ORIGINAL ARTICLE: GENETICS

New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa

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Objective: To characterize the microRNA (miRNA) expression profile in spermatozoa from human fertile individuals and their implications in human fertility.
Design: The expression levels of 736 miRNAs were evaluated using TaqMan arrays. Ontologic analyses were performed to determine the presence of enriched biological processes among their targets.
Setting: University research and clinical institutes.
Patient(s): Ten individuals with normal seminogram, standard karyotype, and proven fertility.
Intervention(s): None.
Main Outcome Measure(s): Expression levels of 736 miRNAs, presence of enriched metabolic routes among their targets, homogeneity of the population, influence of demographic features in the results, presence of miRNA stable pairs, and best miRNA normalizing candidates.
Result(s): A total of 221 miRNAs were consistently present in all individuals, 452 were only detected in some individuals, and 63 did not appear in any sample. The ontologic analysis of the 2,356 potential targets of the ubiquitous miRNAs showed an enrichment of processes related to cell differentiation, development, morphogenesis, and embryogenesis. None of the miRNAs were significantly correlated with age, semen volume, sperm concentration, motility, or morphology. Correlations between samples were statistically significant, indicating a high homogeneity of the population. A set of 48 miRNA pairs displayed a stable expression, a particular behavior that is discussed in relationship to their usefulness as fertility biomarkers. Hsa-miR-532-5p, hsa-miR-374b-5p, and hsa-miR-564 seemed to be the best normalizing miRNA candidates.
Conclusion(s): Human sperm contain a stable population of miRNAs potentially related to embryogenesis and spermatogenesis. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Infertility, microRNA, spermatogenesis, embryogenesis, sperm biomarkers

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In the last decade, many different small RNAs and their main roles have been described in humans (1, 2). Small RNAs cover many different classes of noncoding RNAs, each of them with particular properties and functions. Small RNAs include small interfering RNAs, small nuclear RNAs, small nucleolar RNAs, piwi-interacting RNAs, transcription initiation RNAs, circular RNAs, and microRNAs (miRNAs) (3). These last ones

have emerged as principal regulatory elements of gene expression (3, 4).

MicroRNAs are a family of functional RNA molecules of 22–24 nucleotides (nt) that form imperfect complementary stem-loop structures in the 3′ untranslated region of their target messenger RNA (mRNA). They are synthesized in the nucleus by RNA polymerase-II as primary molecules. These molecules are processed in catalysts groups called DGCR8 and DROSHA to a 60–70-nt miRNA precursor and exported to the cytoplasm via exportin-5′, where they get processed by the DICER and TAR RNA binding protein (TRBP) complex. The miRNA function is performed by miRNA-induced silencing complex (miRISC) that contains the Argonaute family proteins. MicroRNAs can act by decreasing target mRNA levels or inhibiting their translation (5, 6).

Until now, 1,872 miRNAs have been identified in humans (Sanger miRBase v.20.0; www.mirbase.org) (7). It is known that each miRNA has hundreds of potential mRNA targets, and it has been estimated that they can regulate up to 60% of protein-coding genes (8).

MicroRNAs have been shown to play an important role in many biological processes related to the cell cycle, such as cell development, proliferation, differentiation, metabolism, and apoptosis (9–11). Moreover, dysfunction of miRNAs has been associated with cancer (2, 12), neurologic affections (13), and cardiovascular (14–16) or metabolic disorders (17).

In human spermatozoa, the total RNA content is estimated to be 10–400 fg per sperm cell (18–21), and it has been described to contain a complex population of RNAs that include mRNAs, piwi-interacting RNAs, and miRNAs (20, 22–24). Some studies have proposed that these transcripts are not just random remnants from early spermatogenesis stages but constitute a stable population that has been selectively retained (25–27), which suggests that they play an important role in early zygotic development (25).

Regarding the population of miRNAs, some authors have recently identified altered expression profiles in males with different alterations of the seminal parameters, pointing out a fundamental role of these molecules in fertility regulation (28–30). Nevertheless, these previous studies have some limitations related to the number of miRNAs analyzed (28), the reliability of the control samples used (29, 30), or the acquisition of profiles not corresponding to the male gamete content (28, 30). In this sense a complete and reliable profile of the miRNAs present in spermatozoa from fertile and healthy individuals has not yet been established.

With the aim of overcoming these limitations, the present study was designed to deeply characterize the population of miRNAs present in spermatozoa from a set of accurately defined control individuals. We assessed the expression pattern of 736 miRNAs in 10 normozoospermic individuals with proven fertility using real-time quantitative polymerase chain reaction (qPCR). This included the evaluation of the present/absent miRNAs, their relative abundance and stability, and the presence of enriched metabolic routes among their targets. To validate the homogeneity degree of the population analyzed, we evaluated the possible influence of the demographic features (seminal parameters and age) in the results, as well as the similarity of the expression patterns among individuals. Finally, the presence of stable pairs as potential fertility biomarkers and the best miRNA normalizing candidates were also identified.

MATERIALS AND METHODS

Study Population and Sample Collection

Ejaculated samples from 10 healthy donors were obtained. These individuals presented (Supplemental Table 1, available online): [1] normal karyotype, [2] proven fertility, [3] total number of spermatozoa per ejaculate with progressive motility (grades a+b) above 9×10^7, [4] more than 4% of normal forms, and [5] more than 1×10^7 spermatozoa/mL with progressive motility after post-thawing cryosurvival test (World Health Organization 2010 criteria) (31).

The individuals included in this study were recruited by the Laboratorio de Andrología y Banco de Semen from the Instituto Valenciano de Infertilidad (IVI Valencia, Spain). Written informed consent was obtained from all patients. The study was approved by the IVI Valencia and Universitat Autònoma de Barcelona ethics committees.

Sperm RNA Isolation

To eliminate any possible somatic cells present in the ejaculate, semen samples were processed according to the somatic cell lysis (SCL) method (19). Briefly, cells were incubated on ice for 30 minutes in SCL buffer (0.1% sodium dodecyl sulfate and 0.5% Triton X-100 in milliQ water). Optical microscopic examination was used to verify the somatic cell elimination. Otherwise, SCL treatment was repeated until less than one somatic cell per 10,000 spermatozoa was obtained.

Total sperm RNA was isolated using the Trizol method (Life Technologies). Briefly, 1 mL of Trizol reagent plus 0.2 mL of chloroform (Sigma-Aldrich) were added per 1–10 $\times 10^6$ sperm cells. The mixture was centrifuged, and the upper aqueous phase, containing the RNA, was carefully transferred to a 2.0-mL tube. Isopropyl alcohol was added to precipitate the RNA. After centrifugation, the pellet was washed three times with 75% ethanol and dissolved in 10 μL of diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich). Finally, all samples were treated with 1 μL (2 U/μL) per 10 μg RNA of rDNaseI (Life Technologies) to eliminate any trace of DNA and were stored at −80°C until further analysis.

Sperm RNA Quantification

Total RNA concentration and purity were quantified using the Nanodrop-2000 (Thermo Fisher Scientific). Samples of RNA were subjected to three quality controls. First, to exclude the presence of any potential DNA contamination, reverse transcription–polymerase chain reaction (RT-PCR) followed by PCR for Protamine 1 gene (*PRM1*) and for Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) was performed. Accordingly, 5–10 ng of total RNA were converted to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) supplemented with 0.1 μL RNase inhibitor per sample (20 U/μL). After RT, a conventional PCR with exon–exon primers for *PRM1* and