20). The FSH receptor (FSHR) was used to detect the purity of granulosa cells. To exclude the non-specific staining from antibodies, the primary and secondary antibodies were omitted as negative control groups, respectively.

RNA isolation, cDNA synthesis, and real-time quantitative PCR (qPCR)

Total RNA was extracted from granulosa cells using the RNA-easy Isolation Reagent (Vazyme Biotech Co., Ltd, Nanjing), and transcribed into cDNA using the All-in-One™ miRNA qRT-PCR Detection Kit 2.0 (GeneCopoeia, Inc, United States) according to the manufacturer’s protocol. The cDNA synthesis reaction conditions were the following: 37 °C for 60 min and 85 °C for 5 s.

The miR-320a-3p and miR-483-5p primers were purchased by the GeneCopoeia Company. U6 was used as a housekeeping gene. The reaction was performed in a total volume of 20 μl contained 10 μl 2× All-in-One™ qPCR Mix, 2 μl All-in-One™ miRNA qPCR Primer (2 μM), 2 μl Universal Adaptor PCR Primer (2 μM) and 2 μl First-strand cDNA. The cycling conditions used were the following: 95 °C for 600 s, 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72°C for 10 s. The relative quantity of miRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Morphological assessment of oocytes, good quality embryos, and blastocysts

The appearance of prokaryotic zygote 18 to 20 hours after microinjection or artificial insemination is a representative of fertilization. Morphological scores of embryos at day 3 were consistent with the current consensus system (21). High-quality embryos and blastocysts were defined as previous (22).

Statistical analysis

The miR-320a-3p and miR-483-5p levels, expressed as means ± standard deviation (SD), or as median values and the interquartile range (IQR), if appropriate. Linear regression was carried out for the effect of patients’ characteristics information on the miR-320a-3p and miR-483-5p levels in follicular fluid. To evaluate the correlation between miR-320a-3p and miR-483-5p levels and embryo developmental competence, we first subdivided all 195 samples according to their follicular fluid miR-320a-3p and miR-483-5p levels quartile, then the normal fertilized rate, good quality embryo rate and blastocysts rate were compared by ANOVA or Kruskal–Wallis test. Reproductive outcomes of assisted reproductive technology of the miR-320a-3p and miR-483-5p levels were compared with an
unpaired t test or Mann-Whitney test. Statistical analyses were performed using the Statistical Package for Social Sciences program, Version 12.0 (SPSS Inc., Chicago, IL, USA). \( P < 0.05 \) was considered statistically significant.

**Table 1 Association between the levels of miR-320-3p and miR483-5p in granulosa cells from human follicular fluids and normal fertilization rate.**

| Parameters | Q1 | Q2 | Q3 | Q4 | \( p \) value |
|-----------|----|----|----|----|---------------|
| miR-320a-3p | 296/489 | 60.5 | 275/457 | 60.2 | 296/547 | 54.1 | 262/429 | 61.1 | NS |
| Total | 201/358 | 56.1 | 201/361 | 55.7 | 203/405 | 50.1 | 225/368 | 61.1 | NS |
| IVF cycles | 95/131 | 72.5 | 74/96 | 77.1 | 93/142 | 65.5 | 37/61 | 60.7 | NS |

**Results**

**Human granulosa cells in follicular fluids identification**

As shown in Figure 1, most of the cells in the dishes were granulosa cells, which were characterized by a positive FSHR staining. Non-specific staining was not detected.

**Relationship of the miR-320a-3p and miR-483-5p levels in the granulosa cells and embryo developmental competence**

The patients were subdivided into four groups according to the relative expression of miR-320a-3p levels quartile in the granulosa cells: Q1: 0.46-6.17×10\(^3\), \( n=49 \); Q2: 6.41×10\(^3\)-2.35×10\(^5\), \( n=49 \); Q3: 2.63×10\(^5\)-2.34×10\(^6\), \( n=49 \); and Q4: 2.51×10\(^6\)-9.38×10\(^7\), \( n=48 \). The relative expression of miR-483-5p levels quartile: Q1: 0.002-0.18, \( n=49 \); Q2: 0.18-1.13, \( n=49 \); Q3: 1.21-5.80, \( n=49 \); and Q4: 5.81-3.52×10\(^3\), \( n=48 \), respectively.

The relative expression of miR-320a-3p levels across different patient groups were significantly different in the good quality embryo rates, with lowest levels in the Q4 intervals (Table 2, \( P < 0.01 \)). However, the normal fertilized rate for IVF or ICSI and blastocyst rate did not differ (Table 1 and Table 2, \( P > 0.05 \)).

The relative expression of miR-483-5p levels across different patient groups were no difference (\( P > 0.05 \)) in the normal fertilization rates for IVF or ICSI, good quality embryo rate, and blastulation rate, as shown in Table 1 and Table 2.

**Table 2 Association between the levels of miR-320-3p and miR-483-5p in granulosa cells from human follicular fluids and embryo developmental competence**.
| Parameters | miR-320a-3p | miR-483-5p |
|------------|-------------|-------------|
|            | N(%)        | MD(IQR)     | 95% CI       | P-value | MD(IQR)     | 95% CI       | P-value |
| Clinical pregnancy rate | 65.83 | 0.0477* | NS |
| Pregnancy | 106(65.83) | 2.47×10^6 (6.42×10^3-1.91×10^6) | 3.79×10^4,7.43×10^6 | 44.32(0.19-5.56) | -2.27×10^6, 6.48×10^6 |
| Unpregnant | 55 (34.16) | 6.21×10^6 (4.86×10^3-2.72×10^6) | 10.61(0.15-5.28) | |
| Abortion rate | 5.71 | NS | NS |
| Abortion | 6 (5.71) | 4.04×10^5(1.54×10^4-6.50×10^5) | -126.0,58.57 | 4.77(0.06-6.05) | -248.10,331.40 |
| Non-abortion | 99 (94.29) | 2.51×10^6(7.93×10^3-1.94×10^6) | 46.39(0.23-5.20) | |

MD Median, IQR interquartile range, CI confidence interval.

Effect of patients’ clinical characteristics on the miR-320a-3p and miR-483-5p levels in the granulosa cells

The relative expression of miR-320a-3p in the granulosa cells was correlated positively with age (β ± SE: 4.79×10^5 ± 1.61×10^5, P=0.0033) (Table 4, Figure 2). Moreover, both the basal FSH (β ± SE: 7.90×10^5 ± 2.14×10^5, P=0.0003) (Table 4, Figure 2) and ovarian stimulation protocol, including mild stimulation protocol and luteal phase stimulation (β ± SE: 8.27×10^9 ± 2.92×10^9, 6.29×10^9 ± 2.09×10^9, respectively; P=0.006, P=0.004, respectively) (Table 4) significantly and positively affected miR-320a-3p levels in the granulosa cells. The days of stimulation was negatively correlated with the relative expression of miR-320a-3p in the granulosa cells (β...
± SE: -6.85×10^5 ± 3.42×10^5, \( P = 0.0466 \) (Table 4, Figure 2). The relative expression of miR-320a-3p in the granulosa cells was not associated with BMI, basal LH, basal E_2, AMH, AFC and total dose of gonadotropins (Table 1, \( P>0.05 \)).

The relative expression levels of miR-483-5p in the granulosa cells were correlated significantly positively with AMH (\( \beta \pm SE: 17.55 \pm 6.14, P=0.0047 \) (Table 4, Figure 2). However, miR-483-5p levels were also not correlated with other indicators (Table 4, \( P>0.05 \)).

Table 4 Patients' characteristics association with the miR-320a-3p and the miR-483-5p levels in human follicular fluid.
### Variable

| Variable                          | Min-Max  | Mean   | n (%) | SD   | miR-320a-3p | miR-483-5p |
|----------------------------------|----------|--------|-------|------|-------------|------------|
|                                  |          |        |       |      | β ± SE     | P-value    | β ± SE     | P-value    |
| Age (years)                      | 21-46    | 34.39  | 195 (100) | 5.19 | 4.79±10⁵±1.61×10⁵ | 0.0033* | -3.58±4.79 | 0.3084     |
| BMI (kg/m²)                      | 8.93-32.40 | 22.53  | 194 (99.49) | 3.39 | 1.45×10⁵±2.54×10⁵ | 0.5678 | -1.48±5.43 | 0.7850     |
| Female baseline levels           |          |        |       |      | β ± SE     | P-value    | β ± SE     | P-value    |
| Basal FSH (IU/L)                 | 1.25-33.00 | 8.40   | 195 (100) | 3.86 | 7.90×10⁵±2.14×10⁵ | 0.0003* | -3.42±4.72 | 0.4701     |
| Basal LH (IU/L)                  | 0.65-40.02 | 4.76   | 195 (100) | 3.77 | 7.61×10³±2.27×10⁵ | 0.9733 | -0.97±4.85 | 0.8412     |
| Basal E₂ (pg/ml)                 | 2.74-5178 | 76.17  | 195 (100) | 370.11 | -6.09×10²±2.3×10³ | 0.7926 | -7.57±4.94×10² | 0.9878     |
| AMH (ng/ml)                      | 0.06-14.62 | 3.52   | 194 (99.49) | 2.93 | -1.91×10⁵±2.93×10⁵ | 0.5147 | 17.55±6.14 | 0.0047*    |
| Antral follicle count            | 3-52     | 15.74  | 195 (100) | 7.97 | -5.39×10⁴±1.07×10⁵ | 0.6159 | -0.13±2.30 | 0.9535     |
| Days of stimulation              | 5-22     | 9.97   | 195 (100) | 2.48 | -6.85×10⁵±3.42×10⁵ | 0.0466* | 0.31±7.38 | 0.9660     |
| Total dose of gonadotropins (IU) | 900-6450 | 2344.27 | 195 (100) | 842.52 | -1.39×10³±1.01×10³ | 0.1704 | -7.40×10³±2.17×10² | 0.7333     |
| Ovarian stimulation protocol     |          |        |       |      |            |            |            |            |
| Ultra-long protocol              | -        | -      | 62 (31.79) | -   | -           | Ref       | -          |            |
| Long protocol                    | -        | -      | 9 (0.05) | -   | -6.05×10⁹±5.36×10⁹ | 0.263 | -2.57×10⁵±9.61×10⁵ | 0.790      |
| Antagonist protocol              | -        | -      | 75 (0.38) | -   | -7.10×10⁹±6.15×10⁹ | 0.250 | -1.76×10⁴±1.42×10⁴ | 0.217      |
| Progestin-primed ovarian stimulation (PPOS) | -  | -  | 40 (0.21) | - | 2.19×10⁹±4.37×10⁹ | 0.617 | -1.30×10⁴±1.40×10⁴ | 0.354      |
| Mild stimulation protocol        | -        | -      | 5 (0.03) | -   | 8.27×10⁹±2.92×10⁹ | 0.006* | -1.44×10⁵±7.61×10⁵ | 0.851      |
| Luteal phase stimulation         | -        | -      | 4 (0.02) | -   | 6.29×10⁹±2.09×10⁹ | 0.004* | -1.93×10⁵±6.91×10⁵ | 0.781      |

### Discussion

In this study, our results showed that miR-320a-3p expression levels in the granulosa cells were associated with the oocyte potential that had developed to the good quality embryo stage, and with clinical pregnancy for women undergoing IVF or ICSI. Moreover, it was
found to be positively correlated with patient age and basal FSH.

In our study, the expression levels of *miR-320a-3p* in mural granulosa cells was associated with the good quality embryo rate, and the highest expression group of *miR-320a-3p* had a lowest rate of good quality embryo rate (P<0.0001). This could be because of an adverse effect of miRNAs on the quality of the embryo. Moreover, studies showed that follicular fluid miRNAs significantly influence oocyte mature (23), developmental (24) and the quality of the embryo (15). In addition, increasing evidences implicated that a good day-3 embryo is apt to form a high-quality blastocyst (25). Meanwhile, a good day-3 embryo also have an optimistic pregnancy rate (25). Our results demonstrated that there were lower expressed *miR-320a-3p* in pregnant group, compared to unpregnant group (P=0.0477). Older women generally face reduced ovarian function and reduced pregnancy rates (26). Thus *miR-320a-3p* can be used as a non-invasive marker to predict embryonic development quality and clinical pregnancy outcome (14-16, 18).

Furthermore, our results suggested that the expression of *miR-320a-3p* levels were positively correlated with patient's age ($r^2=0.209$, P=0.0033). Findings by Victor A Ansere et al revealed that cellular senescence may contribute to ovarian aging, and subsequently declines in ovarian follicular reserve (27). Indeed, in our study, the *miR-320a-3p* levels were positively associated with basal FSH ($r^2=0.257$, P=0.0003). Studies showed that miRNA is involved in hormone regulation during follicular formation. FSH plays a crucial role in folliculogenesis by a novel pathway of miRNAs (28). With the occurrence of aging, *miR-320a-3p* could regulate the level of basal FSH. Mianmian Yin et al demonstrate that miR-320 regulates steroid production by targeting E2F1 and SF-1 in the follicular development (29). FSH highly capable of forecasting marker of ovarian reserve function (30, 31). Potential associations between *miR-320a-3p* and FSH provide new direction for predicting ovarian response.

In conclusion, the current study showed that the expression levels of *miR-320a-3p* are related to age and embryo development ability for women undergoing IVF/ICSI, suggesting that *miR-320a-3p* can be used as a potential indicator to predict treatment outcomes. A deeper understanding of the specific role of miRNA in the development and maturation of follicles remains to be further studied.

**Conclusion**

The current research showed that changes in the *miR-320a-3p* levels of human follicular fluid were correlated with the good quality embryo rate and clinical pregnancy rate and the patient age, suggesting that *miR-320a-3p* levels have potential use in evaluating embryo development ability and clinical pregnancy. A deeper understanding of the mechanism of *miR-320a-3p* affecting oocyte development ability could promote the future clinical application of *miR-320a-3p*.

**Abbreviations**

IVF, in vitro fertilization  
ICSI, intracytoplasmic sperm injection  
BMI, body mass index BMI  
FSH, follicle-stimulating hormone  
LH, luteinizing hormone  
E2, 17β-estradiol  
AMH, anti-Müllerian hormone  
hCG, human chorionic gonadotrophin

**Declarations**

**Ethics approval and consent to participate**

This project was approved by the Ethics Committee of Reproductive Medicine Center, Tongji Medical College, Huazhong University of Science and Technology ([2020] Ethical Approval (007) Number) on October 16, 2020. Granulosa cells samples were collected with patients’ informed consent.
Consent for publication

Consent for publication have be obtained from that person.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

No interest.

Funding

This work was supported by National Key Research and Development Program of China (2017YFC1002002) and the National Natural Science Foundation of China (NSFC 81871221).

Authors’ contributions

Y.L. carried out experimental work, conducted the statistical analysis and wrote the manuscript. Q.M. prepared samples and helped with experimental work. Q.S. and J.Y. prepared samples. M.Z. and J.L. collected follicular uid samples. H.L. revised the manuscript. L.Z. and W.X. designed experiments, interpreted the data and revised the manuscript. All authors read and approved the manuscript.

Acknowledgements

Thanks to institute of Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology for supporting our experimental samples.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.
2. Kobayashi M, Sawada K, Nakamura K, Yoshimura A, Miyamoto M, Shimizu A, et al. Exosomal miR-1290 is a potential biomarker of high-grade serous ovarian carcinoma and can discriminate patients from those with malignancies of other histological types. J Ovarian Res. 2018;11(1):81.
3. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. J Cell Mol Med. 2014;18(3):371-90.
4. Tscherner A, Gilchrist G, Smith N, Blondin P, Gillis D, LaMarre J. MicroRNA-34 family expression in bovine gametes and preimplantation embryos. Reprod Biol Endocrinol. 2014;12:85.
5. Li XH, Shi AJ, Li J, Yuan HF. Plasma miR-6089 as potential diagnostic biomarker for retinoblastoma. Int Ophthalmol. 2021.
6. Yang Y, Li Y, Yang H, Guo J, Li N. Circulating MicroRNAs and Long Non-coding RNAs as Potential Diagnostic Biomarkers for Parkinson's Disease. Front Mol Neurosci. 2021;14:631553.
7. Ioannidis J, Donadeu FX. Circulating miRNA signatures of early pregnancy in cattle. BMC Genomics. 2016;17:184.
8. Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, et al. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. J Clin Endocrinol Metab. 2013;98(7):3068-79.
9. Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, et al. Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. Fertil Steril. 2014;102(6):1751-61 e1.
10. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem. 2010;56(11):1733-41.
11. Sirotkin AV, Laukova M, Ovcharenko D, Brenaut P, Mlyncek M. Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol. 2010;223(1):49-56.
12. Assou S, Al-edani T, Haouzi D, Philippe N, Lecellier CH, Piquemal D, et al. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. Hum Reprod. 2013;28(11):3038-49.
13. Xu YW, Wang B, Ding CH, Li T, Gu F, Zhou C. Differentially expressed microRNAs in human oocytes. J Assist Reprod Genet. 2011;28(6):559-66.
14. Machtinger R, Rodosthenous RS, Adir M, Mansour A, Racowsky C, Baccarelli AA, et al. Extracellular microRNAs in follicular fluid and their potential association with oocyte fertilization and embryo quality: an exploratory study. J Assist Reprod Genet. 2017;34(4):525-33.
15. Fu J, Qu RG, Zhang YJ, Gu RH, Li X, Sun YJ, et al. Screening of miRNAs in human follicular fluid reveals an inverse relationship between microRNA-663b expression and blastocyst formation. Reprod Biomed Online. 2018;37(1):25-32.
16. Zhao H, Wang L, Wang Y. Circulating microRNAs as candidate biomarkers for the ovarian response during in vitro fertilization. Medicine (Baltimore). 2021;100(6):e24612.
17. Vahdat-Lasemi M, Hosseini S, Jajarmi V, Kazemi B, Salehi M. Intraovarian injection of miR-224 as a marker of polycystic ovarian syndrome declines oocyte competency and embryo development. J Cell Physiol. 2019;234(8):13858-66.
18. Rosenbluth EM, Shelton DN, Wells LM, Sparks AE, Van Voorhis BJ. Human embryos secrete microRNAs into culture media—a potential biomarker for implantation. Fertil Steril. 2011;26(6):307-13.
19. Grondahl ML, Andersen CY, Borgho T, Borg J, Boujida VH, Borup R. Specific genes are selectively expressed between cumulus and granulosa cells from individual human pre-ovulatory follicles. Mol Hum Reprod. 2012;18(12):572-84.
20. Liu Y, Shen Q, Li H, Xiang W, Zhang L. Cell-free mitochondrial DNA increases granulosa cell apoptosis and reduces aged oocyte blastocyst development in the mouse. Reprod Toxicol. 2020;98:278-85.
21. Alpha Scientists in Reproductive M, Embryology ESIGo. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod. 2011;26(6):1270-83.
22. Liu Y, Shen Q, Zhao X, Zou M, Shao S, Li J, et al. Cell-free mitochondrial DNA in human follicular fluid: a promising bio-marker of blastocyst developmental potential in women undergoing assisted reproductive technology. Reprod Biol Endocrinol. 2019;17(1):54.
23. Kim YJ, Ku SY, Kim YY, Suh CS, Kim SH, Choi YM. MicroRNA Profile of Granulosa Cells after Ovarian Stimulation Differs According to Maturity of Retrieved Oocytes. Geburtshilfe Frauenheilkd. 2016;76(6):704-8.
24. Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter P, et al. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. Hum Fertil (Camb). 2014;17(2):90-8.
25. Yin H, Jiang H, He R, Wang C, Zhu J, Luan K. The effects of fertilization mode, embryo morphology at day 3, and female age on blastocyst formation and the clinical outcomes. Syst Biol Reprod Med. 2015;61(1):50-6.
26. Faddy MJ. Follicle dynamics during ovarian ageing. Mol Cell Endocrinol. 2000;163(1-2):43-8.
27. Assou S, Al-edani T, Haouzi D, Philippe N, Lecellier CH, Piquemal D, et al. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. Hum Reprod. 2013;28(11):3038-49.
28. Yao N, Lu CL, Zhao JJ, Xia HF, Sun DG, Shi XQ, et al. A network of miRNAs expressed in the ovary are regulated by FSH. Front Biosci (Landmark Ed). 2009;14:3239-45.
29. Yin M, Wang X, Yao G, Lu M, Liang M, Sun Y, et al. Transactivation of microRNA-320 by microRNA-383 regulates granulosa cell functions by targeting E2F1 and SF-1 proteins. J Biol Chem. 2014;289(26):18239-57.
30. Broer SL, van Disseldorp J, Broeze KA, Dolleman M, Opmeer BC, Bossuyt P, et al. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: an individual patient data approach. Hum Reprod Update. 2013;19(1):26-36.
31. Broer SL, Dolleman M, van Disseldorp J, Broeze KA, Opmeer BC, Bossuyt PM, et al. Prediction of an excessive response in in vitro fertilization from patient characteristics and ovarian reserve tests and comparison in subgroups: an individual patient data meta-analysis. Fertil Steril. 2013;100(2):420-9 e7.
Figure 1

Immunofluorescence staining in human ovarian granulosa cells. The Red (×400) expressed FSHR, the blue (×400) expressed nuclear staining using 4', 6-diamino-2-phenylindole (DAPI). Non-specific staining can be observed with PBS instead of primary or secondary antibodies.
Correlations between the miR-320a-3p in granulosa cells and patient age, basal FSH, days of stimulation, and miR-483-5p and AMH.