Human embryos secrete microRNAs into culture media—a potential biomarker for implantation

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Objective: To determine whether human blastocysts secrete microRNA (miRNAs) into culture media and whether these reflect embryonic ploidy status and can predict in vitro fertilization (IVF) outcomes.
Design: Experimental study of human embryos and IVF culture media.
Setting: Academic IVF program.
Patient(s): 91 donated, cryopreserved embryos that developed into 28 tested blastocysts, from 13 couples who had previously completed IVF cycles.
Intervention(s): None.
Main Outcome Measure(s): Relative miRNA expression in IVF culture media.
Result(s): Blastocysts were assessed by chromosomal comparative genomic hybridization analysis, and the culture media from 55 single-embryo transfer cycles was tested for miRNA expression using an array-based quantitative real-time polymerase chain reaction analysis. The expression of the identified miRNA was correlated with pregnancy outcomes. Ten miRNA were identified in the culture media; two were specific to spent media (miR-191 and miR-372), and one was only present in media before the embryos had been cultured (miR-645). MicroRNA-191 was more highly concentrated in media from aneuploid embryos, and miR-191, miR-372, and miR-645 were more highly concentrated in media from failed IVF/non-intracytoplasmic sperm injection cycles. Additionally, miRNA were found to be more highly concentrated in ICSI and day-5 media samples when compared with regularly inseminated and day-4 samples, respectively.
Conclusion(s): MicroRNA can be detected in IVF culture media. Some of these miRNA are differentially expressed according to the fertilization method, chromosomal status, and pregnancy outcome, which makes them potential biomarkers for predicting IVF success. (Fertil Steril® 2014;101:1493–500. ©2014 by American Society for Reproductive Medicine.)
Key Words: Expression, exosomes, human blastocyst, IVF, media, microRNA, secretion

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MicroRNAs (miRNAs) are small (approximately 22 nucleotides) noncoding RNAs that regulate gene expression and have been implicated in a wide array of biologic processes including early embryo development and stem cell differentiation (1). Recently, miRNAs have been found to be packaged into small vesicles called exosomes and subsequently secreted into the extracellular space (2). Encapsulated miRNAs are protected from degradation and, consequently, can be detected after extended periods of time (3). Although the role of exosomal miRNAs is still being elucidated, growing evidence suggests that packaged miRNAs can reach distant cells and affect gene expression (4).

Regardless of their physiologic role, distinct patterns of secreted miRNAs have been found to correlate with a variety of diseases including cancer (5), diabetes (6), and tissue injury (7, 8). They have been detected in virtually all bodily fluids including breast milk, amniotic fluid, tears, cerebrospinal fluid, peritoneal fluid, blood, pleural fluid, saliva, semen, and urine (9). Consequently, there is great interest in identifying miRNAs within these fluids to be used as biomarkers for the early detection of diseases.

Received January 10, 2013; revised January 16, 2014; accepted January 23, 2014; published online March 12, 2014.
E.M.R. has nothing to disclose. D.N.S. has nothing to disclose. L.M.W. is on the Ferring Pharmaceuticals Regional Advisory Board. A.E.T.S. has nothing to disclose. B.J.V. has nothing to disclose.
Supported in part by the Henrietta Rose and Mary Ellen Molinaro-Blonigan Research Fund, University of Iowa Carver College of Medicine.
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Fertility and Sterility® Vol. 101, No. 5, May 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.01.058

MicroRNAs are highly expressed in rapidly growing and undifferentiated cells such as cancer cells and embryonic stem cells. This led us to discover that miRNAs are highly expressed in human embryos and that *intracellular* miRNA expression patterns differ between euploid and aneuploid embryos (10). Because miRNAs are known to be secreted from cells grown in culture into the surrounding media (4), we first determined whether human embryos secrete miRNAs into in vitro fertilization (IVF) culture media, and if so, whether they were differentially secreted according to embryo chromosomal status. We further hypothesized that secreted miRNAs could be used as biomarkers to determine embryonic health before embryo transfer, with the ultimate goal of improving live-birth rates. Our final goal was to see whether specific patterns of media miRNA correlated with clinical IVF pregnancy outcomes.

MATERIALS AND METHODS

All experiments were performed and monitored via approved protocols under the University of Iowa Institutional Review Board. Specifically, patients donated embryos for research on biomarkers of embryo quality, and the patients were approached prospectively to allow the collection and testing of embryo culture media to find potential markers of implantation. All University of Iowa studies using human embryos are initially approved by the University's IVF ethics committee before institutional review board review.

Embryo Culture

The overall study design (Fig. 1) was to screen culture media from a cohort of donated, IVF embryos for relative and differential miRNA expression using an array-based quantitative real-time PCR (qRT-PCR) method. To confirm the miRNA array findings, the media from an expanded set of embryos were tested for miRNA expression with single miRNA qRT-PCR assays. Finally, miRNAs that were identified as being expressed in the initial experiments were then measured in embryonic conditioned media (hereafter referred to as spent media) samples from single-embryo transfer (SET) cycles to see whether the concentrations correlated with pregnancy outcomes.

Patients who donated excess cryopreserved embryos for this study had used IVF for a variety of infertility diagnoses. However, these were undisclosed because of institutional review board protocol constraints. Only embryos fertilized by intracytoplasmic sperm injection (ICSI) were used in the initial studies to prevent possible sample contamination by accessory sperm. Pronuclear-stage embryos had been cryopreserved by controlled rate freezing 18 to 22 hours after ICSI in 1.5 M 1,2 propanediol (PROH; Sigma) as previously described elsewhere (11). Embryos were thawed by air warming for 40 seconds followed by 10-second exposure to 30°C sterile water. Cryoprotectants were removed in a stepwise dilutional fashion. Surviving embryos were cultured in groups of three to four in 50-μL microdrops of IVC-One (In VitroCare) supplemented with 20% SPS (Serum Protein Substitute; CooperSurgical/Sage) under oil (Cook Medical) in 5.5% to 6.0% CO_2 in air at 37°C. Embryos were moved to fresh drops of IVC-One supplemented with 20% SPS on day 3.

On the morning of day 4, embryos were moved to individual culture in 8 μL of IVC-Three (In VitroCare) supplemented with 20% SPS. Blank media control drops (hereafter referred to as controls) were incubated in the same dishes as those with media drops containing embryos. Before moving the embryos to fresh drops, we rinsed them through a series of five wash drops. All embryos were cultured to the blastocyst stage and were graded according a standardized classification system (12). On the morning of the fifth day of culture, the embryos that had reached at least the early blastocyst stage and had an inner cell mass grade of B or better were chosen for assisted hatching. These embryos were moved to fresh culture media drops, and 6 μL of the spent media were collected and stored at −80°C for miRNA analysis. On the afternoon of day 5 or on the morning of day 6 of culture, hatching blastocysts were selected for biopsy.

Embryo Biopsy and Determination of Chromosomal Makeup of Donated Pronuclear Embryos

To determine the chromosomal makeup of donated embryos, a 10-μm channel was opened in the zona pellucida with a series of three to five laser pulses of 5-ms duration (Octax Microscience, GmbH). Approximately five herniating trophectoderm cells per embryo were aspirated into a biopsy pipette and detached by firing laser pulses at the area of constriction. After several passes through a wash solution, the biopsied cells were placed into a PCR tube with lysis buffer supplied by the Genesis Genetics Institute. The biopsy samples then were shipped on dry ice to their facility for array comparative genomic hybridization (aCGH) by their standard proprietary diagnostic technique using the BlueGnome 24sure V3 microarray platform. Mosaicism was determined by using standard clinical protocols, which includes a cutoff of 25% or greater deviation of fluorescent ratios between the hybridized chromosomes (13).

miRNA Isolation and Detection

To maximize the total amount of RNA available from each spent media sample collected, the direct Cells-to-Ct method was used for reverse transcription (TaqMan Micro RNA Cells-to-CT Kit; Applied Biosystems). The 6 μL of day-5 spent media collected from each sample was placed into an equal amount of Cells-to-Ct lysis buffer with dilute deoxyribonuclease I. After 8 minutes, stop solution was added, and the samples were stored at −80°C. To allow the simultaneous reverse transcription of 754 human miRNAs, three endogenous miRNA controls, and one nonhuman negative control for each sample, two master mixes consisting of the A and B Megaplex RT primer pools, respectively (Human Pools Set v3.0; Applied Biosystems), were made per the manufacturer's Megaplex Pools protocol. We mixed 3 μL of lysate with 4.5 μL of master-mix for each reaction for a total volume of 7.5 μL. Thermal-cycling conditions were as follows: 40 cycles at 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second, then