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hsa-miR-320a-3p and *hsa-miR-483-5p* levels in human granulosa cells: promising bio-markers of live birth after IVF/ICSI

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Abstract

Background: MicroRNAs (miRNAs) are considered potential biomarkers for various diseases. This study investigated whether *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels in human ovarian granulosa cells derived from follicular fluids are associated with embryo developmental competence.

Methods: We collected 195 granulosa cells samples and analyzed the treatment outcomes in patients undergoing in vitro fertilization ($n = 147$) or intracytoplasmic sperm injection ($n = 48$) cycles. The *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels in granulosa cells were measured using quantitative reverse transcription-polymerase chain reaction.

Results: Patients were subdivided into four groups according to the granulosa cells *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels quartiles (Q1–Q4). Embryo developmental competence was compared using the chi-square test. Patients in Q3 were less likely to achieve a normal fertilization rate for in vitro fertilization and blastocyst formation than those in Q1 as they expressed high levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* ($P < 0.05$). Patients in Q3 and Q4 were less likely to achieve a good-quality embryo as they expressed high levels of *hsa-miR-483-5p* and *hsa-miR-320a-3p* ($P < 0.05$). The *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels were not associated with clinical pregnancy. However, multiple regression analysis indicated that in Q3 and Q4 intervals had experienced a decreased chance of live birth due to high expression levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels. The relative *hsa-miR-320a-3p* expression levels in granulosa cells were weakly and positively correlated with the patient age ($P = 0.0033$). Moreover, both the basal follicle stimulating hormone ($P = 0.0003$) and ovarian stimulation protocols ($P = 0.006$ and $P = 0.004$) significantly and positively affected *hsa-miR-320a-3p* levels. The days of stimulation was negatively correlated with the relative *hsa-miR-320a-3p* expression level ($P = 0.047$).

Conclusions: The *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels in human granulosa cells negatively correlated with the good-quality embryo rate and live birth, indicating that *hsa-miR-320a-3p* and *hsa-miR-483-5p* can be used as potential negative indicators to predict good-quality embryos and live births.

Keywords: *hsa-miR-320a-3p*, *hsa-miR-483-5p*, Human granulosa cells, Good-quality embryo, Live birth

Key message

hsa-miR-320a-3p in human granulosa cells can be used as a potential indicator to predict good-quality embryos and live births.

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Background

MicroRNAs (miRNAs) are highly conserved, single-stranded, small, non-coding, and functional RNAs of 19–25 nucleotides, which regulate post-transcriptional RNA levels by binding to the 3'-untranslated region of messenger RNAs (mRNAs) and causing destabilization or translation repression [1]. They are widely expressed in various biological systems. Although many miRNAs are commonly expressed, the selective specific expression of miRNAs is common in tissues, suggesting that the requirement for specific miRNAs in different tissues and specific roles of miRNAs in tissues. Owing to their tissue-specific expression, miRNAs are considered potential biomarkers [2].

Several studies have identified miRNAs that are expressed in ovarian follicle cells [3]. MiRNAs are involved in the regulation of various biological processes, including granulosa cell proliferation, apoptosis [4, 5], and oocyte maturation [5, 6]. Recent studies have reported that the miRNAs expression led to downstream events that will affect fertilization and day 3 embryo morphology [7]. Moreover, miRNAs could be promising biomarkers for ovarian responses during in vitro fertilization (IVF) [8]. Some miRNAs are also differentially expressed according to the fertilization method, chromosomal status, and pregnancy outcome, making them potential biomarkers for predicting IVF success [9]. These findings suggest that miRNAs play important roles in the oocyte development.

The aim of this study was to investigate the relationship between miRNAs (*hsa-miR-320a-3p* and *hsa-miR-483-5p*) in human granulosa cells expression levels and oocyte developmental competence and explored the effect of patient clinical characteristics on miRNAs (*hsa-miR-320a-3p* and *hsa-miR-483-5p*) expression levels in human granulosa cells.

Materials and methods

Patients' characteristics

This study recruited 195 women enrolled in IVF ($n=147$) or ICSI ($n=48$) cycles at the Center for Reproductive Medicine of Tongji Medical College in the Huazhong University of Science and Technology from December 2019 to January 2021. Participants were required to meet the following eligibility requirements: conventional controlled stimulation protocols 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 to 46 years (mean \pm SD: 34.39 ± 5.19 years) and their body mass index (BMI) ranged from 15.80 to 32.40 kg/m² (mean \pm SD: 22.60 ± 3.23 kg/m²). Baseline hormonal

levels including follicle-stimulating hormone (FSH), luteinizing hormone (LH), and 17 β -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 \pm SD: 9.97 ± 2.48 days), and the total dose of gonadotropins received ranged from 900 to 6450 IU (mean \pm SD: 2344.27 ± 842.52 IU).

The controlled ovarian stimulation protocols were 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 cells collection and identification

Granulosa cells were collected from the follicular fluid of 195 patients as described [10]. Briefly, after the isolation of the cumulus-oocyte complexes (COCs) for conventional IVF or ICSI procedures, the follicular fluids were centrifuged and granulosa cells were collected and resuspended in 1 \times phosphate-buffered saline (PBS). Then, it was added to a 50% Percoll gradient (GE Healthcare Life Sciences, Piscataway, NJ, USA) and centrifuged at 400 g for 30 min at 4°C. The cells in the middle layer were collected, resuspended in PBS.

To confirm the purity of granulosa cells, it was seeded and cultured on coverslips at a density of 1×10^5 cells/coverslips for 48 h. Then the granulosa cells were fixed in 4% (v/v) paraformaldehyde for 20 min for immunofluorescence as before [11]. 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-OneTM miRNA quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Detection Kit 2.0 (GeneCopeia, 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 *hsa-miR-320a-3p* and *hsa-miR-483-5p* primers were purchased by the GeneCopeia Company. *hsa-miR-320a-3p* primer forward: 5'-TTGAGAGGGCGAAAA

AAA-3'. *hsa-miR-483-5p* primer forward: 5'-CGGGAG GAAAGAAGGGAGAA-3'. Reverse primers are universal reverse primers in the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Inc. USA). 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. IVF normal fertilization rate = number of 2PN/total number of oocytes × 100%. ICSI normal fertilization rate = number of 2PN/total number of MII oocytes × 100%. Morphological scores of embryos at day 3 were consistent with the current consensus system [12]. Good-quality embryos and blastocysts were defined as previous [13]. Good-quality embryo rate = number of day 3 good-quality embryos/normal fertilization number of cleavage embryos × 100%. Blastulation rate = number of blastocysts at stage 2 and above/total number of cleavage embryos in blastocyst culture × 100%.

Statistical analysis

The *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels, expressed as means ± standard deviation (SD), median values and the interquartile range (IQR), or as OR (95% CI), if appropriate. Linear regression was carried out for the effect of patients' characteristics information on the *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels in human granulosa cells. To evaluate the correlation between *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels and embryo developmental competence, we first subdivided all 195 samples according to their granulosa cells *hsa-miR-320a-3p* and *hsa-miR-483-5p* levels quartile, then the normal fertilization rate, good-quality embryo rate and blastulation rate were compared by Chi-square test. Multi-variable logistic regression analysis was used to analyze clinical pregnancy and live birth. 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.

Results

Identification of granulosa cells from human follicular fluids

As shown in Fig. 1, the 96% cells in the dishes were granulosa cells, which were characterized by a positive FSHR staining. Non-specific staining was not detected. This proves that all the cells isolated were granulosa cells, and directly extracted granulosa cells can be used in subsequent studies.

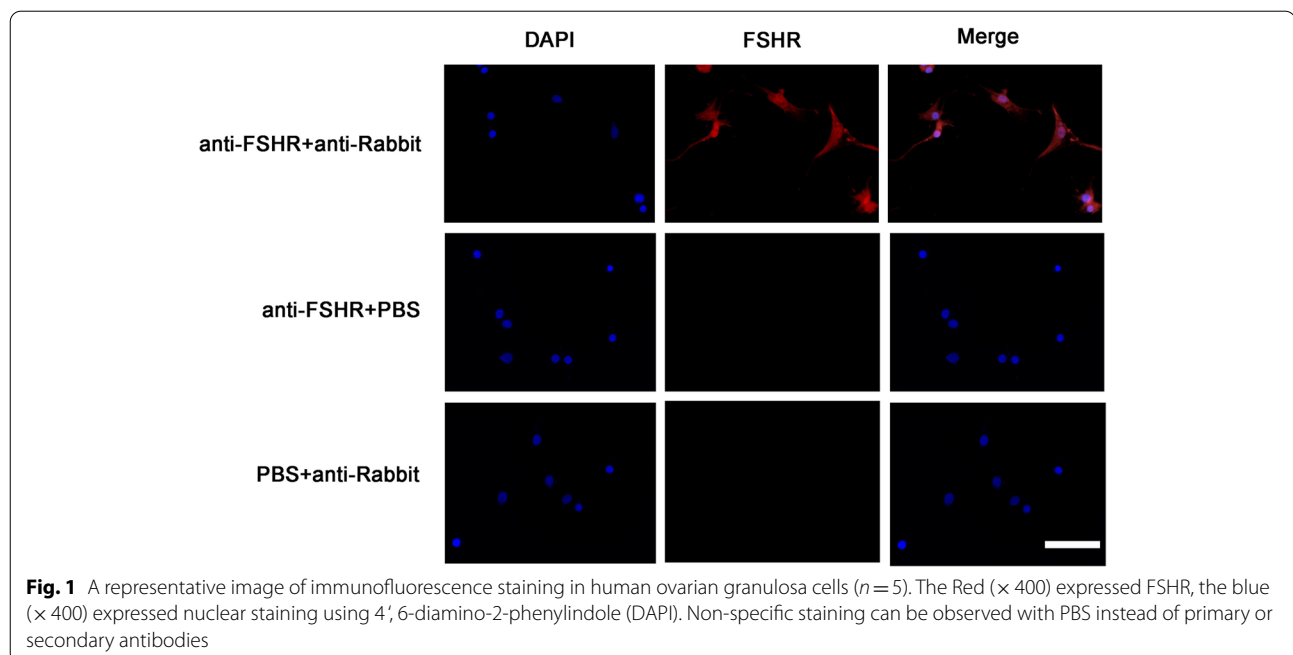


Fig. 1 A representative image of immunofluorescence staining in human ovarian granulosa cells ($n = 5$). The Red ($\times 400$) expressed FSHR, the blue ($\times 400$) expressed nuclear staining using 4',6-diamino-2-phenylindole (DAPI). Non-specific staining can be observed with PBS instead of primary or secondary antibodies

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

The patients were subdivided into four groups according to the relative expression of *hsa-miR-320a-3p* levels quartile in the granulosa cells: Q1: $0.46-6.17 \times 10^3$, $n=49$; Q2: $6.41 \times 10^3-2.35 \times 10^5$, $n=49$; Q3: $2.63 \times 10^5-2.34 \times 10^6$, $n=49$; and Q4: $2.51 \times 10^6-9.38 \times 10^7$, $n=48$. In the Q3 intervals, the normal fertilization rate for IVF was lower compared to Q1 and Q2 (Table 1, $P<0.05$). In the Q3 and Q4 intervals, the good-quality embryo rate was lower than Q2 (Table 1, $P<0.0001$). However, the normal fertilization rate for ICSI and blastulation rate did not differ (Table 1, $P>0.05$). Multiple regression analysis showed that in Q3 and Q4 intervals experienced a decreased chance of live birth when Q1 group was used as reference (Table 2, $P<0.0001$). And there was no difference in clinical pregnancy (Table 2, $P>0.05$).

The patients were subdivided into four groups according to the relative expression of *hsa-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. In the Q3 intervals, the good-quality embryo rate and blastulation rate were lower than Q1 group (Table 1, $P<0.05$). The normal fertilization rate for IVF or ICSI was no significant differences among groups ($P>0.05$), as shown in Table 1. Multiple regression analysis showed that in Q3 and Q4 intervals had a decreased chance of live birth (Table 2, $P<0.0001$). Clinical pregnancy had no differ among four group (Table 2, $P>0.05$).

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

The relative expression of *hsa-miR-320a-3p* in the human granulosa cells were weak positively correlated with age ($\beta \pm SE$: $4.79 \times 10^5 \pm 1.61 \times 10^5$, $P=0.0033$) (Table 3). Moreover, both the basal FSH ($\beta \pm SE$: $7.90 \times 10^5 \pm 2.14 \times 10^5$, $P=0.0003$) (Table 3) and ovarian stimulation

Table 1 Association between the levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* in granulosa cells from human follicular fluids and reproductive outcomes of assisted reproductive technology (ART)

Parameters	Q1	%	Q2	%	Q3	%	Q4	%	P-value
<i>hsa-miR-320a-3p</i>									
Normal fertilization rate									
IVF cycles	201/358	56.1	201/361	55.7	203/405	50.1	225/368	61.1	< 0.05
ICSI cycles	95/131	72.5	74/96	77.1	93/142	65.5	37/61	60.7	NS
Good-quality embryo rate	212/296	71.6	205/275	74.5	214/296	72.3	149/262	56.9	< 0.0001
Blastulation rate	81/212	38.2	65/205	31.7	82/214	38.3	55/149	36.9	NS
<i>hsa-miR-483-5p</i>									
Normal fertilization rate									
IVF cycles	167/341	49.0	258/457	56.5	173/346	50.0	232/348	66.7	NS
ICSI cycles	105/134	78.4	52/74	70.3	83/133	62.4	59/89	66.3	NS
Good-quality embryo rate	225/272	82.7	203/310	65.5	197/256	77.0	155/291	53.3	< 0.0001
Blastulation rate	86/225	38.2	78/203	38.4	55/197	27.9	64/155	41.3	< 0.05

Chi square test was used for statistical analysis

Q quartile, NS not statistically significant, 2PN two pronuclei

Table 2 Multi-variable logistic regression analysis of the levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* in human granulosa cells and clinical outcomes (OR 95% CI)

	Clinical pregnancy		Live birth	
	<i>hsa-miR-320a-3p</i>	<i>hsa-miR-483-5p</i>	<i>hsa-miR-320a-3p</i>	<i>hsa-miR-483-5p</i>
Q1	Reference	Reference	Reference	Reference
Q2	0.328 (0.011-10.20)	7.08 (0.17-297.02)	5.24 (8.58×10^{-5} - 3.20×10^5)	0.21 (1.97×10^{-8} - 2.12×10^6)
Q3	0.069 (0.001-5.46)	0.89 (0.01-84.12)	0.93 (2.14×10^{-5} - 4.04×10^4)	0.096 (2.57×10^{-11} - 3.61×10^8)
Q4	1.59 (0.08-33.53)	11.23 (0.54-234.83)	0.375 (1.17×10^{-12} - 1.21×10^{11})****	0.163 (7.57×10^{-14} - 3.53×10^{11})****

**** $P<0.0001$

Table 3 Patients' characteristics association with the *hsa-miR-320a-3p* and the *hsa-miR-483-5p* levels in granulosa cells from human follicular fluids

Variable	Min-Max	Mean	n (%)	SD	<i>hsa-miR-320a-3p</i>		<i>hsa-miR-483-5p</i>	
					$\beta \pm SE$	P-value	$\beta \pm SE$	P-value
Age (years)	21-46	34.39	195 (100)	5.19	$4.79 \times 10^5 \pm 1.61 \times 10^5$	0.0033*	-3.58 ± 3.51	0.3084
BMI (kg/m ²)	8.93-32.40	22.53	194 (99.49)	3.39	$1.45 \times 10^5 \pm 2.54 \times 10^5$	0.5678	-1.48 ± 5.43	0.7850
Female baseline levels								
Basal FSH (IU/L)	1.25-33.00	8.40	195 (100)	3.86	$7.90 \times 10^5 \pm 2.14 \times 10^5$	0.0003*	-3.42 ± 4.72	0.4701
Basal LH (IU/L)	0.65-40.02	4.76	195 (100)	3.77	$7.61 \times 10^3 \pm 2.27 \times 10^5$	0.9733	-0.97 ± 4.85	0.8412
Basal E ₂ (pg/ml)	2.74-5178	76.17	195 (100)	370.11	$-6.09 \times 10^2 \pm 2.3 \times 10^3$	0.7926	$-7.57 \times 10^{-4} \pm 4.94 \times 10^{-2}$	0.9878
AMH (ng/ml)	0.06-14.62	3.52	194 (99.49)	2.93	$-1.91 \times 10^5 \pm 2.93 \times 10^5$	0.5147	$-3.917 \times 10^{-3} \pm 6.039 \times 10^{-3}$	0.5174
Antral follicle count	3-52	15.74	195 (100)	7.97	$-5.39 \times 10^4 \pm 1.07 \times 10^5$	0.6159	-0.13 ± 2.30	0.9535
Days of stimulation	5-22	9.97	195 (100)	2.48	$-6.85 \times 10^5 \pm 3.42 \times 10^5$	0.0466*	0.31 ± 7.38	0.9660
Total dose of gonadotropins (IU)	900-6450	2344.27	195 (100)	842.52	$-1.39 \times 10^3 \pm 1.01 \times 10^3$	0.1704	$-7.40 \times 10^{-3} \pm 2.17 \times 10^{-2}$	0.7333
Ovarian stimulation protocol								
Ultra-long protocol	-	-	62 (31.79)	-				Ref
Long protocol	-	-	9 (0.05)	-	$-6.05 \times 10^{-9} \pm 5.36 \times 10^{-9}$	0.263	$-2.57 \times 10^{-5} \pm 9.61 \times 10^{-5}$	0.790
Antagonist protocol	-	-	75 (0.38)	-	$-7.10 \times 10^{-9} \pm 6.15 \times 10^{-9}$	0.250	$-1.76 \times 10^{-4} \pm 1.42 \times 10^{-4}$	0.217
Progesterin-primed ovarian stimulation (PPOS)	-	-	40 (0.21)	-	$2.19 \times 10^{-9} \pm 4.37 \times 10^{-9}$	0.617	$-1.30 \times 10^{-4} \pm 1.40 \times 10^{-4}$	0.354
Mild stimulation protocol	-	-	5 (0.03)	-	$8.27 \times 10^{-9} \pm 2.92 \times 10^{-9}$	0.006*	$-1.44 \times 10^{-5} \pm 7.61 \times 10^{-5}$	0.851
Luteal phase stimulation	-	-	4 (0.02)	-	$6.29 \times 10^{-9} \pm 2.09 \times 10^{-9}$	0.004*	$-1.93 \times 10^{-5} \pm 6.91 \times 10^{-5}$	0.781

* $P < 0.05$

protocol, including mild stimulation protocol and luteal phase stimulation ($\beta \pm SE$: $8.27 \times 10^{-9} \pm 2.92 \times 10^{-9}$, $6.29 \times 10^{-9} \pm 2.09 \times 10^{-9}$, respectively; $P = 0.006$, $P = 0.004$, respectively) (Table 3) significantly and positively affected *hsa-miR-320a-3p* levels in the human granulosa cells. The days of stimulation were negatively correlated with the relative expression of *hsa-miR-320a-3p* in the human granulosa cells ($\beta \pm SE$: $-6.85 \times 10^5 \pm 3.42 \times 10^5$, $P = 0.0466$) (Table 3). The relative expression of *hsa-miR-320a-3p* in the human granulosa cells were not associated with BMI, basal LH, basal E₂, AMH, AFC and total dose of gonadotropins (Table 3, $P > 0.05$).

The relative expression of *hsa-miR-483-5p* in the human granulosa cells were not associated with age, BMI, female baseline levels, AFC, days of stimulation, total dose of gonadotropins and ovarian stimulation protocol (Table 3, $P > 0.05$).

Discussion

In this study, our results indicated that *hsa-miR-320a-3p* and *hsa-miR-483-5p* expression levels in the human granulosa cells were negatively associated with good-quality embryos and live births. Moreover, *hsa-miR-483-5p* levels were negatively associated with

blastulation. Further studies revealed that *hsa-miR-320a-3p* levels positively correlated with patient age and basal follicle stimulating hormone (FSH) levels.

Follicular fluid content can be used as a noninvasive marker to predict oocyte quality. In our study, we found that a significantly difference in normal fertilization rate those with high *hsa-miR-320a-3p* expression levels during IVF cycles. Further, multi-variable logistic regression analysis indicated that the high expression levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* in granulosa cells seemed to reduce the number of good-quality embryos and live births ($P < 0.0001$). Notably, patients with higher levels of *hsa-miR-483-5p* exhibited a decreasing trend in blastulation. These results suggested a negative effect of *hsa-miR-320a-3p* and *hsa-miR-483-5p* on oocyte development and pregnancy outcomes. Additionally, *hsa-miR-320a-3p* and *hsa-miR-483-5p* have been reported to plays important roles in inhibiting cell proliferation and migration [14–16]. These processes have been proven to affect oocyte development [17]. *hsa-miR-320a-3p* and *hsa-miR-483-5p* in granulosa cells may also acts as apoptosis factors and decrease oocyte development via a paracrine mechanism. Consequently, higher levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p*

in granulosa cells may reduce the developmental competency of oocytes.

Furthermore, our results suggested that *hsa-miR-320a-3p* expression is weakly and positively correlated with patient age ($r^2=0.209$, $P=0.0033$). Anseré et al. revealed that cellular senescence may contribute to ovarian aging, and the subsequent decline in ovarian follicular reserve [18]. In our study, the *hsa-miR-320a-3p* levels were positively associated with basal FSH levels ($r^2=0.257$, $P=0.0003$). It is a useful predictor of ovarian reserve [19, 20], indicating an association between *hsa-miR-320a-3p* and ovarian reserve function. However, no obviously relationship was observed between *hsa-miR-320a-3p* and AMH, AFC or BMI, which have also been reported to affect ovarian functions [21, 22]. These conflicting results may be due to several factors, such as the cause of infertility and ovarian stimulation protocols. The potential associations between *hsa-miR-320a-3p* and FSH may provide a new direction to predict ovarian reserve function.

Many positive regulatory indicators predict the ART outcomes in human granulosa cells, such as circRNA [23], AQP7 [24] and telomerase activity [25]. As negative regulatory indicators, *hsa-miR-320a-3p* and *hsa-miR-483-5p*, can be combined with positive regulatory indicators to make the prediction results more reliable.

Conclusion

The current study indicated that the expression levels of *hsa-miR-320a-3p* and *hsa-miR-483-5p* in granulosa cells are negatively associated with good-quality embryos and live births in women undergoing IVF/ICSI. Notably, patients with higher levels of *hsa-miR-483-5p* exhibited a decreasing trend in blastulation. These results suggest that *hsa-miR-320a-3p* and *hsa-miR-483-5p* could be used as potential indicators to predict the quality of embryos and live births.

Abbreviations

IVF: In vitro fertilization; ICSI: Intracytoplasmic sperm injection; BMI: Body mass index; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; E2: 17 β -estradiol; AMH: Anti-Müllerian hormone; hCG: Human chorionic gonadotrophin; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

Acknowledgements

Thanks to institute of Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology for supplying our experimental samples. We would like to thank Editage (www.editage.com) for English language editing.

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. J.Y. and Q.S. prepared samples. M.Z. and J.L. collected granulosa cells 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.

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).

Availability of data and materials

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

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

Not applicable.

Competing interests

No interest.

Author details

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Received: 22 July 2022 Accepted: 9 November 2022

Published online: 21 November 2022

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