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

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Research

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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/m^2 to 32.40 kg/m^2 (mean ± SD: 22.53 ± 3.39 kg/m^2). Baseline hormonal levels including follicle-stimulating hormone [FSH], luteinizing hormone [LH], and 17β-estradiol [E2] and anti-Mülerian 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 ℃ for 60 min and 85 ℃ 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 ^{TM} qPCR Mix, 2 μl All-in-One ^{TM} 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 ℃ for 600 s, 40 cycles at 95 ℃ for 10 s, 60 ℃ for 20 s and 72℃ for 10 s. The relative quantity of miRNA expression was calculated using the $2^{-\triangle CT}$ method.

Morphological assessment of oocytes, good qualityembryos, 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									*P*-value
	Q1		Q2		Q3		Q4		
	Number of 2PN/number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	
miR-320a-3p									
Total	296/489	60.5	275/457	60.2	296/547	54.1	262/429	61.1	NS
IVF cycles	201/358	56.1	201/361	55.7	203/405	50.1	225/368	61.1	NS
ICSI cycles	95/131	72.5	74/96	77.1	93/142	65.5	37/61	60.7	NS
miR-483-5p									
Total	272/475	57.3	310/531	58.4	256/479	53.4	291/437	66.6	NS
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

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									*P*-value
	Q1	%	Q2	%	Q3	%	Q4	%	
miR-320a-3p									
Good quality embryo rate	212/296	71.6	205/275	74.5	214/296	72.3	149/262	56.9	< 0.0001
Blastocysts rate	81/212	38.2	65/205	31.7	82/214	38.3	55/149	36.9	NS
miR-483-5p									
Good quality embryo rate	225/272	82.7	203/310	65.5	197/256	77.0	155/291	53.3	NS
Blastocysts rate	86/225	38.2	78/203	38.4	55/197	27.9	64/155	41.3	NS

Relationship of the *miR-320a-3p* levels in the granulosa cells and assisted reproductive technology outcomes

The median quantity and the IQR of the *miR-320a-3p* levels in 195 human granulosa cells samples from pregnancy and unpregnant groups were 2.47×10^6 (IQR: 6.42×10^3-1.91×10^6) and 6.21×10^6 (IQR: 4.86×10^3-2.72×10^6). The median quantity and the IQR of the *miR-483-5p* levels from pregnancy and unpregnant groups, abortion and non-abortion groups were 44.32 (IQR: 0.19-5.56) and 10.61 (IQR: 0.15-5.28), 4.77 (IQR: 0.06-6.05) and 46.39 (IQR: 0.23-5.20), respectively.

The relative expression levels of *miR-320a-3p* were associated negetively with clinical pregnancy rate (Table 3, $P<0.05$). No correlation was observed between the relative expression of *miR-320a-3p* levels and abortion rate (Table 3, $P>0.05$). The relative expression of *miR-483-5p* levels were not associated with clinical pregnancy rate and abortion rate (Table 3, $P>0.05$).

Table 3 Association between the levels of *miR-320-3p* and *miR-483-5p* in granulosa cells from human follicular fluids and reproductive outcomes of assisted reproductive technology.

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 ($\beta \pm$ SE: $4.79\times10^5 \pm 1.61\times10^5$, $P=0.0033$) (Table 4, Figure 2). Moreover, both the basal FSH ($\beta \pm$ SE: $7.90\times10^5 \pm 2.14\times10^5$, $P=0.0003$) (Table 4, Figure 2) and ovarian stimulation protocol, including mild stimulation protocol and luteal phase stimulation ($\beta \pm$ SE: $8.27\times10^{-9} \pm 2.92\times10^{-9}$, $6.29\times10^{-9} \pm 2.09\times10^{-9}$, 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 (β ± SE: 17.55 ± 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	Min-Max	Mean	n (%)	SD	*miR-320a-3p*		*miR-483-5p*	
					$\beta \pm$ SE	*P*-value	$\beta \pm$ SE	*P*-value
Age (years)	21-46	34.39	195 (100)	5.19	$4.79\times10^{5}\pm1.61\times10^{5}$	0.0033*	-3.58 ± 3.51	0.3084
BMI (kg/m^2)	8.93-32.40	22.53	194 (99.49)	3.39	1.45×10^{5} ± 2.54×10^{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	7.90×10^{5} ± 2.14×10^{5}	0.0003*	-3.42 ± 4.72	0.4701
Basal LH (IU/L)	0.65-40.02	4.76	195 (100)	3.77	7.61×10^{3} ± 2.27×10^{5}	0.9733	-0.97 ± 4.85	0.8412
Basal E_2 (pg/ml)	2.74-5178	76.17	195 (100)	370.11	-6.09×10^{2} ± 2.3×10^{3}	0.7926	-7.57×10^{-4}± 4.94×10^{-2}	0.9878
AMH (ng/ml)	0.06-14.62	3.52	194 (99.49)	2.93	-1.91×10^{5} ± 2.93×10^{5}	0.5147	17.55 ± 6.14	0.0047*
Antral follicle count	3-52	15.74	195 (100)	7.97	-5.39×10^{4} ± 1.07×10^{5}	0.6159	-0.13 ± 2.30	0.9535
Days of stimulation	5-22	9.97	195 (100)	2.48	-6.85×10^{5} ± 3.42×10^{5}	0.0466*	0.31 ± 7.38	0.9660
Total dose of gonadotropins (IU)	900-6450	2344.27	195 (100)	842.52	-1.39×10^{3} ± 1.01×10^{3}	0.1704	-7.40×10^{-3} ± 2.17×10^{-2}	0.7333
Ovarian stimulation protocol								
Ultra-long protocol	-	-	62 (31.79)	-				Ref
Long protocol	-	-	9 (0.05)	-	-6.05×10^{-9} ± 5.36×10^{-9}	0.263	-2.57×10^{-5} ± 9.61×10^{-5}	0.790
Antagonist protocol	-	-	75 (0.38)	-	-7.10×10^{-9} ± 6.15×10^{-9}	0.250	-1.76×10^{-4} ± 1.42×10^{-4}	0.217
Progestin-primed ovarian stimulation (PPOS)	-	-	40 (0.21)	-	2.19×10^{-9} ± 4.37×10^{-9}	0.617	-1.30×10^{-4} ± 1.40×10^{-4}	0.354
Mild stimulation protocol	-	-	5 (0.03)	-	8.27×10^{-9} ± 2.92×10^{-9}	0.006*	-1.44×10^{-5} ± 7.61×10^{-5}	0.851
Luteal phase stimulation	-	-	4 (0.02)	-	6.29×10^{-9} ± 2.09×10^{-9}	0.004*	-1.93×10^{-5} ± 6.91×10^{-5}	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-320-3p*.

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-Mülerian 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.

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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 fluid 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.

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Figures

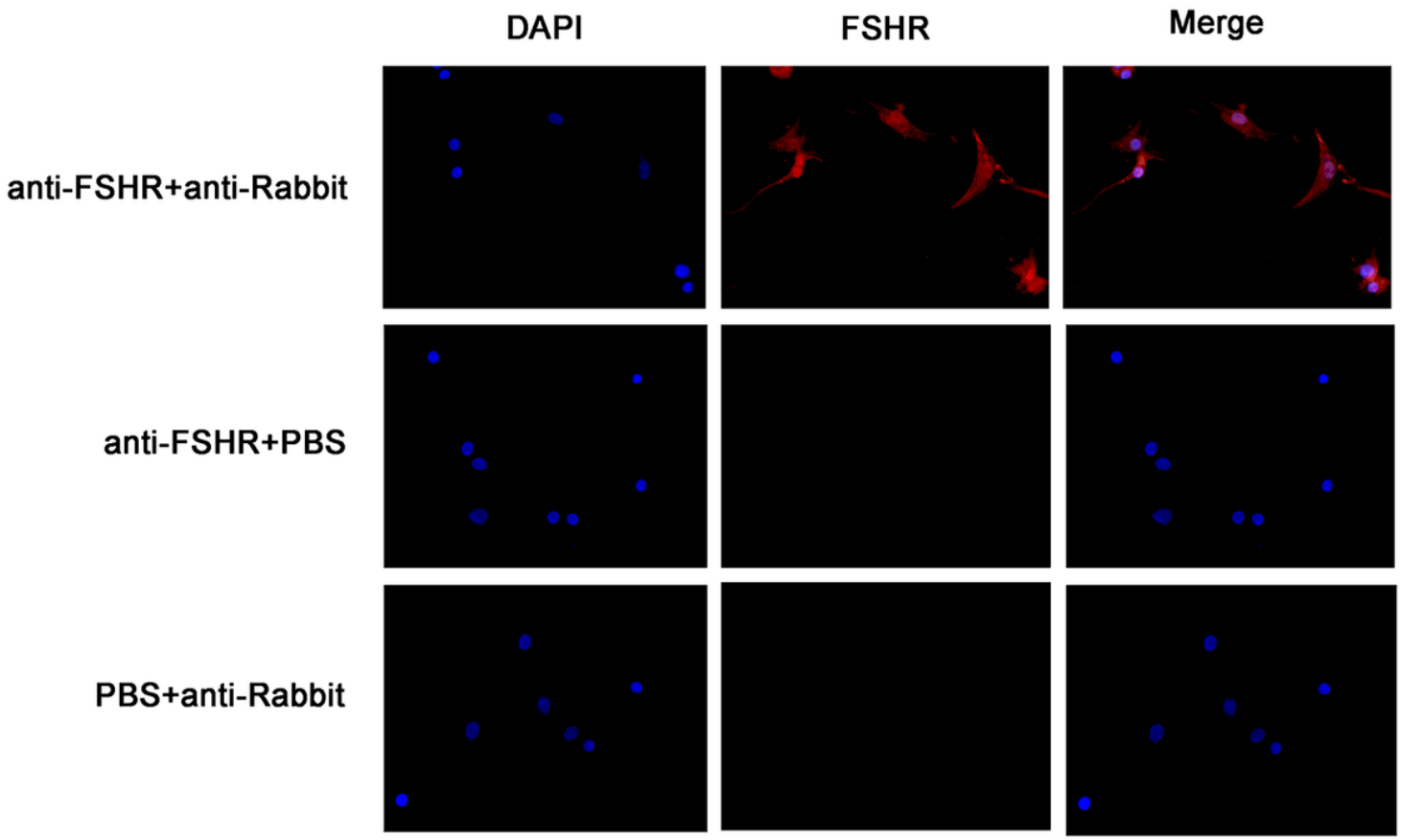

Figure 1

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.

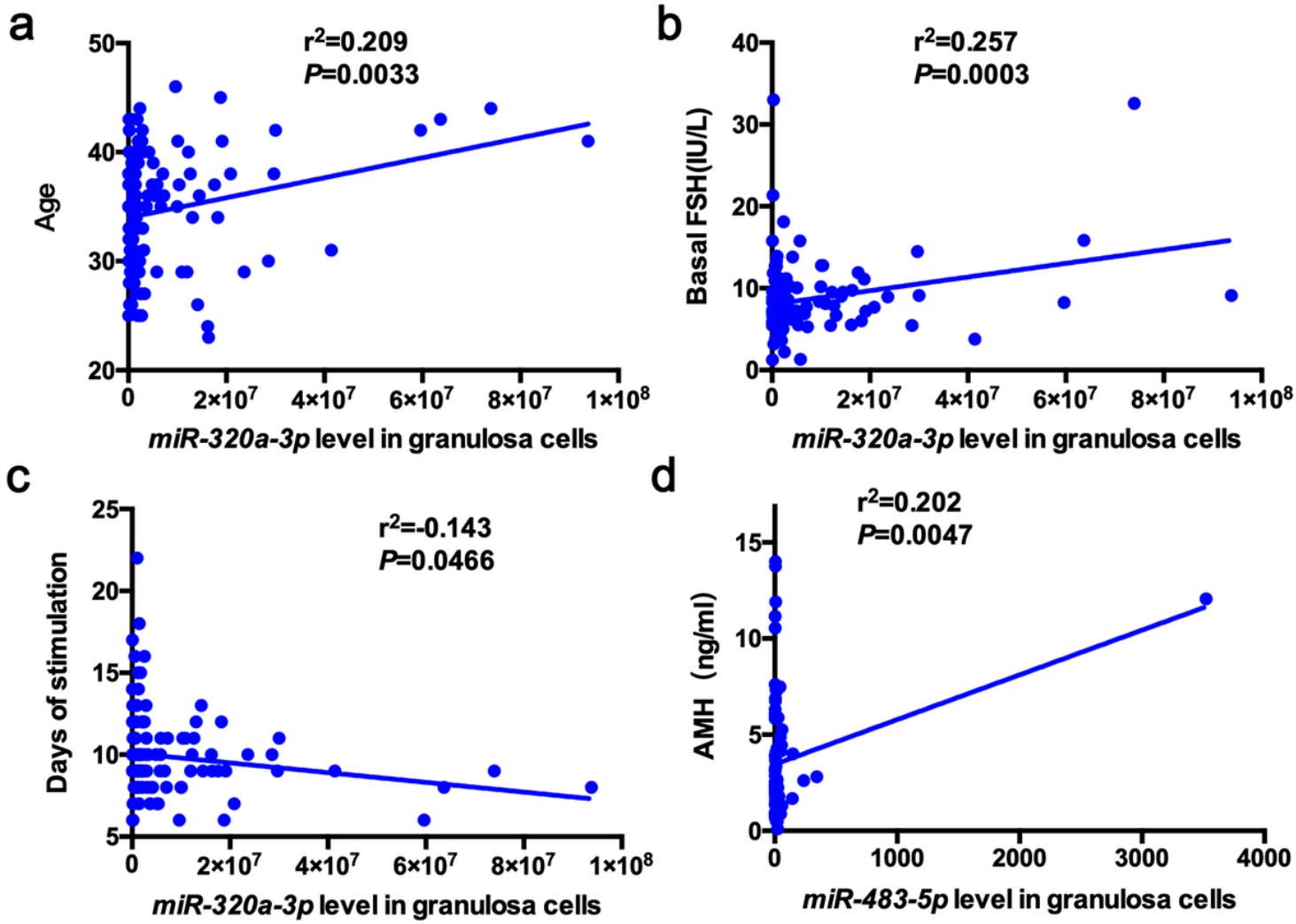

Figure 2

Correlations between the miR-320a-3p in granulosa cells and patient age, basal FSH, days of stimulation, and miR-483-5p and AMH.