MicroRNA Expression Profiles Analysis in Sperm Reveals hsa-mir-191 is an Auspicious Omen of in Vitro Fertilization

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Research article

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Abstract

Background: MicroRNAs(miRNAs) are a class of non-coding small RNAs, which play important roles in many physiological processes by regulating gene expression. Previously studies show that the expression level of total miRNAs increased during the progression of mouse embryonic development, and some miRNAs control the regulatory network in the development progression. However, there are few studies focused on effects of miRNA on the early human embryonic development. The relationship of between miRNAs and early human embryogenesis is still unknown. Results In this study, 102 male sperm samples that were treated with assisted reproductive technology but with normal sperm index were collected for small RNA sequencing, and the relationships between differentially expressed small RNA and fertilization rate, blastocyst rate and high-quality embryo rate were analyzed. The results showed that the sperm samples with high expression of hsa-mir-191 had higher fertilization rate, blastocyst rate and high-quality embryo rate. Using hsa-mir-191 as a single indicator to predict high-quality embryo rate, the ROC curve has an AUC of 0.686. At the same time, we also found that the expression of hsa-mir-191 has a certain correlation with sperm abnormality rate (cor = 0.29, $p < 0.01$). In addition, we also evaluated the relationship between hsa-mir-34c and human early embryo development in these 102 sperm samples, and obtained negative results. Conclusions These findings suggest that higher hsa-mir-191-5p expression with better human early embryonic development, and indicate that the hsa-mir-191-5p could be a potential marker to access the quality of IVF.

Background

MicroRNAs are a class of non-coding small RNAs of 19–24 nt in length, which, after transcription, bind to the 3’untranslated region of the target gene, causing down-regulation of the target gene. Previous studies have shown that the expression characteristics of miRNA are closely related to cell morphology, disease progression, cell differentiation, and gamete maturation[1–3]. However, there are few studies on the relationship between miRNA and human early embryo development.

The scientific view of the role of sperm content in the reproductive process is constantly evolving. Early studies suggested that sperm only played a role in transmitting the paternal genome during the reproductive process. However, more and more studies have shown that the role of sperm in the reproductive process is diversified. The sperm content also contains many coding and non-coding RNAs[4], which play an important role in epigenetics[5, 6].

Epigenetics plays an important role in the early development of embryos, especially miRNAs carried by sperm. Previous studies have also shown that miRNAs in mouse sperm respond to benzo-a-pyrene exposure and reflect gene expression in early developing mouse embryos[7, 8]. Yeung et al. have also reported that mmu-mir–34c carried by sperm is essential for the first cleavage in mice[9]. The pri-miR–181c carried by sperm is processed and matured, and then delivered to the oocyte to regulate the expression of development-related genes[10]. The miR–34 family in bovine sperm is also required for the development of bovine female gametes or male gametes[11]. Increasing the expression of miR–34c in

somatic cell nuclear transfer(SCNT) embryos can not only affect the early development of bovine embryos, increase the cleavage rate of developing embryos, but also change the quality of the resulting SCNT embryos[12]. However, all these studies use animal models, such as mice or cattle, to assess the role of miRNAs in early embryonic development.

In this work, we collect 102 human sperm with various of implant development parameter like fertilization rate (FR), effective embryos rate (EER) and high quality embryos rate (HQER). Our results reveal that a series of miRNAs are involved in embryo preimplantation development. Meanwhile, we evaluate the role of controversial miR−34 family, which are the most previously reported sperm-carrying miRNAs that play an important role in the early embryonic development of mice/bovines, in in vitro fertilization of human beings. These results will help us to further understand of sperm-borne miRNAs' function in embryo development.

Results

Overview of small RNA library sequencing in spermatozoa and samples grouping

To evaluate the role of miRNAs in spermatozoa in the process of embryo during in vitro fertilization, the miRNA profiling in the spermatozoa of 102 patients with in vitro fertilization were investigated by small RNA deep sequencing. Quality control (QC) assessment showed that about 50% reads were filtered after QC. 50% of reads after QC could be mapped to the human reference genome (hg19). However, most of the samples only have 5% mappable reads that could be annotated for known miRNA database (Figure1A). We identified a total of 797 miRNAs out of 2042 known human miRNAs. Hierarchy cluster analysis indicated that the miRNA expressions were significantly different in 102 samples (Figure1B). Samples were divided into different groups based on FR, EER and HEQR to detect differentially expressed microRNAs. Based on FR, we divided the sequencing results into four groups: patients with FR < 20%, accounting for 11.01%; patients with FR between 20%−60%, accounting for 11.01%; patients with FR between 60%−80%, accounting for 29.36%; patients with FR > 80%, accounting for 48.62%. Based on EER, we divided the sequencing results into four groups: patients with EER < 20%, accounting for 25.71%; patients with EER between 20%−60%, accounting for 31.43%; patients with EER between 60%−80%, accounting for 16.19%; patients with EER > 80%, accounting for 26.67%. Based on HQER, we divided the sequencing results into three groups: patients with HQER < 10%, accounting for 38.53%; patients with HQER between 10%−70%, accounting for 34.86%; patients with HQER > 20%, accounting for 26.61% (Figure 1C).We demonstrated that most patients have high FR but not enough embryo. This result may indicate that there is no direct link between fertility and embryo development.

Analysis of differentially expressed miRNAs

A total of 11 miRNAs were identified as differentially expressed miRNAs patterns with inter-group difference of FR (p<0.05), including hsa-mir−191−5p, hsa-mir−320a, hsa-mir−320b, hsa-mir−190b, hsa-mir−423−5p, hsa-mir−20a−5p, hsa-mir−548ay−5p, hsa-mir−153, hsa-mir−548d−5p, hsa-mir−1 and hsa-mir−618 (Figure 2A). A total of 10 miRNAs were identified as differentially expressed miRNAs patterns

with inter-group difference of EER (p<0.05), including hsa-mir–191–5p, hsa-mir–891a, hsa-mir–101–3p, hsa-mir–345–5p, hsa-mir–664a–3p, hsa-mir–19a–3p, hsa-mir–92b–3p, hsa-mir–153, hsa-mir–22–5p and hsa-mir–497–5p (Figure 2B). A total of 4 miRNAs were identified as differentially expressed miRNAs patterns with inter-group difference of HQER (p<0.05), including hsa-mir–191–5p, hsa-mir–200b–3p, hsa-mir–891a and hsa-mir–400a–3p (Figure 2C). Overlapping three sets of differentially expressed miRNAs, we identified only one miRNA, hsa-mir–191–5p, which is differentially expressed in the FR, EER and HQER groups (Figure 2D). This result indicated hsa-mir–191–5p may be a key factor in both fertility and embryo development.

Comparison of miR–191–5p expression in different FR, EER and HQER groups

We used the independent T test to compare the differences of hsa-mir–191–5p in the FR, EER and HQER groups. The results showed that the expression of hsa-mir–191–5p in the highest FR group was significantly higher than that in the lowest FR group (p< 0.01) (Figure 3A). The expression of hsa-mir–191–5p was significantly higher in the highest EER group (p<0.05) (Figure 3B), but there was no significant difference between the other EER groups. In the highest HQER group, the expression of miR–191–5p was significantly higher than that of lowest HQER group (p<0.01) (Figure 3C), but there was also no significant difference between the other HQER groups. This suggested that hsa-mir–191–5p expression in in vitro fertilization patients with high FR, EER and HQER was higher than those with low FR, EER and HQER. In order to further clarify the function of hsa-mir–191–5p during fertilization and embryonic development, we conducted the ROC analysis. The results showed that the area under the ROC curve for FR, EER and HQER groups predicted by hsa-mir–191–5p was 0.612, 0.637 and 0.686, respectively, which indicated that miR–191–5p could be used to predict whether the patients belonged to the highest FR, EER and HQER group, especially in the high HQER group with an AUC close to 0.7 (Figure 3D,E,F). In addition, it further indicated that high expression of hsa-mir–191–5p could lead to better in vitro fertilization results, although its low expression did not indicate that the in vitro fertilization results would be poor.

Analysis of correlations between hsa-mir–191–5p and sperm routine parameters

We investigated the correlation between hsa-mir–191–5p and 3 sperm routine parameters: sperm density, sperm morphology and sperm viability. The results showed that the correlation between hsa-mir–191–5p and sperm density as well as sperm viability was not significant (Figure 4A and C, p> 0.05), while the correlation between hsa-mir–191–5p and sperm morphology was significant (Figure 4B, p< 0.01), but the correlation coefficient was only 0.29, indicating a weak correlation between them. As was shown above, hsa-mir–191–5p may be one of the key molecules to maintain normal sperm morphology.

Overview evaluation of the function of sperm-carrying hsa-mir–34c in early development of human embryos

The function of sperm-carrying mmu-mir–34c in early embryonic development has been discussed in mice, but its function in early development of human embryos has not been studied. In this paper, our

results showed that there was no significant difference in hsa-mir–34c among different groups of FR, EER and HQER (Figure 5A). At the same time, we also investigated the correlation between hsa-mir–34c and three sperm routine parameters, namely sperm density, sperm morphology and sperm viability (Figure 5B). The results showed that hsa-mir–34c had a weak negative linear relationship with sperm morphology ($p<0.05$), but not linearly related to sperm density and sperm viability. These evidences do not indicate that sperm-carrying hsa-mir–34c plays an important role in early development of human embryos. However, hsa-mir–34c may have a certain function during the normal development of sperm morphology.

Discussion

The process of artificial assisted reproductive technology is affected by many factors, and the clinical success rate is only about 30%. This brings economic, physical and mental burdens to the majority of patients, which in turn have a greater impact on the outcome of in vitro fertilization. If high-quality embryos can be selected before embryo transfer, a reasonable interval between embryo development and implantation can be given to reduce the rejection of the uterus and improve the success rate of IVF (ART, Assisted Reproductive Technology). Recent studies have shown that sperm content plays an important role in the development of early embryos. Using sperm content as a biomarker, preferential selection of high-quality sperm can effectively improve early embryonic development and further affect pregnancy outcomes. In this study, high-throughput sequencing was used to deeply sequence the miRNAs carried by a large number of IVF-treated male sperm. The results showed that sperm with high expression of hsa-mir–191 had better early embryo development. This makes it possible to improve the success rate of artificial assisted reproduction through miRNA-based sperm screening.

Hsa-mir–191 is considered to belong to the same family as hsa-mir–425, which is located in the first intron of the DALRD3 gene on human chromosome 3 (3p21.31), encoding four mature miRNAs, hsa-mir–191–5p, hsa-mir–191–3p, hsa-mir–425–5p and hsa-mir–425–3p[13]. Since hsa-mir–191 is present at 381 bases upstream of hsa-mir–425, both miRNAs are generally transcribed simultaneously. However, current studies have shown that hsa-mir–191 expression is significantly higher than hsa-mir–425 in various tissues[14, 15]. It has been reported that hsa-mir–191 is abnormally expressed in several cancers and various other diseases such as type 2 diabetes, Crohn's disease, pulmonary hypertension and Alzheimer's disease. However, only a few reports have shown that hsa-mir–191 is involved in the reproductive process of humans.

Sharma S et al. have reported that hsa-mir–191–5p targets the SOX4 gene and is a key signaling pathway in the development of the embryo[16]. Our results show that the level of hsa-mir–191–5p in sperm is closely related to the process of fertilization and the early development of the embryo. In addition, studies by Grinchuk OV et al. have also shown the association of hsa-mir–191, hsa-mir–425, DALRD3 and NDUFAF3 with spermatogenesis. These genes have significant co-expression relationships in sperm cells of normal individuals, whereas their direct co-expression relationship is not significant in patients with teratozoospermia[17]. Other studies have found that hsa-mir–191 has a higher

concentration in the IVF/ICSI cycle failure medium than the successful medium[18]. This further illustrates the important role of hsa-mir–191 in embryonic development.

The miR–34 family is the most studied miRNA in spermatozoa that may affect early embryonic development. Previous contradictory studies in mice have shown that the parental mmu-mir–34c is essential for the first cleavage[9]. However, it has also been reported that cleavage of mmu-mir–34c knockout male mice is normal[19]. The effect of hsa-mir–34c in human sperm on early embryo development has not been reported. In the present study, we demonstrate that there is no difference in the expression of human hsa-mir–34c between different FR, EER and HQER groups, and there was no strong correlation between the amount of expression and sperm routine parameters. This makes us more inclined that hsa-mir–34c in human sperm is not indispensable in early embryonic development.

Conclusions

This study shows the differences in the miRNA expression profiles of FR, EER, and HQER groups, and suggests that hsa-mir–191–5p expression in in vitro fertilization patients with high FR, EER and HQER was higher than those with low FR, EER and HQER, highlights the possible role of hsa-mir–191–5p in IVF and embryonic development, and could play a key role in maintain normal sperm morphology. All these results suggest that hsa-mir–191–5p would be a potential biomarker to improve the success rate of IVF.

Methods

Samples collection

We recruited 102 couples at Shanghai Jiai Genetics and IVF Institute from May 2011 to December 2012. All couples were in the first IVF cycle. Male participants collected semen samples by masturbation 3 days after sexual abstinence. The sample was added with PureSperm System, and sperm cells were obtained after centrifugation at 500 g for 20 minutes at 25 °C. All semen samples were analyzed for primary semen parameters, including sperm density, motility, viability and morphology, according to the WHO Semen Analysis Manual (5th edition, 2010) to ensure that the recruited males provided normal semen samples.

RNA library preparation and sequencing

We extracted total cellular RNA using TRIzol reagent (Takara) and constructed a small RNA library using approximately 200 ng of total cellular RNA. High-throughput RNA sequencing was performed using Hiseq X Ten (PE150). Cutadapt was used to edit the adapter and filter low quality reads. Reads that could not match adaptor or less than 17 nt in length were discarded. In order to assess the expression level of miRNA, only reads that exactly match the 5’ initiation site of the annotated miRNA, and those with $\leq$ 2 nt deletion at the 3’ terminus or those from the pri-miRNA were counted as miRNA. The reads of individual miRNA were divided by the total reads aligned to the human genome and expressed as RPM for normalization. All data used to obtain the conclusions are presented in the paper. Sequencing data has

been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE110190.

Statistical analysis

All statistics analysis was performed using R statistical software. As for the ratios linked to sequencing data, a density plot adding rugs was provided to show their distributions. Heatmap were performed for the expression value of the entire samples using gplots package. Analysis of variance was used to identity the differentially expressed genes in various FR groups, EER groups and HQER groups, respectively. Genes were considered statistically significant if their *p* value was less than 0.05. Moreover, Venn diagram was generated to discover the common differentially expressed genes in three groups by R package VennDiagram. To determine the difference of the hsa-mir–191–5p expression value between different FR (as well as EER and HQER) subgroups, we performed a series of *student's t tests* and displayed them with boxplots. In addition, *ROC* curve also was utilized in this process to validate the results by package Daim. To confirm this finding, we further performed regression analysis between the expression levels of hsa-mir–191–5p and FR (as well as EER and HQER) value and the Pearson test was used to test the linear relationship.

List Of Abbreviations

IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; QC: quality control; FR: fertilization rate; EER: effective embryos rate; HQER: high quality embryos rate; WHO: world health organization; RPM: reads of exon model per million mapped reads; *ROC:* receiver operating characteristic curve; DALRD3: DALR anticodon binding domain containing 3; NDUFAF3: NADH Dehydrogenase [Ubiquinone] 1 Alpha Subcomplex Assembly Factor 3; GEO: Gene Expression Omnibus; hsa: human sapiens; mmu: mus musculus; SCNT: somatic cell nuclear transfer; miR or miRNA: microRNA;

Declaration

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Institute of Planned Parenthood Research. All the participants in this study provided written informed consent. Sperm samples were collected from human and used in the study according to the approved protocols.

Consent for publication

Not applicable

Availability of data and material

The partial sequencing datasets generated for this study have been deposited in the Gene Expression Omnibus(GEO) under accession number GSE110190, and other sequencing datasets used in the current

study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HX and XW sorted out the data; ZKW,JHL and ZMX processed Samples; MHM and GWC preserved samples; XDL and JW analyze the data; HJS and KW helped to drafted the manuscript; TCZ and XXS conceived of the study, and participated in its design and coordination and drafted the manuscript.

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Not applicable

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Figures

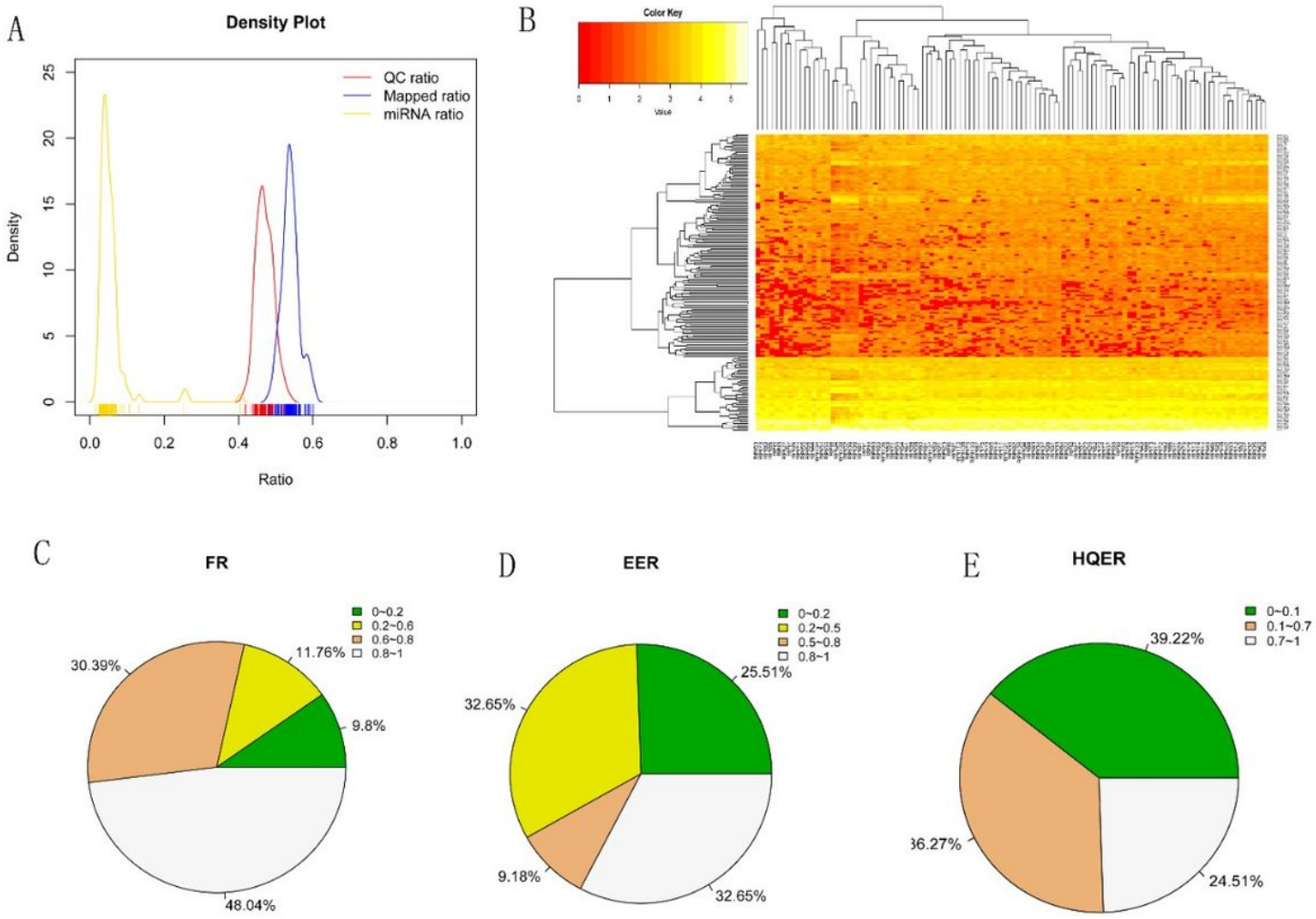


Figure 1

miRNA profile of IVF patient's sperm: a sequencing data quality control (red line), mapping (blue line) and miRNA annotation (yellow line). The ordinate indicates the number of samples, and the abscissa

indicates the ratio of the reads obtained by the corresponding analysis to the total reads before analysis. b Heat map of miRNA expression characteristics. Yellow represents low expression and red represents high expression. The abscissa of the heat map shows the sample name and the ordinate shows the miRNA name. c-e Patients were grouped according to FR, EER and HQER.

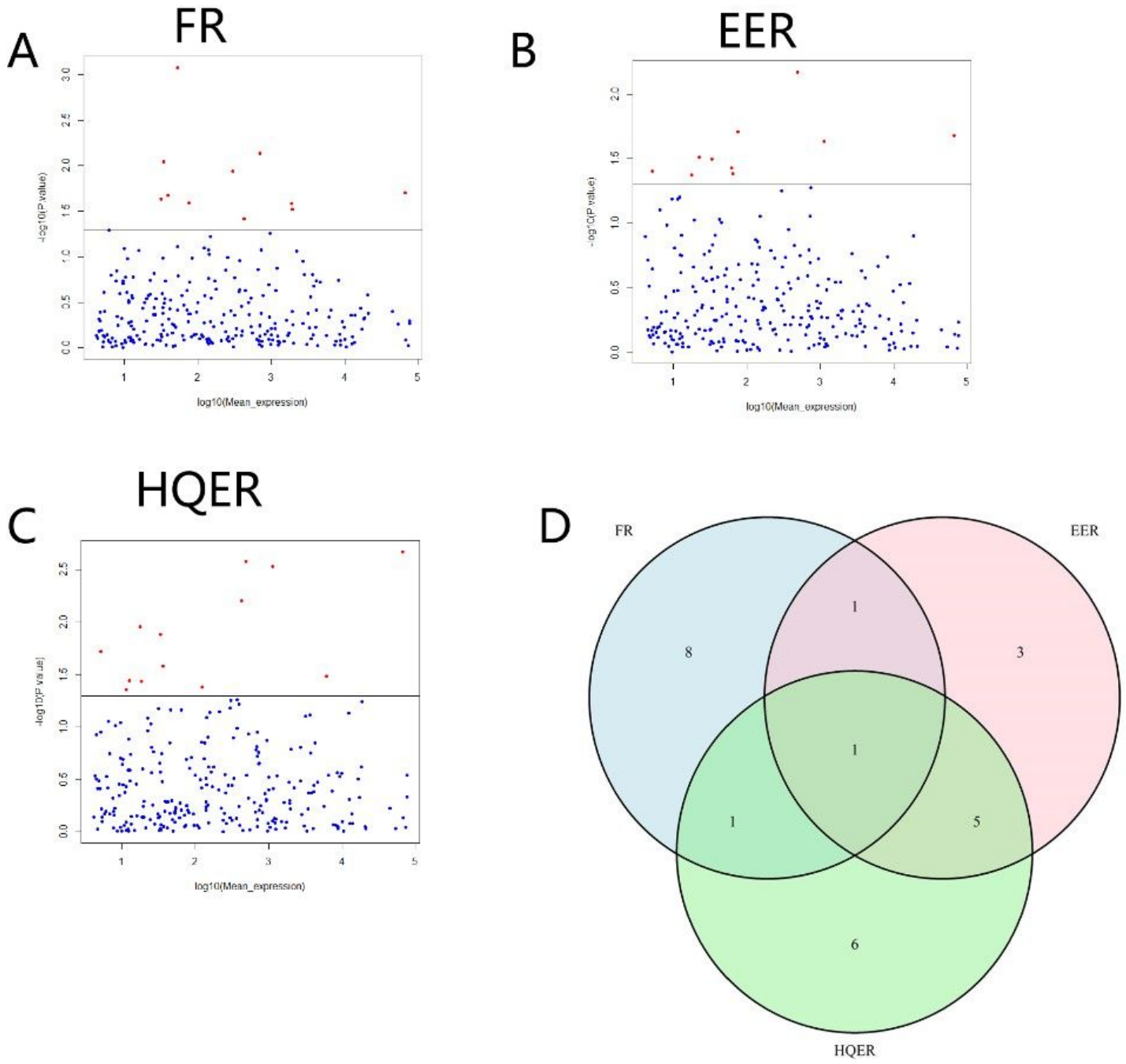


Figure 2

Differential expression analysis of miRNA in sperm: (a-c) Differentially expressed miRNAs were screened in different subgroups of FR, EER and HQER, with red dots indicating differentially expressed miRNAs and blue dots indicating non-differentiated expressed miRNAs. d hsa-mir-191 differentially expressed in FR,

EER and HQER (in vitro fertilization embryo development). The p value corresponding to the baseline in the figure is 0.05, and the red dots above the baseline is the difference point.

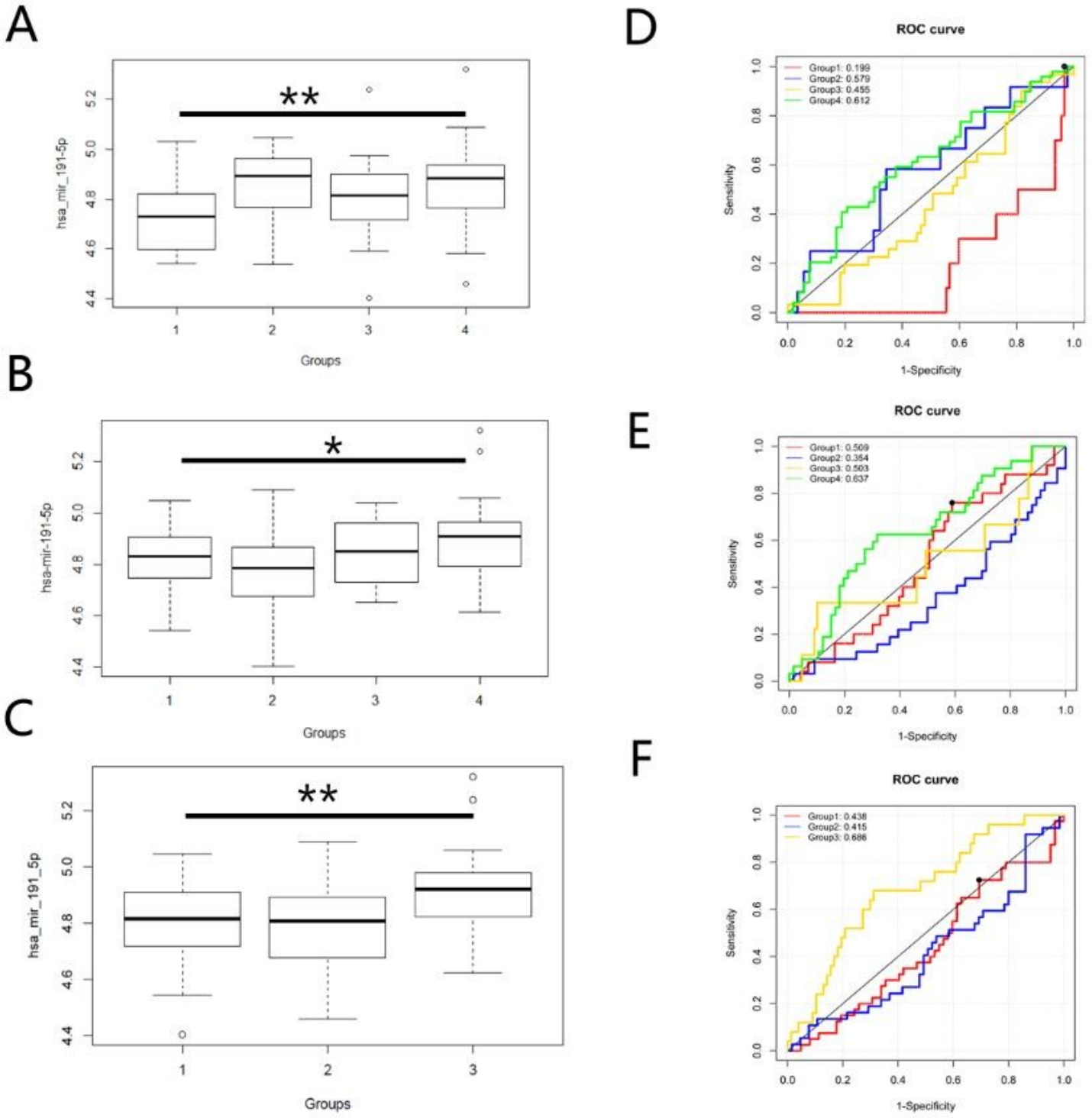
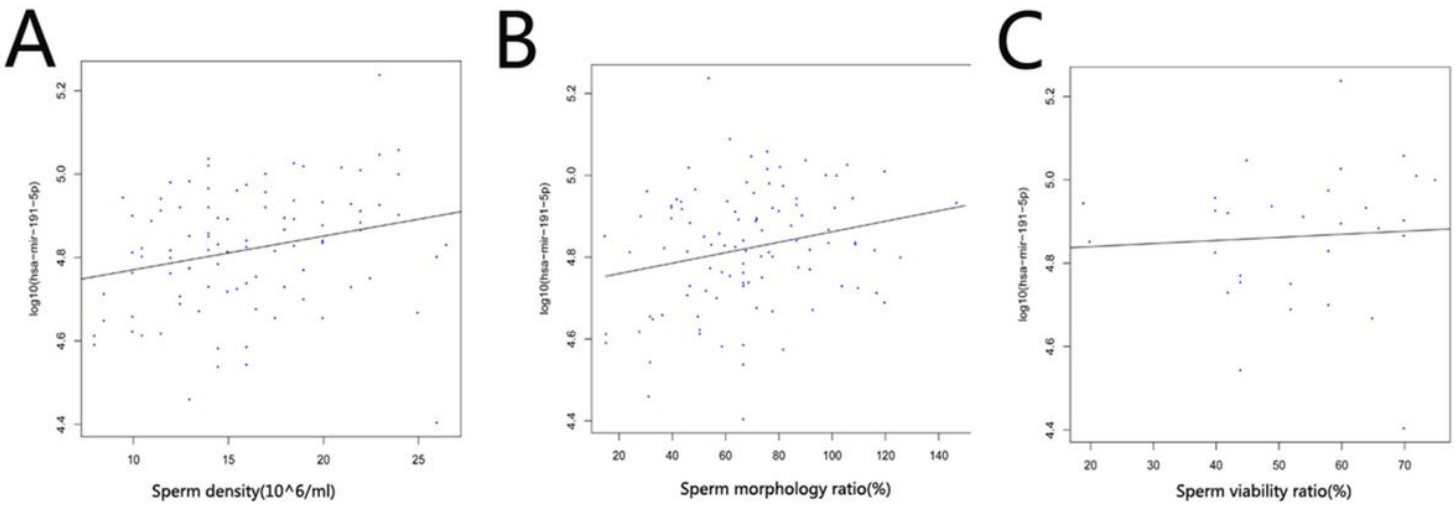


Figure 3

Comparison of hsa-mir-191-5p expression: a Differential expression of hsa-mir-191-5p in sperm samples from patients with different FR groups. b Differential expression of hsa-mir-191-5p in sperm samples from patients with different EER groups. c Differential expression of hsa-mir-191-5p in sperm samples from patients with different HQER groups. d Using the hsa-mir-191-5p as a single predictor, the ROC

curves of patients in different FR groups were obtained. e Using the hsa-mir-191-5p as a single predictor, the ROC curves of patients in different EER groups were obtained. f Using the hsa-mir-191-5p as a single predictor, the ROC curves of patients in different HQER groups were obtained.



hsa-mir-191-5p and Sperm density		
	cor	*p*.value
Pearson's product-moment corelation	0.19093	0.05457
hsa-mir-191-5p and Sperm morphology		
	cor	*p*.value
Pearson's product-moment corelation	0.29352	0.0037
hsa-mir-191-5p and Sperm viability		
	cor	*p*.value
Pearson's product-moment corelation	0.13228	0.4859

Figure 4

Analysis of the relationship between the expression of hsa-mir-191-5p and clinical sperm routine parameters: a The relationship between the expression level of hsa-mir-191-5p and sperm density was calculated by the Pearson method. b The relationship between the expression level of hsa-mir-191-5p and sperm morphology was calculated by the Pearson method. c The relationship between the expression level of hsa-mir-191-5p and sperm viability was calculated by the Pearson method.

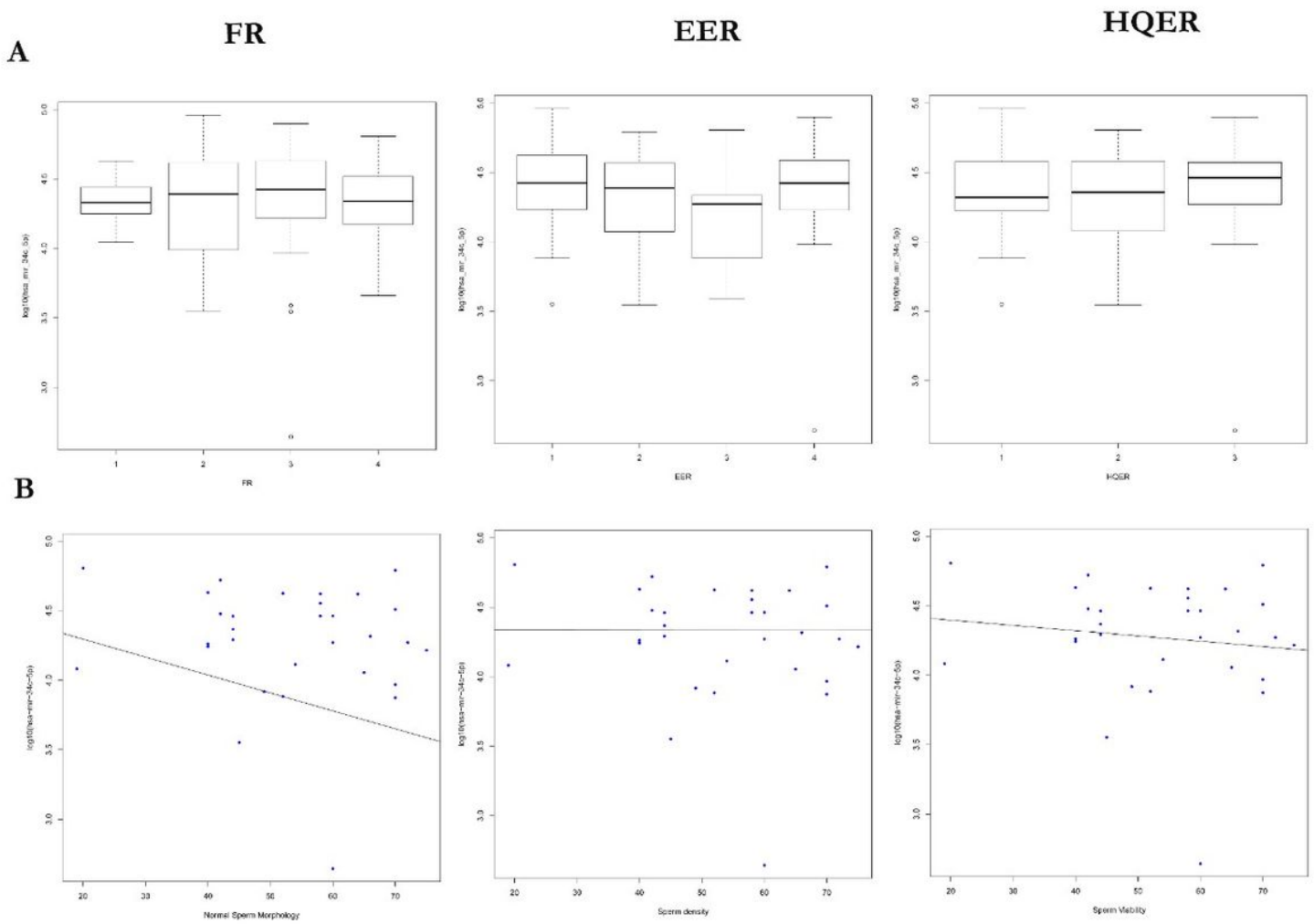


Figure 5

Expression characteristics of hsa-mir-34c: a Differential expression of hsa-mir-34c between different FR, EER and HQER. b Correlation analysis between hsa-mir-34c and sperm density, morphology and viability.