Universitat Autònoma de Barcelona

Departament de Biologia Cel·lular, Fisiologia i Immunologia

Contingut i funció dels miRNAs de l'espermatozoide humà: implicacions reproductives

Memòria presentada per Albert Salas-Huetos

per optar al grau de Doctor per la Universitat Autònoma de Barcelona

dins el programa del doctorat en Biologia Cel·lular

Autor:

Albert Salas-Huetos

Directors:

Dra. Ester Anton Martorell i Dr. Joan Blanco Rodríguez

Bellaterra, 26 de novembre de 2015

Capítol 5. Publicacions

Publicació I

Títol: Análisis de la expresión de 4 micro-ARN en espermatozoides y su implicación en la fertilidad masculina

Autors: Albert Salas-Huetos, Joan Blanco, Francesca Vidal i Ester Anton

Revista: Revista Internacional de Andrología, **2012**;10(3):92-97

Factor d'impacte: 0.256 (2012), Q4, *Andrology*

Citat en:

1 article

Rev Int Androl. 2012;**10(3)**:92-97

Revista Internacional de Andrología

www.elsevier.es/andrologia

ORIGINAL

Análisis de la expresión de 4 micro-ARN en espermatozoides y su implicación en la fertilidad masculina

Albert Salas-Huetos, Joan Blanco, Francesca Vidal y Ester Anton*

Unitat de Biologia Cel·lular, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra, Cerdanyola del Vallès, Barcelona, España

Recibido el 3 de mayo de 2012; aceptado el 25 de mayo de 2012

PALABRAS CLAVE
Infertilidad masculina;
Espermatozoides;
Transcriptoma;
Mi-ARN

Resumen

Introducción: Los micro-ARN (mi-ARN) son moléculas de 22-24 nucleótidos implicadas en la regulación de la expresión génica de numerosos procesos. Recientemente, se han identificado perfiles alterados de expresión de mi-ARN en diferentes casos de infertilidad idiopática, sugiriendo un papel relevante de estas moléculas en la regulación de la fertilidad.
Objetivo: Caracterizar los patrones de expresión de 4 mi-ARN en ácido ribonucleico espermático procedente de individuos fértiles e infértiles.
Material y métodos: Se obtuvo la fracción espermática de 4 muestras de semen de individuos fértiles y 4 muestras de individuos que consultaban por infertilidad. Se aisló el ácido ribonucleico utilizando el método TRIzol® y seguidamente se efectuó un tratamiento con DNasa. La ausencia de ácido desoxirribonucleico en las muestras extraídas se confirmó mediante una reacción en cadena de la polimerasa para el gen de la protamina 1. Seguidamente, con cebadores específicos para los mi-ARN hsa-miR-23a, hsa-miR-744, hsa-let-7f y hsa-miR-1, y el normalizador Mamm-U6, se realizaron reacciones en cadena de la polimerasa a tiempo real mediante tecnología TaqMan. La cuantificación de las diferencias de expresión se realizó mediante el cálculo del estadístico *Fold Change* y la utilización de un intervalo de confianza del 95%.
Resultados: Para cada individuo, se obtuvo una media de $2,6 \times 10^{-5}$ng de ácido ribonucleico/espermatozoide con una pureza de 1,72 (± 0,05) y sin trazas de ácido desoxirribonucleico. El análisis TaqMan reveló la presencia de hsa-miR-23a, hsa-miR-744 y hsa-let-7f en los espermatozoides de todos los individuos estudiados. La expresión de hsa-let-7f mostró una reducción significativa en 3 de los 4 individuos problema respecto a los individuos control.
Conclusión: Los espermatozoides humanos contienen los mi-ARN hsa-miR-23a, hsa-miR-744 y hsa-let-7f. Este último muestra una reducción significativa de expresión en 3 de los 4 individuos infértiles analizados sugiriendo la implicación de alteraciones en la expresión de mi-ARN como causa subyacente de algunos casos de infertilidad masculina.

*Autor para correspondencia
Correo electrónico: ester.anton@uab.cat (E. Anton).

KEYWORDS
Male infertility;
Spermatozoa;
Transcriptome;
miRNAs

Analysis of the expression of four microRNAs in spermatozoa and their role in male fertility

Abstract
Introduction: MicroRNAs (miRNAs) are 22-24nt molecules involved in the gene expression regulation of many biological processes. Several authors have recently identified altered miRNA expression profiles in cases of male idiopathic infertility, suggesting its fundamental role in fertility regulation.
Objective: To characterize the expression of four miRNAs in sperm ribonucleic acid from fertile and infertile men.
Material and methods: Four ejaculated samples from fertile donors and four samples from idiopathic infertile patients were obtained. Total RNA was isolated using the *TRIzol* method followed by a DNase treatment. To confirm proper ribonucleic acid purification, a polymerase chain reaction for the Protamine-1 gene (PRM-1) was performed. Afterwards, real-time polymerase chain reaction with specific primers for the miRNAs hsa-miR-23a, hsa-miR-744, hsa-let-7f and hsa-miR-1, together with the normalization control Mamm-U6, were performed. Differences in expression were quantified by the Fold-Change statistic and 95% confidence intervals.
Results: For each individual, an average of 2.6×10^{-5}ng ribonucleic acid/spermatozoa was obtained, with a purity of 1.72 (±0.05) and no traces of deoxyribonucleic acid. *TaqMan* analysis confirmed the presence of hsa-miR-23a, hsa-miR-744 y hsa-let-7f in all the samples. The expression of hsa-let-7f was significantly lower in three of the four samples from men with idiopathic infertility.
Conclusion: Human spermatozoa contain the miRNAs hsa-miR-23a, hsa-miR-744 and hsa-let-7f. Hsa-let-7f expression was significantly reduced in three out of four infertile patients, suggesting the involvement of alterations in miRNA expression as the underlying cause of some cases of male infertility.

Introducción

Los micro-ARN (mi-ARN) son moléculas de ácido ribonucleico (ARN) funcionales, formadas por una cadena de 22-24 nucleótidos que forman estructuras semicomplementarias en las regiones 3' no traducidas de los ARN mensajeros a los que regulan[1,2].

Los mi-ARN se sintetizan en el núcleo en forma de moléculas precursoras primarias. Éstas son procesadas por grupos catalizadores llamados DGCR8 y DROSHA hasta las moléculas precursoras secundarias, de 60-70 nucleótidos. Éstas son exportadas al citoplasma, vía exportina-5', y procesadas por los complejos TRBP y DICER. Después del procesamiento, los mi-ARN se unen a proteínas de la familia Argonauta, formando los complejos *miRNA-induced silencing complex*[3]. Estos complejos regulan la expresión génica, inhibiendo o activando la traducción de determinados ARN mensajeros[4,5].

En humanos, se han identificado hasta 1.527 mi-ARN[6] (miRBase, versión 18.0, http://microrna.sanger.ac.uk), muchos de ellos relacionados con la regulación del ciclo celular[7,8], el desarrollo embrionario[9] y la regulación de la gametogénesis, tanto masculina[10] como femenina[11].

Por otra parte, alteraciones en los perfiles de expresión de mi-ARN se han vinculado con procesos biológicos anormales como el cáncer[8,12,13], afecciones cardíacas[14], trastornos neuronales[15] o alteraciones respiratorias[16], entre otros. Algunos autores también han identificado perfiles alterados de expresión de mi-ARN en tejido testicular de individuos con infertilidad idiopática[17,18], sugiriendo un papel relevante de estas moléculas en la regulación de la fertilidad. Lian et al[17,18] identificaron pacientes con azoospermia no obstructiva con alteraciones de la expresión de mi-ARN en células de la línea germinal. McCallie et al[18] observaron que blastocistos transferibles obtenidos de parejas con síndrome de ovario poliquístico e infertilidad por factor masculino mostraban una disminución significativa en la expresión de mi-ARN en comparación con los blastocistos de donantes fértiles.

Está descrito que el transcriptoma del espermatozoide humano contiene más de 5.000 ARN mensajeros[9], más de 35 mi-ARN[19] y unos 1.100 *piwi-interacting RNAs*[19]. *También se ha demostrado que* algunos de estos transcritos son necesarios para el desarrollo del zigoto y el embrión[20-25], indicando que la presencia de los diferentes tipos de ARN en el espermatozoide maduro no sería el resultado de un proceso estocástico[23].

En este estudio hemos caracterizado los patrones de expresión de 4 mi-ARN en ARN espermático procedente de individuos fértiles e infértiles. Este trabajo marca el punto de partida de un proyecto global destinado a determinar si los patrones de mi-ARN presentes en espermatozoides de individuos con infertilidad idiopática son diferentes a los patrones de mi-ARN en espermatozoides de individuos fértiles.

Material y métodos

Se analizaron muestras de semen procedentes de 4 pacientes infértiles y 4 individuos fértiles o control. Las muestras control fueron facilitadas por el Laboratorio de Andrología y Banco de Semen del Instituto Valenciano de Infertilidad de Valencia (casos C1-C4). Los individuos que conformaban esta población presentaban: cariotipo normal, fertilidad probada, recuentos superiores a 90 × 10^6 espermatozoides totales con motilidad progresiva, más del 14% de formas normales (criterios estrictos de Kruger[26]) y recuentos superiores a 10 × 10^6 espermatozoides/ml con motilidad progresiva después del test de supervivencia posdescongelación (criterios de la Organización Mundial de la Salud, 2010[27]). Las muestras de semen de individuos que consultaban por infertilidad procedían de la Unidad de Reproducción Asistida del Centro Médico Teknon de Barcelona, y se les atribuyó un origen idiopático después del examen andrológico rutinario (exploración física, historia reproductiva, perfil hormonal y seminal) (casos P1-P4, tabla 1). Los protocolos utilizados fueron aprobados por los comités de ética de los centros colaboradores.

La fracción espermática fue separada del resto de células del eyaculado mediante la lisis de las células somáticas utilizando una solución de 0,1% dodecilsulfato sódico y 0,5% Tritón X-100 en H_2O miliQ, según el protocolo descrito por Goodrich et al[28].

El ARN total de los espermatozoides fue extraído mediante el método TRIzol® (Invitrogen™, LifeTechnologies, EE. UU.), basado en el protocolo de Chomczynski y Sacchi[29,30], y seguidamente purificado mediante un tratamiento con DNasa recombinante (Ambion®, LifeTechnologies, EE. UU.). La calidad y cantidad de ARN extraído se determinó utilizando un espectrofotómetro UV/visible (Nanodrop-2000; ThermoScientific Inc., EE. UU.).

Una fracción de cada una de las muestras de ARN obtenidas fue retrotranscrita a cDNA mediante reacción en cadena de la polimerasa con transcriptasa inversa (RT-PCR) y posteriormente amplificada por PCR con cebadores exón-exón para el gen de la protamina 1 sintetizados por Roche Applied® (tabla 2). Con esta estrategia se puede verificar la integridad de los transcritos obtenidos y diferenciar los productos resultantes de la amplificación de ARN de los resultantes de la amplificación de ácido desoxirribonucleico (ADN).

Una vez confirmada la ausencia de ADN, se realizaron RT-PCR adicionales utilizando el kit TaqMan MicroRNA Reverse Transcription (Applied Biosystems, LifeTechnologies, EE. UU.), para los siguientes mi-ARN: hsa-miR-23a, hsa-miR-744, hsa-let-7f y hsa-miR-1, y el gen normalizador Mamm-U6. El cDNA se diluyó con los cebadores correspondientes (TaqMan MicroRNA Assays, Applied Biosystems, LifeTechnologies, EE. UU.), y se realizó la PCR a tiempo real con el kit TaqMan Universal PCR Master MixII (Applied Biosystems, LifeTechnologies, EE. UU.). Se analizaron 3 réplicas para cada muestra mediante la plataforma 7900HT Fast Real-Time PCR (Applied Biosystems, LifeTechnologies, EE. UU.).

La cuantificación de las diferencias de expresión se determinó mediante el cálculo del estadístico *Fold Change* obtenido a partir de la variable Ct normalizada (△Ct) por Mamm-U6. El objetivo de la normalización es reducir la variación técnica dentro de un conjunto de datos y, por tanto, permitir una mejor apreciación de la variación de expresión. La variable Ct indica el ciclo de PCR en que los mi-ARN son detectados, siendo un reflejo de sus niveles de expresión. El valor de *Fold Change* se calculó a partir de la relación numérica entre cada uno de los △Ct obtenidos en individuos problema y la media de los valores △Ct de los 4 individuos control. Los valores de *Fold Change* < 1 son indicativos de una menor expresión, mientras que valores > 1

Tabla 1 Características de las muestras problema analizadas

Muestras	Concentración (espz./ml)[a]	Motilidad (% PR + % NP)[a]	Morfología (% FN)[b]	
P1	27,1 × 10^6	40	8	Astenoteratozoospermia
P2	120,6 × 10^6	50	30	Normozoospermia
P3	47,2 × 10^6	60	10	Teratozoospermia
P4	41,7 × 10^6	45	31	Normozoospermia

[a]: criterios de la Organización Mundial de la Salud 2010[27]; [b]: criterios de Kruger[26]; espz./ml: espermatozoides por mililitro; FN: formas normales; NP: no progresivos; PR: progresivos.

Tabla 2 Cebadores y tamaños de los productos resultantes en la detección de protamina 1 mediante PCR convencional

Gen (n.º acceso GenBank)	Secuencias cebador (dirección 5'-3')	Posición secuencias cebador	Tamaño amplicón cADN	Tamaño amplicón ADN
PRM-1 (NM_002761.2)	For. CAGAGTTCCACCTGCTCACA Rev. AGAACTTTTACGGTGGTAGG	14-34 415-435	331pb	422 pb (Chr16:11,374,707-11,375,129)

ADN: ácido desoxirribonucleico; PCR: reacción en cadena de la polimerasa; PRM-1: protamina 1.

Tabla 3 Cantidad y pureza del ácido ribonucleico recuperado en las muestras control (C1-C4) y problema (P1-P4)

	Muestras	ARN/espz. (ng)	Pureza
Controles	C1	$1,52 \times 10^{-5}$	1,67
	C2	$1,88 \times 10^{-5}$	1,72
	C3	$2,26 \times 10^{-5}$	1,75
	C4	$1,19 \times 10^{-5}$	1,73
Problemas	P1	$3,63 \times 10^{-5}$	1,71
	P2	$2,65 \times 10^{-5}$	1,63
	P3	$3,04 \times 10^{-5}$	1,77
	P4	$4,07 \times 10^{-5}$	1,81
Total	media (± DE)	$2,6 \times 10^{-5}$ ($\pm 2,5 \times 10^{-5}$)	1,72 (± 0,05)

ARN/ espz.: cantidad de ARN por espermatozoide; DE: desviación estándar.

indican un incremento de expresión. Se consideraron significativas las diferencias de expresión cuando el valor de △Ct se situaba fuera del intervalo de confianza del 95% de △Ct en las muestras control.

Resultados

Las extracciones de ARN total realizadas en espermatozoides mediante la utilización del método TRIzol® permitieron obtener una media de $2,6 \times 10^{-5}$ ng de ARN (equivalente a 26 fg por espermatozoide). El grado medio (± DE) de pureza de la muestras fue de 1,72 (± 0,05) (tabla 3).

Los resultados de las PCR para el gen de protamina 1 confirmaron la presencia de este transcrito en las muestras analizadas y descartaron contaminaciones de ADN genómico, ya que solamente aparecieron amplificados los productos con medidas correspondientes a cADN (fig. 1).

Los resultados del ensayo TaqMan para los 4 mi-ARN identificaron la presencia de 3 de ellos tanto en las muestras problema como control (fig. 2): hsa-miR-23a, hsa-miR-744 y hsa-let-7f. En cambio, ninguna de las muestras mostró resultados de Ct para miR-1 compatibles con la presencia de esta molécula en espermatozoides humanos. Dos de los 3 mi-ARN identificados, hsa-miR-23a y hsa-miR-744, mostraron niveles de expresión equivalentes en muestras control y problema, mientras que hsa-let-7f presentó una reducción significativa de expresión en 3 de los 4 individuos problema. La reducción de expresión resultó significativa en los individuos P1 (astenoteratozoospérmico), P3 (teratozoospérmico) y P4 (normozoospérmico), no siendo significativa en el individuo P2 (normozoospérmico).

Discusión

El método de extracción de ARN utilizado ha permitido la obtención de muestras de ARN espermático con una media de 26 fg de ARN por espermatozoide, coincidentes con las descritas en la bibliografía (entre 20 y 50 fg de ARN)[23,31]. Los valores de pureza de ARN obtenidos se consideran adecuados, teniendo en cuenta la alta proporción de TRIzol® utilizado y la reducida cantidad de ARN presente en los espermatozoides[31,32]. Esta alta proporción de TRIzol® desplaza la curva de absorbancia hacia 270 nm, situación que provoca una disminución de la ratio 260/280[33]. Además, el elevado grado de empaquetamiento del ADN espermático dificulta la recuperación del ARN e influye negativamente en las purezas obtenidas[34].

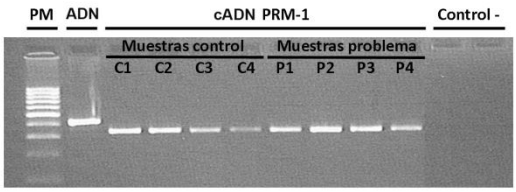


Figura 1 Análisis electroforético al 2% de los productos de la PCR de protamina 1 (PRM-1). Carril 1: peso molecular (PM) de 100 a 1.000pb. Carril 2: control positivo de ADM (422pb). Carriles 3-10: cADN procedentes de espermatozoides de los individuos estudiados (331pb); la falta de intrones (91pb) permite ver la diferencia respecto el control positivo (ADN). Carriles 11 y 12: controles negativos.

Por otro lado, los resultados de las PCR de protamina 1 han confirmado que la extracción de ARN seguida por el tratamiento con DNasa recombinante permite la obtención de un ARN íntegro y libre de contaminaciones de ADN. Esta estrategia con cebadores exón-exón fue descrita previamente por varios autores[28,35,36] y su aplicabilidad en este tipo de estudio está extensamente aceptada.

Los 4 mi-ARN analizados (hsa-miR-23a, hsa-miR-744, hsa-let-7f y hsa-miR-1) se han descrito con anterioridad en diferentes tejidos humanos[17,37], pero solamente hsa-miR-23a lo ha sido en espermatozoides[19]. Nuestro estudio, además de confirmar la presencia del hsa-miR-23a en espermatozoides humanos, constata, por primera vez, la presencia del hsa-miR-744 y hsa-let-7f en estas células.

El presente estudio ha puesto de manifiesto una disminución significativa de la expresión del mi-ARN hsa-let-7f en 3 de los 4 individuos infértiles (individuos P1, P3 y P4). De estos 3 individuos, 2 de ellos (P1 y P3) presentaban altera-

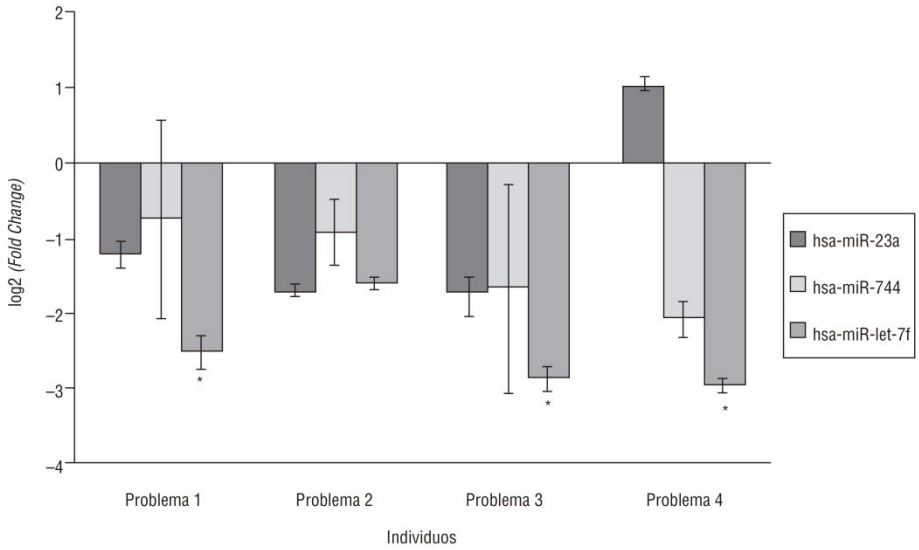


Figura 2 Diferencia de expresión de los 3 mi-ARN detectados en los individuos problema respecto a la media de los individuos control.
* Diferencia de expresión significativa (la media se sitúa fuera del intervalo de confianza de la expresión normalizada en las muestras control).

ciones del seminograma. Aunque la función del mi-ARN hsa-let-7f todavía es desconocida en humanos, en distintas especies animales se ha descrito que la familia let-7 está estrechamente relacionada con las funciones de desarrollo embrionario[38,39]. En conjunto, nuestros resultados indican que diferencias en el patrón de expresión de ciertos mi-ARN podrían ser la causa subyacente de algunos casos de infertilidad masculina.

Conclusiones

Los espermatozoides humanos contienen los mi-ARN hsa-miR-23, hsa-miR-744 y hsa-let-7f. Este último muestra una reducción significativa de expresión en 3 de los 4 individuos infértiles, sugiriendo la implicación de alteraciones en la expresión de mi-ARN como la causa subyacente de algunos casos de infertilidad masculina.

Responsabilidades éticas

Protección de personas y animales: los autores declaran que para esta investigación no se han realizado experimentos en seres humanos ni en animales.

Confidencialidad de los datos: los autores declaran que han seguido los protocolos de su centro de trabajo sobre la publicación de datos de pacientes y que todos los pacientes incluidos en el estudio han recibido información suficiente y han dado su consentimiento informado por escrito para participar en dicho estudio.

Derecho a la privacidad y consentimiento informado: los autores declaran que en este artículo no aparecen datos de pacientes.

Financiación

El trabajo se ha desarrollado mediante la financiación de los proyectos FIS PS-09-00330, 2009/SGR00282 y Ayudas Merk Serono 2011FE16. Albert Salas-Huetos es el beneficiario de una beca predoctoral PIF de la UAB 456-01-1/E2010.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

Agradecimientos

Este trabajo ha sido posible gracias a la aportación de muestras del Instituto Valenciano de Infertilidad de Valencia y la Unidad de Reproducción Asistida del Centro Médico Teknon de Barcelona. Los autores agradecen la colaboración del Dr. Nicolás Garrido del Instituto Valenciano de Infertilidad de Valencia y del Dr. Mark Grossmann, la Dra. Carme Pons y el Dr. Javier Nadal de la Unidad de

Reproducción Asistida del Centro Médico Teknon de Barcelona.

Bibliografía

1. Ambros V. microRNAs: tiny regulators with great potential. Cell. 2001;107:823-6.
2. Boyd SD. Everything you wanted to know about small RNA but were afraid to ask. Lab Invest. 2008;88:569-78.
3. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. Nat Rev Mol Cell Biol. 2010;11:252-63.
4. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev. 2004;18:504-11.
5. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007;318:1931-4.
6. miRBase.org [Internet]. University of Manchester [actualizado 3 Nov 2011; citado 26 Abr 2012]. Disponible en: http://www.mirbase.org
7. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res. 2005;65:6029-33.
8. Lu J, Getz G, Miska EA, Álvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834-8.
9. Boerke A, Dieleman SJ, Gadella BM. A possible role for sperm RNA in early embryo development. Theriogenology. 2007;68 Suppl 1:S147-55.
10. He Z, Kokkinaki M, Pant D, Gallicano GI, Dym M. Small RNA molecules in the regulation of spermatogenesis. Reproduction. 2009;137:901-11.
11. Toloubeydokhti T, Bukulmez O, Chegini N. Potential regulatory functions of microRNAs in the ovary. Semin Reprod Med. 2008;26:469-78.
12. Hwang HW, Mendell JT. MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer. 2006;94:776-80.
13. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006;6:259-69.
14. Van Rooij E, Olson EN. MicroRNAs: powerful new regulators of heart disease and provocative therapeutic targets. J Clin Invest. 2007;117:2369-76.
15. Fiore R, Siegel G, Schratt G. MicroRNA function in neuronal development, plasticity and disease. Biochim Biophys Acta. 2008;1779:471-8.
16. Tan Z, Randall G, Fan J, Camoretti-Mercado B, Brockman-Schneider R, Pan L, et al. Allele-specific targeting of microRNAs to HLA-G and risk of asthma. Am J Hum Genet. 2007;81:829-34.
17. Lian J, Zhang X, Tian H, Liang N, Wang Y, Liang C, et al. Altered microRNA expression in patients with non-obstructive azoospermia. Reprod Biol Endocrinol. 2009;7:13.
18. McCallie B, Schoolcraft WB, Katz-Jaffe MG. Aberration of blastocyst microRNA expression is associated with human infertility. Fertil Steril. 2010;93:2374-82.
19. Krawetz SA, Kruger A, Lalancette C, Tagett R, Antón E, Draghici S, et al. A survey of small RNAs in human sperm. Hum Reprod. 2011;26:3401-12.
20. Wykes SM, Visscher DW, Krawetz SA. Haploid transcripts persist in mature human spermatozoa. Mol Hum Reprod. 1997;3:15-19.
21. Kramer JA, Krawetz SA. RNA in spermatozoa: implications for the alternative haploid genome. Mol Hum Reprod. 1997;3:473-8.
22. Miller D. RNA in the ejaculate spermatozoon: a window into molecular events in spermatogenesis and a record of the unusual requirements of haploid gene expression and post-meiotic equilibration. Mol Hum Reprod. 1997;3:669-76.
23. Krawetz SA. Paternal contribution: new insights and future challenges. Nat Rev Genet. 2005;6:633-42.
24. Lalancette C, Miller D, Li Y, Krawetz SA. Paternal contributions: new functional insights for spermatozoal RNA. J Cell Biochem. 2008;104:1570-9.
25. Liu WM, Pang RT, Chiu PC, Wong BP, Lao K, Lee KF, et al. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. Proc Natl Acad Sci USA. 2012;109:490-4.
26. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. Fertil Steril. 1988;49:112-7.
27. Organización Mundial de la Salud. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5.ª ed. Cambridge: University Press; 2010.
28. Goodrich R, Johnson G, Krawetz SA. The preparation of human spermatozoal RNA for clinical analysis. Arch Androl. 2007;53:161-7.
29. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987;162:156-9.
30. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc. 2006;1:581-5.
31. Dadoune JP. Spermatozoal RNAs: what about their functions? Microsc Res Tech. 2009;72:536-51.
32. Linschooten JO, Van Schooten FJ, Baumgartner A, Cemeli E, Van Delft J, Anderson D, et al. Use of spermatozoal mRNA profiles to study gene-environment interactions in human germ cells. Mutat Res. 2009;667:70-6.
33. Krebs S, Fischaleck M, Blum H. A simple and loss-free method to remove TRIzol contaminations from minute RNA samples. Anal Biochem. 2009;387:136-8.
34. Oliva R. Protamines and male infertility. Hum Reprod Update. 2006;12:417-35.
35. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: Delivering spermatozoan RNA to the oocyte. Nature. 2004;429:154.
36. Kempisty B, Depa-Martynow M, Lianeri M, Jedrzejczak P, Darul-Wasowicz A, Jagodzinski PP. Evaluation of protamines 1 and 2 transcript contents in spermatozoa from asthenozoospermic men. Folia Histochem Cytobiol. 2007;45 Suppl 1:S109-13.
37. Berezikov E, Thuemmler F, Van Laake LW, Kondova I, Bontrop R, Cuppen E, et al. Diversity of microRNAs in human and chimpanzee brain. Nat Genet. 2006;38:1375-7.
38. Tennessen JM, Thummel CS. Developmental timing: let-7 function conserved through evolution. Curr Biol. 2008;18:R707-8.
39. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000;408:86-9.

Publicació II

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

Autors: Albert Salas-Huetos, Joan Blanco, Francesca Vidal, Josep M. Mercader, Nicolas Garrido, and Ester Anton

Revista: Fertility and Sterility, **2014**;102(1):213-222

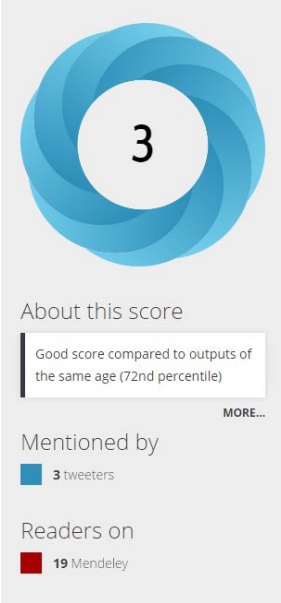
Factor d'impacte: 4.590 (2014), Q1, *Reproductive Biology*

Citat en:

6 articles

1 llibre

Altimetric score:



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

Albert Salas-Huetos, M.Sc.,[a] Joan Blanco, Ph.D.,[a] Francesca Vidal, Ph.D.,[a] Josep M. Mercader, Ph.D.,[b] Nicolás Garrido, Ph.D.,[c] and Ester Anton, Ph.D.[a]

[a] Unitat de Biologia Cel·lular, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Spain; [b] Joint Institution for Research in Biomedicine–Barcelona Supercomputing Center Program on Computational Biology, Barcelona Supercomputing Center, Barcelona, Spain; and [c] Laboratorio de Andrología y Banco de Semen, Instituto Valenciano de Infertilidad Valencia, Valencia, Spain

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;102:213–22. ©2014 by American Society for Reproductive Medicine.)
Key Words: Infertility, microRNA, spermatogenesis, embryogenesis, sperm biomarkers

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/salashuetosa-expression-profile-function-microrna-human-spermatozoa/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Received December 27, 2013; revised February 28, 2014; accepted March 18, 2014; published online April 29, 2014.
A.S.-H. has nothing to disclose. J.B. has nothing to disclose. F.V. has nothing to disclose. J.M.M. has nothing to disclose. N.G. has nothing to disclose. E.A. has nothing to disclose.
This work was supported by the projects 2011FE16 (Ayudas Merck Serono 2011; Investigación Clínica en Fertilidad, Spain), FIS/PS09-00330 (Ministerio de Ciencia e Innovación, Gobierno de España, Spain), 2009/SGR00282 (Agència de Gestió d'Ajuts Universitaris i de Recerca, Generalitat de Catalunya, Catalonia, Spain). A.S.-H. is a recipient of Professor Investigador en Formació grant 456-01-1/E2010 (Universitat Autònoma de Barcelona), and J.M.M. was supported by a Sara Borrell Fellowship from the Instituto Carlos III.
Reprint requests: Ester Anton, Ph.D., Unitat de Biologia Cel·lular, Facultat de Biociències, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès) 08193, Spain (E-mail: ester.anton@uab.cat).

Fertility and Sterility® Vol. 102, No. 1, July 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.03.040

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

GAPDH were performed. The *PRM1* primers (*PRM1* forward: 5′-cagagttccacctgctcaca-3′; *PRM1* reverse: 5′-agaacttt tacggtggtagg-3′) and the *GAPDH* primers (*GAPDH* forward: 5′-cgaccactttgtcaagctca-3′; *GAPDH* reverse: 5′-aggggtcta catggcaactg-3′) were designed using Primer3 v.3.0.0 software (http://primer3.wi.mit.edu) (32) and supplied by TIB MOLBIOL. Second, to exclude the presence of somatic RNAs, a PCR for the surface receptor *CD45* (present in all differentiated nucleated hematopoietic cells but absent in spermatozoa) was performed using the primers *CD45* forward: 5′-ccttgaacccgaacatgagt-3′ and *CD45* reverse: 5′-atctttgagggg gattccag-3′, previously described by Jodar et al. (33). Third, to assess the presence of miRNAs and confirm the lack of somatic RNA, we ran a small-RNA and a nano-RNA chip, respectively, in the Agilent-2100 Bioanalyzer (Agilent Technologies) using 25 ng of RNA per sample.

microRNA Evaluation

Samples of RNA were reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies) supplemented with 0.1 μL RNase inhibitor per sample (20 U/μL) and the Multiplex RT Human Primer Pool A+B (Life Technologies). These stem-loop RT primers are designed to specifically bind to at the 3′ end of mature miRNA, thus discarding the retrotranscription of precursor miRNAs (34).

Reverse transcription reactions were performed in a volume of 7.5 μL containing 0.8 μL Megaplex RT Primers (10×), 0.2 μL deoxyribonucleotide triphosphates (dNTPs) with deoxythymidine triphosphates (100 mM), 1.5 μL MultiScribe reverse transcriptase (50 U/μL), 0.8 μL RT buffer (10×), 0.9 μL $MgCl_2$ (25 mM), 0.1 μL RNase inhibitor (20 U/μL), 0.2 μL nuclease-free water (Sigma-Aldrich), and 3 μL RNA (15–20 ng/μL). The thermal cycling profile was 40 cycles of 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second, and finally 85°C for 5 minutes (Eppendorf Mastercycler ep Gradient S 96). Complementary DNA was stored at −20°C until further analysis.

Before the qPCR reaction, cDNA was preamplified using TaqMan PreAmp Master Mix (Life Technologies) and Megaplex PreAmp Primers (Life Technologies). Reactions were performed in a final volume of 25 μL containing 12.5 μL TaqMan PreAmp Master Mix (2×), 2.5 μL Megaplex PreAmp Primers (10×), 7.5 μL nuclease-free water (Sigma-Aldrich), and 2.5 μL cDNA. The thermal cycling profile was 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, 12 cycles of 95°C for 15 seconds and 60°C for 4 minutes, and finally, 99°C for 10 minutes (Eppendorf Mastercycler ep Gradient S 96). Preamplified cDNA was diluted with 75 μL of 0.1× TE (Life Technologies) and stored at −20°C.

Quantitative PCR was performed using a mix of 9 μL of PreAmp product, 450 μL TaqMan Universal PCR Master Mix, No AmpErase UNG (2×) (Life Technologies), and 441 μL nuclease-free water (Sigma-Aldrich). Quantitative PCR assays were performed using the TaqMan Array Human MicroRNA A+B Card Set v3.0 kit (Life Technologies). Each card contains a total of 384 TaqMan MicroRNA Assays (Life Technologies), enabling a total quantitation of 736 human miRNAs. Every card also comprises three endogenous controls (Mamm-U6, RNU-44, and RNU-48) and a negative control (ath-miR-159a). A total of 100 μL of the final mix was loaded on each port. The arrays were analyzed in a 7900HT Fast Real-Time PCR System (Life Technologies).

Data Analysis

Data from qPCR were processed by SDS v.2.3 and RQ Manager v.1.2 software (Life Technologies). MicroRNA expressions (Ct values) were classified as Determined (Ct $\geq$15 and <35), Undetermined (Ct $\geq$35), and Unreliable. On the basis of the Ct value classification, we performed a hierarchical cluster analysis using a Euclidean metric with averages method. This hierarchical cluster was used to assess the degree of similarity among samples.

All determined Ct values were normalized to obtain the normalized threshold cycle (normCt) values using the mean-centering restricted (MCR) method. This method, specifically created to normalize miRNA expression from high-throughput qPCR data, uses the mean expression of all the miRNAs found across all samples to correct any technical variance (35). To determine the top observed miRNA families, we used the newly designed computational Tool for Annotations of miRNAs (TAM v.1.3; http://cmbi.bjmu.edu.cn/tam) (36).

Statistical analyses were performed using the freely available R statistical computing environment v.2.14.2 (www.r-project.org) (37) and an additional package for high-throughput analysis of qPCR data v.1.13.1 (HTqPCR package at http://www.bioconductor.org) (38). All tests were performed considering the ubiquitous miRNAs (expressed in all the samples studied). *P* values of <.05 were considered statistically significant, and the strict post hoc Bonferroni correction was used to prevent false discoveries.

DIANA-microT v.3.0 software (39, 40) allowed the identification of the target genes for 168 of the 221 ubiquitous miRNAs and 11 of the 63 constantly absent miRNAs in all 10 samples. Those targets with an miRNA target genes value >19 were selected (strict threshold), which is indicative of a high likeliness of being a real target. We evaluated the enrichment of biological process by using DAVID Bioinformatics Resources v.6.7 (41, 42) and using the human genome background (30,000 gene total). *P* values <.05 were considered significant after correcting for Bonferroni.

The nonparametric Spearman test was used to evaluate the correlation between each miRNA expression level and individual age, semen sample volume, sperm concentration, sperm motility, and sperm morphology. This test was also used to perform sample-to-sample correlations as a way to check interindividual differences at the miRNA expression level. To represent the relative distances between samples according to the normCt values we performed a heatmap using the Lattice Graphics package v.0.20-15 (Lattice Graphics package at http://www.bioconductor.org) (43).

To evaluate the presence of stable miRNA pairs, we performed single miRNA–miRNA correlations using the Spearman correlation test.

Finally, to identify the best normalizing candidates, we evaluated which miRNAs best correlated to the mean

expression of each card by using the concordance correlation restricted (CCR) method (35). Briefly, the mean expression of all ubiquitous miRNAs per sample and plate was calculated. Then, using the Pearson method, the concordance correlation coefficient (CCC) for each miRNA was calculated comparing the miRNA Ct value with the mean expression of all ubiquitous miRNAs per sample and plate. The miRNAs with better CCC were thus established as the best normalizer candidates. Afterward, Ct values were normalized using the selected miRNAs. The normCt values from both methods (MCR and CCR) were compared by the Spearman correlation test.

RESULTS

Results of the total human sperm RNA isolation from the 10 samples evaluated are presented in Supplemental Table 2. Purity and RNA quantities ranged 1.84–1.97 and 373–3,697 ng, respectively. Taking into account the RNA recovered and the total number of spermatozoa processed, we established an average (±SD) content of RNA per spermatozoa of 55 ± 22 fg.

PRM1 and *GAPDH* quality control PCRs confirmed the presence of intact *PRM1* and *GAPDH* transcripts and the absence of DNA in all samples (Supplemental Fig. 1A and B). Small-RNA chips confirmed the presence of transcripts comprising 4–150 nt that could correspond to small RNAs (Supplemental Fig. 2A). Nano-RNA chips displayed the absence of the 18S and 28S ribosomic RNA peaks in the sperm sample electropherograms (Supplemental Fig. 2B). The control PCR for *CD45* confirmed the lack of transcripts of this somatic marker in all the samples analyzed (Supplemental Fig. 1C).

In the evaluation of the miRNAs present in spermatozoa, from the 736 miRNAs screened, 221 were consistently present in all 10 individuals (Supplemental Table 3). In contrast, 452 miRNAs were only detected in some individuals, and 63 miRNAs did not appear in any sample (Supplemental Table 4). From 5 to 14 miRNAs were noninformative or unreliable (Fig. 1A). The ubiquitous miRNAs with lower normCt values corresponded to the most abundant miRNAs. Table 1 shows the top 10 miRNAs that display higher average expression levels. On the other hand, the SD of the normCt values of the miRNAs present in all 10 samples allowed the identification of the most stable miRNA. Table 1 shows the top 10 miRNAs with lower SD values. The dendrogram obtained from the hierarchical cluster analysis based on the presence (Determined), absence (Undetermined), and noninformative (Unreliable) miRNAs in each individual revealed two clusters that differed by a percentage lower than 10% (Fig. 1B).

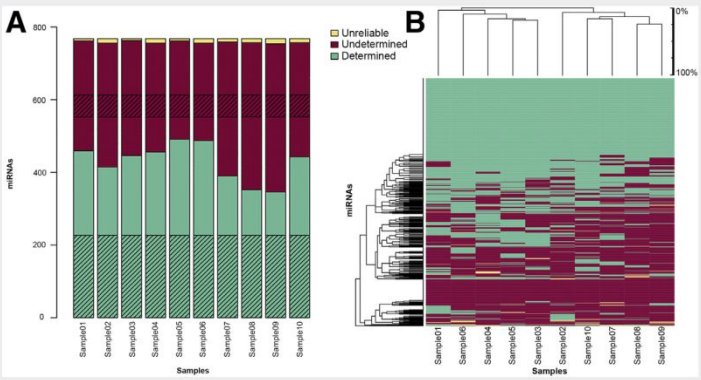
DIANA-microT allowed the identification of 2,356 potential gene targets for the ubiquitous miRNA and 597 for the constantly absent miRNAs (strict selection criteria). The ontologic analysis of these genes showed an enrichment of biological processes related to cell differentiation and development, morphogenesis, and embryogenesis, among others, in the group of targets of the ubiquitous miRNAs (Table 2A), whereas the targets predicted for the constantly absent miRNAs did not show any significant relationship to spermatogenesis or embryogenesis (Table 2B).

In relation to the correlations performed between miRNA normCt values and the age and seminal parameters of the individuals analyzed, we did not find any miRNA significantly correlated with age, ejaculate volume, sperm concentration, motility, or morphology.

All Spearman correlations performed among samples considering the expression level of the 221 ubiquitous miRNAs seemed to be statistically significant (ranging from 0.932 to 0.611; Supplemental Fig. 3). A heatmap showing the relative similarity between samples is provided in Supplemental Figure 4.

In the evaluation of the possible presence of miRNA stable pairs, we found 48 cases that displayed statistically significant correlation coefficients ($\rho \geq 0.915$ or

FIGURE 1



(A) miRNA Ct expression values classified as Determined, Undetermined, or Unreliable. The miRNAs fraction of each category present or absent in all individuals has been indicated by stripes. (B) Hierarchical clustering of the 10 individuals based on the miRNA Ct values obtained. Samples are represented by column (n = 10) and assays by row (n = 768).

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

TABLE 1

Top 10 most expressed miRNAs and top 10 most stable miRNAs.

Most expressed		Most stable	
miRNA	normCt mean	miRNA	normCt SD
hsa-miR-34b-3p	−7.551	hsa-miR-663b	0.335
hsa-miR-375	−6.134	hsa-miR-564	0.365
hsa-miR-191-5p	−6.024	hsa-miR-744-5p	0.426
hsa-miR-19b-3p	−5.328	hsa-miR-1282	0.438
hsa-miR-200c-3p	−5.309	hsa-miR-935	0.452
hsa-miR-132-3p	−5.138	hsa-let-7d-5p	0.483
hsa-miR-30c-5p	−4.940	hsa-miR-543	0.494
hsa-miR-891a	−4.625	hsa-miR-572	0.511
hsa-miR-30b-5p	−4.421	hsa-miR-1180	0.513
hsa-miR-1233-3p	−4.143	hsa-miR-638	0.528

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

≤ -0.915). The top 10 best-correlated pairs are presented in Table 3.

We also searched for miRNA candidates for normalization, because this could be useful for future studies in which only a subset of miRNAs is tested. For this we applied the CCR method (35), which allowed us to determine the three miRNAs that best correlated to the mean expression of each card: hsa-miR-532-5p and hsa-miR-374b-5p (from plate A) and hsa-miR-564 (from plate B). The normalized data of the ubiquitous miRNAs Ct values using these three miRNAs were highly correlated to the data obtained using the MCR method (35) (significant P value $<.001$ and a ρ value $= 0.977$).

DISCUSSION

We have described the miRNA expression profile in spermatozoa from fertile donors, demonstrating the high homogeneity of the population analyzed both in terms of the miRNAs present/absent as well as the expression levels detected. Our data also show a lack of correlation between the miRNA content and the seminal parameters or age of the individuals analyzed and relates this cargo to biological processes, such as cell differentiation, embryonic development, and morphogenesis.

One of the most significant challenges in quantifying the miRNA expression levels in spermatozoa was the development of an optimum strategy that eliminates any interference derived from the RNA purification protocols, the methodology of RT-PCR and qPCR, and the normalization and analysis of the results (35). The methodology applied in this study required the use of a sperm selection protocol to ensure that the results related specifically to the sperm fraction (19). The RNA isolation method allowed us to obtain the total human sperm RNA with an optimal quantity and purity. As a first quality control, the absence of DNA was confirmed by performing a *PRM1* and a *GAPDH* PCR that amplified cDNA products with lengths corresponding to mRNA. This procedure also ensured the proper integrity of the transcripts obtained. Additionally, the use of nanoelecrophoresis chips confirmed the presence of small RNAs with sizes corresponding to miRNAs and the correct isolation of the sperm fraction by ruling out the presence of ribosomic RNA peaks (spermatozoa are essentially void of ribosomic RNA [22]). These results were also corroborated by the *CD45* PCR that discarded the presence of this somatic marker among the transcripts obtained.

Regarding the qPCR-based strategy for miRNAs, we used a methodology that combines stem-loop RT primers and TaqMan miRNA Assays. This approach is very efficient and specific for the detection and quantification of mature miRNAs only, has a high sensitivity (ability to discriminate miRNAs that differ by one nucleotide), and the results are easily interpretable (34). The main limitation of this procedure is related to the fact that it only allows the evaluation of the expression levels of the 736 miRNAs included in the TaqMan plates. However, it is important to note that this panel allows a wide coverage of the miRNAs described in the Sanger miRBase database v.14.0 (www.mirbase.org), because they correspond to the most abundant and well-characterized miRNAs in humans.

Overall, the methodology used in this study has provided an accurate and reliable basis to ensure the proper characterization of the miRNA profile in human sperm.

miRNA profile

Among the 221 miRNAs consistently detected in all individuals, 18 have been previously described to display a direct role related to spermatogenesis (hsa-let-7a-5p, hsa-miR-7-1-3p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-26a-5p, hsa-miR-30a-5p, hsa-miR-34b-5p, hsa-miR-34b-3p, hsa-miR-99a-5p, hsa-miR-100-5p, hsa-miR-122-5p, hsa-miR-146b-5p, hsa-miR-184, hsa-miR-193b-5p, hsa-miR-374b-5p, hsa-miR-429, hsa-miR-512-3p, and hsa-miR-1275), 4 have been related to epididymis sperm maturation (hsa-miR-890, hsa-miR-891a, hsa-miR-892a, and hsa-miR-892), 1 has been described to be involved in the renewal of spermatogonial stem cells (hsa-miR-21-5p), and 4 have been associated to embryonic development (hsa-miR-26b-5p, hsa-miR-125b-5p, hsa-miR-150-5p, and hsa-miR-372) (28, 29, 44–53) (Supplemental Table 3). On the other hand, 122 miRNAs have not previously been detected in human spermatozoa by other authors (24, 29, 44) (Supplemental Table 3). Regarding the 63 miRNAs not observed in any sample, 2 of them (hsa-miR-19a-5p and hsa-miR-221-5p) had previously been found in human spermatozoa (24, 29, 44) (Supplemental Table 4). The miRNAs that we detected were significantly enriched in miRNAs previously detected in other studies (χ^2 P value $= 4.38 \times 10^{-9}$).

This study shows a significant increase in the number of miRNAs identified compared with other studies that evaluated miRNAs expression profiles using RNAseq (24) or miRNA Microarray (44) strategies. Although RNA-seq allows a complete analysis of the transcriptome, it has been described to be less accurate for the expression analysis of specific parts of the transcriptome (54, 55). Furthermore, this methodology requires the use of RNA purification protocols that can entail a loss of small RNA molecules (RNeasy MinElute Cleanup Kit; www.qiagen.com). Regarding the microarray approach, although the number of miRNAs that can be tested (n = 1,349) is higher than that provided by the

TABLE 2

Enriched biological functions associated with (A) the 2,356 predicted genes for the ubiquitous miRNAs and (B) the 597 predicted genes for the constantly absent miRNAs in all samples.

A. Biological functions	Spearman correlation *P* value	Bonferroni correction
Regulation of cell development	7.80×10^{-14}	3.10×10^{-10}
Cell fate commitment	5.30×10^{-12}	2.10×10^{-8}
Embryonic morphogenesis	2.40×10^{-10}	9.60×10^{-7}
Cell morphogenesis	2.70×10^{-10}	1.10×10^{-6}
Cell projection organization	3.20×10^{-10}	1.30×10^{-6}
Positive regulation of developmental process	3.70×10^{-10}	1.50×10^{-6}
Cell morphogenesis involved in differentiation	5.50×10^{-10}	2.20×10^{-6}
Cell projection morphogenesis	6.70×10^{-10}	2.70×10^{-6}
Cellular component morphogenesis	9.10×10^{-10}	3.60×10^{-6}
Cell part morphogenesis	2.00×10^{-9}	7.80×10^{-6}
Regulation of cell motion	3.70×10^{-9}	1.50×10^{-5}
Negative regulation of cell differentiation	6.50×10^{-9}	2.60×10^{-5}
Positive regulation of cell differentiation	1.10×10^{-8}	4.30×10^{-5}
Chordate embryonic development	1.10×10^{-8}	4.30×10^{-5}
Regulation of cell migration	1.30×10^{-8}	5.00×10^{-5}
Embryonic development ending in birth or egg hatching	1.70×10^{-8}	6.60×10^{-5}
Chromatin modification	1.70×10^{-8}	6.70×10^{-5}
Embryonic organ development	2.40×10^{-8}	9.50×10^{-5}
Cell motion	1.00×10^{-7}	4.20×10^{-4}
Regulation of cell morphogenesis	1.40×10^{-7}	5.50×10^{-4}
Regulation of cell proliferation	1.80×10^{-7}	7.10×10^{-4}
Tissue morphogenesis	3.10×10^{-7}	1.20×10^{-3}
Regulation of locomotion	4.00×10^{-7}	1.60×10^{-3}
Regulation of cell morphogenesis involved in differentiation	7.30×10^{-7}	2.90×10^{-3}
Positive regulation of cell motion	4.60×10^{-6}	1.80×10^{-2}
Regulation of cell projection organization	5.30×10^{-6}	2.10×10^{-2}
In utero embryonic development	5.80×10^{-6}	2.30×10^{-2}
Chromatin organization	7.30×10^{-6}	2.90×10^{-2}
B. Biological functions	**Spearman correlation *P* value**	**Bonferroni correction**
Transcription	3.00×10^{-11}	6.50×10^{-8}
Regulation of transcription from RNA polymerase II promoter	3.60×10^{-8}	7.90×10^{-5}
Transmembrane receptor protein tyrosine kinase signaling pathway	5.30×10^{-8}	1.20×10^{-4}
Regulation of transcription	1.50×10^{-7}	3.30×10^{-4}
Positive regulation of transcription, DNA dependent	2.00×10^{-7}	4.40×10^{-4}
Positive regulation of RNA metabolic process	2.50×10^{-7}	5.50×10^{-4}

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

TABLE 2

Continued.

B. Biological functions	Spearman correlation *P* value	Bonferroni correction
Positive regulation of gene expression	6.30×10^{-7}	1.40×10^{-3}
Positive regulation of transcription from RNA polymerase II promoter	1.00×10^{-6}	2.20×10^{-3}
Positive regulation of transcription	1.90×10^{-6}	4.20×10^{-3}
Eye development	4.70×10^{-6}	1.00×10^{-2}
Positive regulation of macromolecule biosynthetic process	5.40×10^{-6}	1.20×10^{-2}
Camera-type eye development	7.50×10^{-6}	1.60×10^{-2}
Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9.30×10^{-6}	2.00×10^{-2}
Positive regulation of biosynthetic process	1.10×10^{-5}	2.40×10^{-2}
Enzyme linked receptor protein signalling pathway	1.40×10^{-5}	3.10×10^{-2}
Positive regulation of cellular biosynthetic process	1.70×10^{-5}	3.70×10^{-2}
Positive regulation of nitrogen compound metabolic process	2.00×10^{-5}	4.20×10^{-2}
Transmission of nerve impulse	2.20×10^{-5}	4.70×10^{-2}

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

Taqman Arrays (n = 736), a better sensitivity and specificity of the qPCR-based strategy has been highlighted by some authors (56, 57). Additionally, the high characterization level of the population used in our study and the homogeneity degree of the parameters included in the recruitment of the "fertile" individuals are factors that most likely influenced the quality of the results.

Among the most abundant miRNAs (top 10 expressed), the miRNA hsa-miR-34b-3p stands out. Although this miRNA has been related to myotonic dystrophy type 2 in a study in humans (58), the miR-34 family has been more extensively studied (24, 29) and associated with the regulation of the E2F-pRb pathway during male meiosis (59). Other top 10 expressed miRNAs include hsa-miR-132-3p, related to cell cycle progression through *MYC* activation (60), and hsa-miR-191-5p, a miRNA highly expressed in testis and linked to morphologic sperm differentiation (61). On the other hand, hsa-miR-375, hsa-miR-19b-3p, and hsa-miR-200c-3p play an important role in aging and cancer (62–65), whereas hsa-miR-891a and hsa-miR-1233-3p have not yet been related to any biological function. Finally, among the top 10 expressed miRNAs, hsa-miR-30b-5p and hsa-miR-30c-5p have only been previously related with cancer progression (66, 67).

Regarding the most stable miRNAs (top 10 stable), hsa-miR-744 is directly related to the regulation of transforming growth factor B1 (68), which is involved in cell proliferation processes, cell differentiation, and apoptosis (69). The hsa-miR-638 has been detected in human embryos and linked to their development, although this miRNA also has been

TABLE 3

Correlation values of the most stable miRNAs pairs.

miRNAs stable pairs		ρ value	P value
hsa-miR-20a-5p	hsa-miR-106a-5p	0.988	9.31×10^{-8}
hsa-miR-152	hsa-miR-218-5p	0.988	9.31×10^{-8}
hsa-miR-512-3p	hsa-miR-517a-3p	0.988	9.31×10^{-8}
hsa-miR-942	hsa-miR-1208	0.988	9.31×10^{-8}
hsa-miR-30c-5p	hsa-miR-616-5p	0.976	1.47×10^{-6}
hsa-miR-197-3p	hsa-miR-1291	−0.976	1.47×10^{-6}
hsa-miR-10a-5p	hsa-miR-519a-3p	−0.964	7.32×10^{-6}
hsa-miR-10a-5p	hsa-miR-628-3p	−0.964	7.32×10^{-6}
hsa-miR-92a-3p	hsa-miR-636	0.964	7.32×10^{-6}
hsa-miR-100-5p	hsa-miR-125b-5p	0.964	7.32×10^{-6}

Note: Only the top 10 best correlations are displayed (Bonferroni-adjusted significance level = 2.26×10^{-4}).

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

associated with cancer (70). The remaining top 10 stable miRNAs also play an important role in different kinds of cancer growth and progression: hsa-miR-663b (71), hsa-miR-564 (72), hsa-miR-935 (73), hsa-miR-let-7d (74), hsa-miR-543 (75), and hsa-miR-572 (76). Hsa-miR-1180 and hsa-miR-1282 have not yet been related to any notable biological role.

Concerning the miRNA families observed in this study, most of the miR-30 and miR-10 family members have been detected in the fertile donors analyzed. The miR-30 family has been associated with the Hedgehog pathway in Zebrafish (77), an extensively studied signaling pathway related to embryo development that is conserved from *Drosophila* to humans (78, 79). This family has also been related to the transcription factor *Xlim1*, located within the LIM homeobox 1 (*Lhx1*), which is involved in tissue patterning and control of neuronal morphogenesis and differentiation in *Xenopus* embryos (80, 81). Additionally, it has been suggested that it can play an important role in human endometrium development (82). As mentioned above, two members of this miR-30 family (hsa-miR-30b-5p and hsa-miR-30c-5p) were among the top 10 expressed miRNAs. Regarding the miR-10 family, they belong to the *HOX* development cluster (83), which is also conserved throughout evolution in mammals. It contains five miRNAs that have been identified in our study (miR-10a, miR-10b, miR-196a-1, miR-196a-2, and miR-196b) and were previously described in spermatozoa by other authors (84). There is evidence that several targets of miR-10 are regulators of the homeobox(*HOX*) genes themselves. For example, it has been described in Zebrafish that miR-10 regulates *hoxb1a*, and *hoxb3a* targets (85) and our gene ontology analysis predicted *HOXA3*, *HOXB13*, and *HOXB3* as among the target genes for the miR-10 family (see below). Moreover, we also found a large part of the let-7 family, as well as most of the miR-8, miR-15, miR-17, miR-28, miR-99, miR-188, miR-515, and miR-743 families, which have been extensively characterized in different species and tissues (61, 86–95). In humans, these families have been related to processes including gamete differentiation (let-7) (61, 86, 87), cell growth promotion (miR-8) (89), cell cycle control (miR-15 and miR-17) (90, 93), mammalian development and stem cell differentiation (miR-17) (88), cancer regulation (miR-28, miR-99, and miR-515) (91, 92, 94), and nervous system control (miR-188) (95).

Gene Ontology

The gene ontology analysis performed in our study over the targets of the miRNAs detected in all samples reinforces the bibliographic results that have been mentioned in previous paragraphs. Our findings display enriched biological processes related to cell differentiation, development, morphogenesis, and embryogenesis (Table 2A), which supports the participation of the sperm miRNA cargo in these processes.

Among the targets, the most frequently predicted genes (more than 15 times) were *PTPRD*, *FIGN*, *MLL*, *CPEB2*, *KLF12*, and *TNRC6B*. Interestingly, these transcripts are also candidates to play an important role in processes related to spermatogenesis and embryogenesis. Protein Tyrosine Phosphatase Receptor Type Delta (*PTPRD*) gene (predicted 27 times) is a member of the protein tyrosine phosphatase family that is related with a variety of cellular processes, including cell growth, differentiation, and mitotic cycle regulation (96–98). *FIGN* (predicted 20 times) or Fidgetin belongs to the superfamily of ATPases Associated with diverse cellular Activities (AAA-ATPases) proteins with important functions in mammalian development (99). Their main activity has been associated with microtubule depolymerization, a process involved in the mitotic spindle architecture and dynamics during anaphase A (100). Mixed-Lineage Leukemia (*MLL*) gene (predicted 20 times) is a positive regulator of gene transcription involved in the epigenetic preservation of transcriptional memory (101). The protein encoded by transcript *CPEB2* (predicted 17 times) presents a primary sequence highly similar to the Cytoplasmic Polyadenylation Element Binding (CPEB) protein, which is active during meiosis and controls the translation of Synaptonemal Complex Proteins (SCPs). Because the *CPEB2* protein is expressed at a later stage of spermatogenesis (round spermatids), some authors have proposed its role as a translation regulator of mRNAs required for spermiogenesis (102). Kruppel-Like Factor 12 (*KLF12*) or Activator Protein-2 (*AP-2*) (predicted 17 times) is a transcription factor involved in gene expression regulation during development of vertebrates and carcinogenesis (103). Finally, Trinucleotide Repeat-Containing 6B (*TNRC6B*) (predicted 27 times), which interacts with *EIF2C2* (104), has been associated with Argonaute proteins (105) that are involved in miRNAs biosynthesis.

Overall our study reveals a significant increase of potential targets involved in processes related to spermatogenesis and embryogenesis as well as miRNA synthesis. These results support the hypothesis that miRNAs are not just remnants of the spermatogenesis process and that their presence is not the result of stochastic processes (22, 24, 26, 106–108).

Homogeneity

A good control population must be homogeneous regarding their features and behavior to prevent false-positive associations. To address this aspect we performed several analyses that allowed us to determine the degree of homogeneity of the population included in our study and thus its suitability as a proper control group. Regarding the clustering of the samples according to the presence/absence of miRNAs, the differences observed between the resulting two main groups are negligible, supporting a high homogeneity among

samples. This interpretation was also validated by detecting highly significant correlation coefficients between pairs of samples based on the miRNA expression values. These findings concur with the fact that we used strict selection criteria to recruit the individuals of the study. Additionally, the use of an accurate and efficient protocol for the processing of the samples eliminates artefactual distortions from the technique. In this sense, the uniformity of the results also agrees with the lack of correlation that was found between the miRNA expression profiles and the age and seminogram features of the individuals. However, we cannot rule out that these nonsignificant results could be influenced by statistical power limitations considering that the sample size would make it difficult to detect modest or weak associations.

Stable Pairs

In human reproduction there is a debate about the real usefulness of classic semen analysis to assess male fertility (109). For this reason some recent research has been focused on the identification of new fertility markers, with the transcriptome one of the potential candidates. Accordingly, the expression level of single sperm transcripts has been proposed as markers of male fertility by several authors (33, 110, 111) (*ADCY10*, *AKAP4*, *ANXA2*, *BRD2*, *CAPN11*, *FAM71F1*, *FSCN3*, *GGN*, *GSG1*, *GTSF1L*, *mtND2*, *mtND3*, *OAZ3*, *PRM1*, *PRM2*, *SPACA4*, *SPATA3*, *SPATS1*, *TMEM225*, *UBQLN3*, and *WBSCR28*). Additionally, a suggested new alternative has been the use of stable pairs of transcripts that present a relatively constant expression (18, 112). Lalancette et al. (18) described 30 transcript pairs with stable expression in 24 sperm samples and proposed the relative expression of these genes as fertility biomarkers. More recently, another work compared the expression level of some pair of transcripts in spermatozoa from fertile male and individuals with fibrous sheath dysplasia, identifying some disrupted pairs in the infertile population (112). In our work we have assessed the presence of stable pairs of miRNAs in the individuals analyzed. As a result, a set of 48 miRNA pairs with a very high statistical correlation was detected. This group of miRNA pairs defines a set of candidates with a potential role as fertility biomarkers that can be considered in further studies. Of note, the most stable miRNA pair (hsa-miR-20a-5p/hsa-miR-106a-5p; $\rho = 0.988$) is formed by members of the same family (miR-17 family), which has been previously related to mammalian development and stem cell differentiation (82).

Normalization and Normalizing Candidates

The normalization method is critical to perform a reliable analysis of gene expression to correct any distortion caused by the methodologic processing. In our study, data obtained from qPCR was normalized using the MCR method because a high number of assays were included in each card. However, this method is not feasible for single tests or when only a small number of miRNAs is evaluated. To overcome this limitation we have applied the CCR method to select the best three normalizing miRNA candidates (hsa-miR-532-5p, hsa-miR-374b-5p, and hsa-miR-564). The high correlation between the normCt values resulting from the application of both approaches (CCR and MCR methods) has demonstrated the validity of these three miRNAs as proper normalizing assays for further studies that evaluate the expression of miRNAs in human spermatozoa.

Future Directions

All the data obtained in this study constitute a wide and comprehensive characterization of the miRNA expression profile present in fertile individuals. We believe that this information is of great significance, not only because it defines the "normal sperm miRNA cargo" that will be transmitted to the embryo but also because it provides a control reference that can be used in further studies performed in infertile individuals. These comparisons may allow determination of the contribution of miRNAs as a possible underlying cause of idiopathic male infertility and be indicative of an altered reproductive prognosis.

Acknowledgment: The authors thank the Laboratorio de Andrología y Banco de Semen of the Instituto Universitario IVI Valencia (Valencia, Spain) for providing the semen samples; and the staff of the Unitat de Genòmica (Edifici Cúster [PCB], Centres Científics i Tecnològics, Universitat de Barcelona, Spain), the staff of the Servei de Genòmica Bioinformàtica (Institut de Biotecnologia i Biomedicina, Parc de Recerca UAB, Universitat Autònoma de Barcelona, Spain), and the staff of the Servei d'Estadística Aplicada (Edifici D, Serveis Informàtics UAB, Universitat Autònoma de Barcelona, Spain) for their technical assistance.

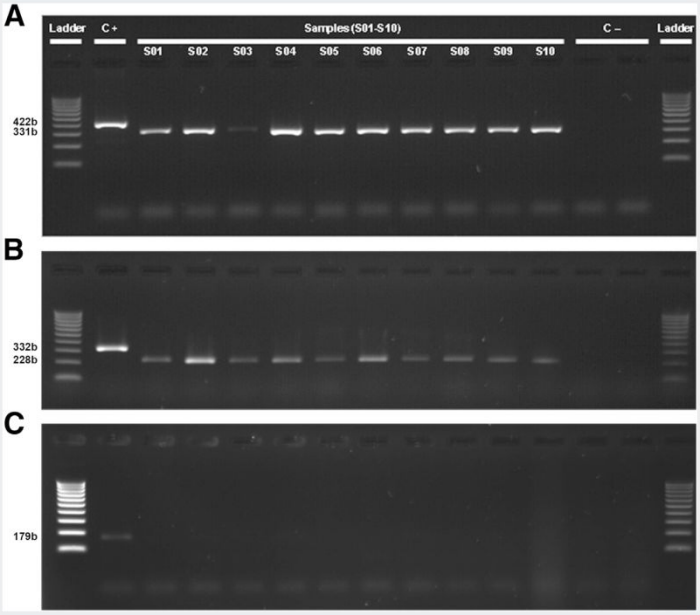
REFERENCES

1. Castel SE, Martienssen RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat Rev Genet 2013;14:100–12.
2. Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011;12:861–74.
3. Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. Science 2005;309:1519–24.
4. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
5. Boyd SD. Everything you wanted to know about small RNA but were afraid to ask. Lab Invest 2008;88:569–78.
6. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. Nat Rev Mol Cell Biol 2010;11:252–63.
7. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res 2004;32:D109–11.
8. Friedman RC, Farh KKH, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19:92–105.
9. Hermeking H. MicroRNAs in the p53 network: micromanagement of tumour suppression. Nat Rev Cancer 2012;12:613–26.
10. Bowen T, Jenkins RH, Fraser DJ. MicroRNAs, transforming growth factor beta-1, and tissue fibrosis. J Pathol 2013;229:274–85.
11. Leonardo TR, Schultheisz HL, Loring JF, Laurent LC. The functions of microRNAs in pluripotency and reprogramming. Nat Cell Biol 2012;14:1114–21.
12. Chen PS, Su JL, Hung MC. Dysregulation of microRNAs in cancer. J Biomed Sci 2012;19:90.
13. Abe M, Bonini NM. MicroRNAs and neurodegeneration: role and impact. Trends Cell Biol 2013;23:30–6.
14. Hata A. Functions of microRNAs in cardiovascular biology and disease. Annu Rev Physiol 2013;75:69–93.

15. Hynes CJ, Clancy JL, Preiss T. miRNAs in cardiac disease: sitting duck or moving target? IUBMB Life 2012;64:872–8.
16. Ono K, Kuwabara Y, Han J. MicroRNAs and cardiovascular diseases. FEBS J 2011;278:1619–33.
17. Rottiers V, Naar AM. MicroRNAs in metabolism and metabolic disorders. Nat Rev Cell Biol 2012;13:239–50.
18. Lalancette C, Platts AE, Johnson GD, Emery BR, Carrell DT, Krawetz S. Identification of human sperm transcripts as candidate markers of male fertility. J Mol Med (Berl) 2009;87:735–48.
19. Goodrich RJ, Johnson GD, Krawetz S. The preparation of human spermatozoal RNA for clinical analysis. Arch Androl 2007;53:161–7.
20. Salas-Huetos A, Blanco J, Vidal F, Anton E. Analysis of the expression of four microRNAs in spermatozoa and their implication in male fertility. Rev Int Andrología 2012;10:92–7.
21. Dadoune JP. Spermatozoal RNAs: what about their functions? Microsc Res Tech 2009;72:536–51.
22. Hamatani T. Human spermatozoal RNAs. Fertil Steril 2012;97:275–81.
23. Boerke A, Dieleman S, Gadella B. A possible role for sperm RNA in early embryo development. Theriogenology 2007;68(Suppl 1):S147–55.
24. Krawetz S, Kruger A, Lalancette C, Tagett R, Anton E, Draghici S, et al. A survey of small RNAs in human sperm. Hum Reprod 2011;26:3401–12.
25. Krawetz S. Paternal contribution: new insights and future challenges. Nat Rev Genet 2005;6:633–42.
26. Lalancette C, Miller D, Li Y, Krawetz S. Paternal contributions: new functional insights for spermatozoal RNA. J Cell Biochem 2008;104:1570–9.
27. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet 2002;360:772–7.
28. Wu W, Hu Z, Qin Y, Dong J, Dai J, Lu C, et al. Seminal plasma microRNAs: potential biomarkers for spermatogenesis status. Mol Hum Reprod 2012; 18:489–97.
29. Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, et al. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. Fertil Steril 2013;99:1249–55.
30. Lian J, Zhang X, Tian H, Liang N, Wang Y, Liang C, et al. Altered microRNA expression in patients with non-obstructive azoospermia. Reprod Biol Endocrinol 2009;7:13.
31. Cooper TG. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization Press; 2010.
32. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365–86.
33. Jodar M, Kalko S, Castillo J, Ballescà JL, Oliva R. Differential RNAs in the sperm cells of asthenozoospermic patients. Hum Reprod 2012;27:1431–8.
34. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
35. Wylie D, Shelton J, Choudhary A, Adai AT. A novel mean-centering method for normalizing microRNA expression from high-throughput RT-qPCR data. BMC Res Notes 2011;4:555.
36. Lu M, Shi B, Wang J, Cao Q, Cui Q. TAM: a method for enrichment and depletion analysis of a microRNA category in a list of microRNAs. BMC Bioinformatics 2010;11:419.
37. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2010.
38. Dvinge H, Bertone P. HTqPCR: high-throughput analysis and visualization of quantitative real-time PCR data in R. Bioinformatics 2009;25:3325–6.
39. Maragkakis M, Reczko M, Simossis V, Alexiou P, Papadopoulos GL, Dalamagas T, et al. DIANA-microT web server: elucidating microRNA functions through target prediction. Nucleic Acids Res 2009;37:W273–6.
40. Maragkakis M, Alexiou P, Papadopoulos GL, Reczko M, Dalamagas T, Giannopoulos G, et al. Accurate microRNA target prediction correlates with protein repression levels. BMC Bioinformatics 2009;10:295.
41. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4:44–57.
42. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37:1–13.
43. Sarkar D. Lattice: multivariate data visualization with R. New York: Springer; 2008.
44. Liu T, Cheng W, Gao Y, Wang H, Liu Z. Microarray analysis of microRNA expression patterns in the semen of infertile men with semen abnormalities. Mol Med Rep 2012;6:535–42.
45. Jung YH, Gupta MK, Shin JY, Uhm SJ, Lee HT. MicroRNA signature in testes-derived male germ-line stem cells. Mol Hum Reprod 2010;16:804–10.
46. Wu W, Qin Y, Li Z, Dong J, Dai J, Lu C, et al. Genome-wide microRNA expression profiling in idiopathic non-obstructive azoospermia: significant up-regulation of miR-141, miR-429 and miR-7-1-3p. Hum Reprod 2013; 28:1827–36.
47. Niu Z, Goodyear SM, Rao S, Wu X, Tobias JW, Avarbock MR, et al. MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 2011;108:12740–5.
48. Ma YL, Zhang P, Wang F, Moyer MP, Yang JJ, Liu ZH, et al. Human embryonic stem cells and metastatic colorectal cancer cells shared the common endogenous human microRNA-26b. J Cell Mol Med 2011;15:1941–54.
49. Wang C, Yang C, Chen X, Yao B, Yang C, Zhu C, et al. Altered profile of seminal plasma microRNAs in the molecular diagnosis of male infertility. Clin Chem 2011;57:1722–31.
50. Lu YC, Chen H, Fok KL, Tsang LL, Yu MK, Zhang XH, et al. CFTR mediates bicarbonate-dependent activation of miR-125b in preimplantation embryo development. Cell Res 2012;22:1453–66.
51. Lin YC, Kuo MW, Yu J, Kuo HH, Lin RJ, Lo WL, et al. c-Myb is an evolutionary conserved miR-150 target and miR-150/c-Myb interaction is important for embryonic development. Mol Biol Evol 2008;25:2189–98.
52. Medeiros LA, Dennis LM, Gill ME, Houbaviy H, Markoulaki S, Fu D, et al. Mir-290-295 deficiency in mice results in partially penetrant embryonic lethality and germ cell defects. Proc Natl Acad Sci U S A 2011;108: 14163–8.
53. Belleannée C, Calvo E, Thimon V, Cyr DG, Légaré C, Garneau L, et al. Role of microRNAs in controlling gene expression in different segments of the human epididymis. PLoS One 2012;7:e34996.
54. Kogenaru S, Qing Y, Guo Y, Wang N. RNA-seq and microarray complement each other in transcriptome profiling. BMC Genomics 2012;13:629.
55. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, et al. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 2010;16:991–1006.
56. Chen Y, Gelfond JA, McManus LM, Shireman PK. Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics 2009;10:407.
57. Koshiol J, Wang E, Zhao Y, Marincola F, Landi MT. Strengths and limitations of laboratory procedures for microRNA detection. Cancer Epidemiol Biomarkers Prev 2010;19:907–11.
58. Greco S, Perfetti A, Fasanaro P, Cardani R, Capogrossi MC, Meola G, et al. Deregulated microRNAs in myotonic dystrophy type 2. PLoS One 2012;7: e39732.
59. Bao J, Li D, Wang L, Wu J, Hu Y, Wang Z, et al. MicroRNA-449 and microRNA-34b/c function redundantly in murine testes by targeting E2F transcription factor-retinoblastoma protein (E2F-pRb) pathway. J Biol Chem 2012;287:21686–98.
60. Pede V, Rombout A, Vermeire J, Naessens E, Mestdagh P, Robberecht N, et al. CLL cells respond to B-Cell receptor stimulation with a microRNA/mRNA signature associated with MYC activation and cell cycle progression. PLoS One 2013;8:e60275.
61. McIver SC, Roman SD, Nixon B, McLaughlin EA. miRNA and mammalian male germ cells. Hum Reprod Update 2012;18:44–59.
62. Grillari J, Hackl M, Grillari-Voglauer R. miR-17-92 cluster: ups and downs in cancer and aging. Biogerontology 2010;11:501–6.
63. Hackl M, Brunner S, Fortschegger K, Schreiner C, Micutkova L, Muck C, et al. miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging. Aging Cell 2010;9:291–6.
64. Kong KL, Kwong DLW, Chan THM, Law SYK, Chen L, Li Y, et al. MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. Gut 2012;61:33–42.

65. Radisky DC. miR-200c at the nexus of epithelial-mesenchymal transition, resistance to apoptosis, and the breast cancer stem cell phenotype. Breast Cancer Res 2011;13:110.
66. Gao W, Shen H, Liu L, Xu J, Xu J, Shu Y. MiR-21 overexpression in human primary squamous cell lung carcinoma is associated with poor patient prognosis. J Cancer Res Clin Oncol 2011;137:557–66.
67. Xi Y, Formentini A, Chien M, Weir DB, Russo JJ, Ju J, et al. Prognostic values of microRNAs in colorectal cancer. Biomark Insights 2006;2:113–21.
68. Martin J, Jenkins RH, Bennagi R, Krupa A, Phillips AO, Bowen T, et al. Post-transcriptional regulation of transforming growth factor beta-1 by microRNA-744. PLoS One 2011;6:e25044.
69. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753–91.
70. Lin Y, Zeng Y, Zhang F, Xue L, Huang Z, Li W, et al. Characterization of microRNA expression profiles and the discovery of novel microRNAs involved in cancer during human embryonic development. PLoS One 2013;8:e69230.
71. Takada S, Yamashita Y, Berezikov E, Hatanaka H, Fujiwara S, Kurashina K, et al. MicroRNA expression profiles of human leukemias. Leukemia 2008;22:1274–8.
72. Rokah OH, Granot G, Ovcharenko A, Modai S, Pasmanik-Chor M, Toren A, et al. Downregulation of miR-31, miR-155, and miR-564 in chronic myeloid leukemia cells. PLoS One 2012;7:e35501.
73. Lui WO, Pourmand N, Patterson BK, Fire A. Patterns of known and novel small RNAs in human cervical cancer. Cancer Res 2007;67:6031–43.
74. Childs G, Fazzari M, Kung G, Kawachi N, Brandwein-Gensler M, McLemore M, et al. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. Am J Pathol 2009;174:736–45.
75. Feifei N, Mingzhi Z, Yanyun Z, Huanle Z, Fang R, Mingzhu H, et al. MicroRNA expression analysis of mammospheres cultured from human breast cancers. J Cancer Res Clin Oncol 2012;138:1937–44.
76. Sand M, Skrygan M, Sand D, Georgas D, Hahn SA, Gambichler T, et al. Expression of microRNAs in basal cell carcinoma. Br J Dermatol 2012;167:847–55.
77. Ketley A, Warren A, Holmes E, Gering M, Aboobaker A, Brook JD. The miR-30 microRNA family targets smoothened to regulate hedgehog signalling in zebrafish early muscle development. PLoS One 2013;8:e65170.
78. Marigo V, Roberts DJ, Lee SMK, Tsukurov O, Levi T, Gastier JM, et al. Cloning, expression, and chromosomal location of SHH and IHH: two human homologues of the Drosophila segment polarity gene hedgehog. Genomics 1995;28:44–51.
79. Chang DT, López A, von Kessler DP, Chiang C, Simandl BK, Zhao R, et al. Products, genetic linkage and limb patterning activity of a murine hedgehog gene. Development 1994;120:3339–53.
80. Hobert O, Westphal H. Functions of LIM-homeobox genes. Trends Genet 2000;16:75–83.
81. Taira M, Jamrich M, Good PJ, Dawid IB. The LIM domain-containing homeo box gene Xlim-1 is expressed specifically in the organizer region of Xenopus gastrula embryos. Genes Dev 1992;6:356–66.
82. Ye L, Evans J, Gargett CE. Lim1/LIM1 is expressed in developing and adult mouse and human endometrium. Histochem Cell Biol 2012;137:527–36.
83. Tanzer A, Amemiya CT, Kim CB, Stadler PF. Evolution of microRNAs located within Hox gene clusters. J Exp Zool B Mol Dev Evol 2005;304:75–85.
84. Lemons D, McGinnis W. Genomic evolution of Hox gene clusters. Science 2006;313:1918–22.
85. Woltering JM, Durston AJ. MiR-10 represses HoxB1a and HoxB3a in zebrafish. PLoS One 2008;3:e1396.
86. Pasquinelli A, Reinhart B, Slack F, Martindale M, Kuroda M, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 2000;408:86–9.
87. Tennessen JM, Thummel CS. Developmental timing: let-7 function conserved through evolution. Curr Biol 2008;18:R707–8.
88. Foshay KM, Gallicano GI. miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. Dev Biol 2009;326:431–43.
89. Hyun S, Lee JH, Jin H, Nam J, Namkoong B, Lee G, et al. Conserved MicroRNA miR-8/miR-200 and its target USH/FOG2 control growth by regulating PI3K. Cell 2009;139:1096–108.
90. Klein U, Lia M, Crespo M, Siegel R, Shen Q, Mo T, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell 2010;17:28–40.
91. Scapoli L, Palmieri A, Lo Muzio L, Pezzetti F, Rubini C, Girardi A, et al. MicroRNA expression profiling of oral carcinoma identifies new markers of tumor progression. Int J Immunopathol Pharmacol 2010;23:1229–34.
92. Sun D, Lee YS, Malhotra A, Kim HK, Matecic M, Evans C, et al. miR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res 2011;71:1313–24.
93. Trompeter HI, Abbad H, Iwaniuk KM, Hafner M, Renwick N, Tuschl T, et al. MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. PLoS One 2011;6:e16138.
94. Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafà R, et al. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. Gastroenterology 2012;142:886–96.
95. Lee K, Kim JH, Kwon OB, An K, Ryu J, Cho K, et al. An activity-regulated microRNA, miR-188, controls dendritic plasticity and synaptic transmission by downregulating neuropilin-2. J Neurosci 2012;32:5678–87.
96. Clark O, Schmidt F, Coles CH, Tchetchelnitski V, Stoker AW. Functional analysis of the putative tumor suppressor PTPRD in neuroblastoma cells. Cancer Invest 2012;30:422–32.
97. Paul S, Lombroso PJ. Receptor and nonreceptor protein tyrosine phosphatases in the nervous system. Cell Mol Life Sci 2003;60:2465–82.
98. Denu JM, Dixon JE. Protein tyrosine phosphatases: mechanisms of catalysis and regulation. Curr Opin Chem Biol 1998;2:633–41.
99. Bar-Nun S, Glickman MH. Proteasomal AAA-ATPases: structure and function. Biochim Biophys Acta 2012;1823:67–82.
100. Mukherjee S, Valencia JDD, Stewman S, Metz J, Monnier S, Rath U, et al. Human fidgetin is a microtubule severing en\e and minus-end depolymerase that regulates mitosis. Cell Cycle 2012;11:2359–66.
101. Del Rizzo PA, Trievel RC. Substrate and product specificities of SET domain methyltransferases. Epigenetics 2011;6:1059–67.
102. Kurihara Y, Tokuriki M, Myojin R, Hori T, Kuroiwa A, Matsuda Y, et al. CPEB2, a novel putative translational regulator in mouse haploid germ cells. Biol Reprod 2003;69:261–8.
103. Zhu CH, Huang Y, Broman MT, Domann FE. Expression of AP-2 alpha in SV40 immortalized human lung fibroblasts is associated with a distinct pattern of cytosine methylation in the AP-2 alpha promoter. Biochim Biophys Acta 2001;1519:85–91.
104. Till S, Lejeune E, Thermann R, Bortfeld M, Hothorn M, Enderle D, et al. A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. Nat Struct Mol Biol 2007;14:897–903.
105. Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Lührmann R, et al. Identification of novel argonaute-associated proteins. Curr Biol 2005;15:2149–55.
106. Carrell DT, Hammoud SS. The human sperm epigenome and its potential role in embryonic development. Mol Hum Reprod 2010;16:37–47.
107. Miller D, Ostermeier GC, Krawetz S. The controversy, potential and roles of spermatozoal RNA. Trends Mol Med 2005;11:156–63.
108. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 2004;429:154.
109. Lewis SEM. Is sperm evaluation useful in predicting human fertility? Reproduction 2007;134:31–40.
110. Malcher A, Rozwadowska N, Stokowy T, Kolanowski T, Jedrzejczak P, Zietkowiak W, et al. Potential biomarkers of nonobstructive azoospermia identified in microarray gene expression analysis. Fertil Steril 2013;100:1686–94.
111. Garrido N, García-Herrero S, Meseguer M. Assessment of sperm using mRNA microarray technology. Fertil Steril 2013;99:1008–22.
112. Lima-Souza A, Anton E, Mao S, Ho WJ, Krawetz SA. A platform for evaluating sperm RNA biomarkers: dysplasia of the fibrous sheath—testing the concept. Fertil Steril 2012;97:1061–6.

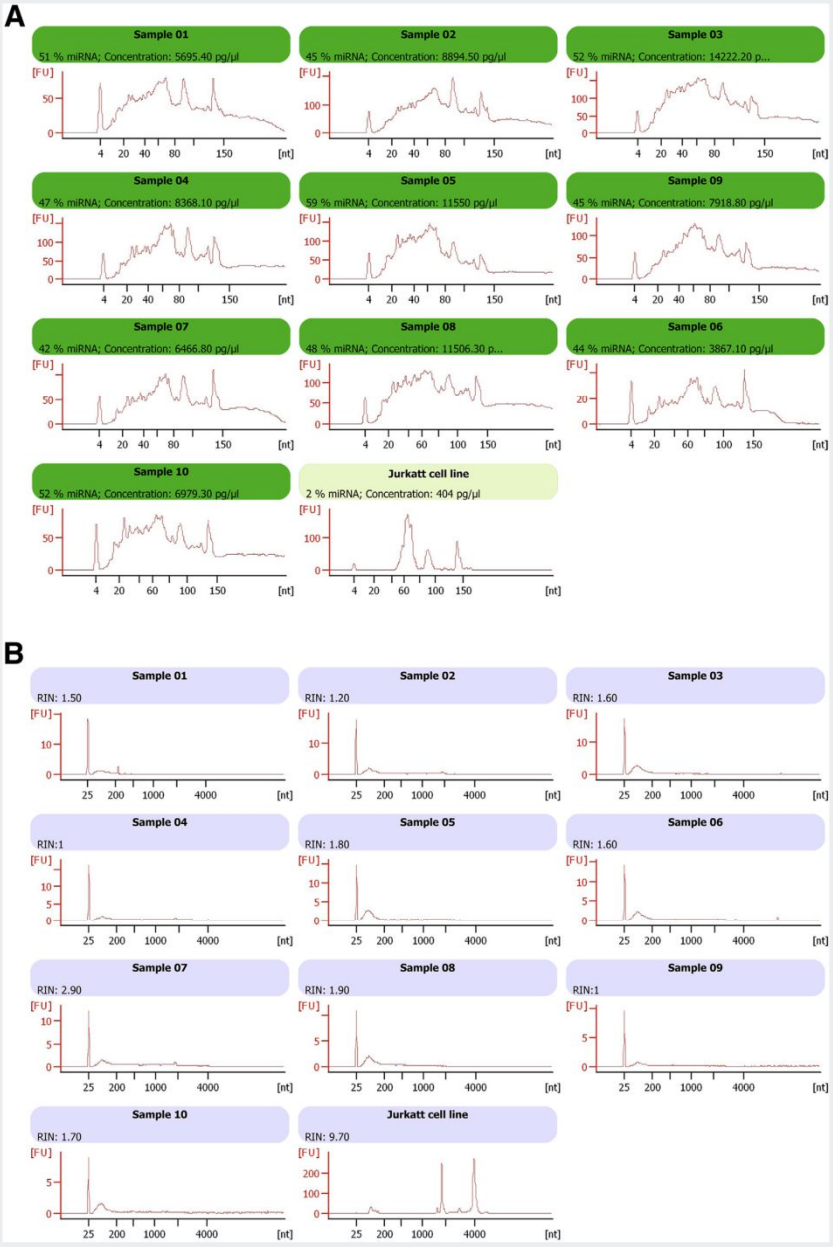
SUPPLEMENTAL FIGURE 1



Electrophoretic gel of PCR products corresponding to (A) *PRM1* amplification. Ladder: molecular marker (0.1–1 kb). C+ = Genomic DNA as a positive control (422b). S01-S10 = Sperm cDNAs showing an intronless product obtained from RT (331b). C− = negative controls (RT-PCR without RNA and *PRM1* PCR without cDNA). (B) *GAPDH* amplification. Ladder: molecular marker (0.1–1kb). C+ = Genomic DNA as a positive control (332b). S01-S10 = Sperm cDNAs showing an intronless product obtained from RT (228b). C− = negative controls (RT-PCR without RNA and *GAPDH* PCR without cDNA). (C) *CD45* amplification. Ladder: molecular marker (0.1–1kb). C+ = Complementary DNA obtained from peripheral blood lymphocytes as a positive control (179b). S01-S10 = Sperm cDNAs showing the absence of CD45 marker. C− = negative controls (RT-PCR without RNA and *CD45* PCR without cDNA).

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

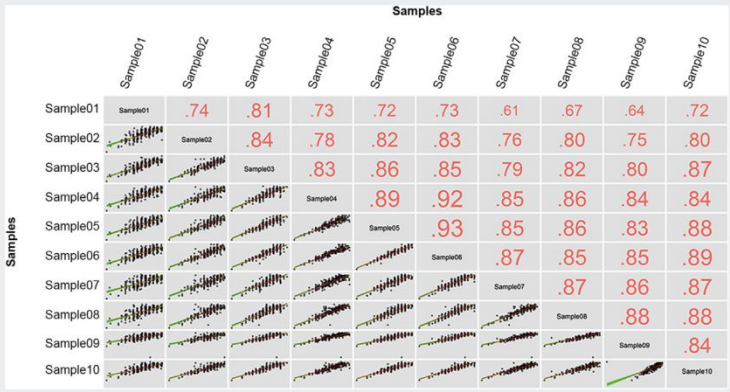
SUPPLEMENTAL FIGURE 2



Electrophoretic analysis results of the sperm RNA samples using the Agilent 2100 Bioanalyzer. (A) Small-RNA chip (RNAs ranging from 4 to 150 nt). (B) Nano-RNA chip (RNAs ranging from 25 to 200 nt). The last electropherogram (in A and B) was obtained from Jurkatt cells RNA (somatic cell control).

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

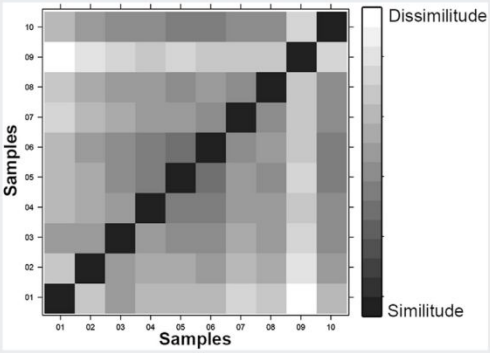
SUPPLEMENTAL FIGURE 3



Results of the Spearman correlations performed between samples regarding the ubiquitous miRNA normCt expression values.

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.

SUPPLEMENTAL FIGURE 4



Heatmap showing the relative similitude between samples according to miRNA normCt values.

Salas-Huetos. miRNA expression profile in human sperm. Fertil Steril 2014.