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MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment

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Objective: To assess whether extracellular microRNAs (miRNAs) can be accurately profiled from spent blastocyst culture media (SBM) and used as embryonic biomarkers.
Design: Prospective cohort study.
Setting: Private and academic in vitro fertilization centers.
Patient(s): Inner cell mass-free trophectoderm (TE) samples and their relative SBM from five good-quality human blastocysts.
Intervention(s): Protocol for miRNA purification and analysis based on quantitative polymerase chain reaction set and validated on human embryonic stem cells (hESCs) and on SBM with and without biological variability.
Main Outcomes Measure(s): Analysis of miRNAs in culture media in relation with TE cells and comparison of miRNA profiles between implanted and unimplanted euploid blastocysts.
Result(s): Culture media from embryos in the cleavage, morula, and blastocyst stages were collected to investigate the presence of miRNAs. The SBM were prospectively collected from euploid implanted (n = 25) and unimplanted blastocysts (n = 28) for comparison. We hypothesized that human embryos secrete miRNAs in culture media that can be used as biomarkers. The comparative analysis of TE and SBM samples revealed that 96.6% (57 of 59; 95 CI, 88.3–99.6) of the miRNAs detected in the SBM were expressed from TE cells, suggesting a TE origin. The culture media collected from cleavage and morula stage embryos showed a pattern similar to blanks, suggesting that miRNAs profiling from spent culture media applies only for blastocysts. MicroRNAs analysis of SBM from euploid implanted and unimplanted blastocysts highlighted two miRNAs (miR-20a, miR-30c) that showed increased concentrations in the former and were predicted in silico to be involved in 23 implantation-related pathways.
Conclusion(s): MicroRNAs secreted from human blastocysts in culture media can be profiled with high reproducibility, and this approach can be further explored for noninvasive embryo selection. (Fertil Steril® 2016;105: 225–35. ©2016 by American Society for Reproductive Medicine.)
Key Words: Biomarkers of implantation, blastocyst-endometrial dialogue, blastocyst evaluation, embryo quality, microRNA

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MicroRNAs (miRNAs) are evolutionarily conserved, single-stranded, noncoding RNA molecules of ~22 nucleotides in length. Over 1,000 miRNAs have been identified in the human genome (miRBase, www.mirbase.org), and they are considered to be major transcriptional/posttranscriptional regulators of gene expression. A miRNA can modulate the levels of many target genes at once through partial sequence complementation, usually at the 3′ untranslated regions (UTRs) of mRNAs, and subsequent interference with mRNA stability and/or protein translation (1, 2). MicroRNAs are secreted in membrane-bound exosomes and microvesicles, are bound to stabilizing proteins, and can be detected in virtually all body fluids, including blood, urine, saliva, tears, breast milk, semen, amniotic fluid, cerebrospinal fluid, peritoneal fluid, and pleural fluid as well as in culture media collected from different cell lines (3–5). It is believed that miRNAs that are secreted from donor cells can be internalized into recipient cells, where they can exert their genetic regulatory function. Chen et al. (6) tested blood serum samples and found that miRNAs were highly stable and able to withstand extreme environmental conditions such as freezing and thawing; thus, they may be effective biomarkers. Consequently, there is great interest in identifying miRNAs within biofluids that can be used as noninvasive biomarkers for the early detection of diseases. For example, miR-141, miR-499, and miR-122 are associated with prostate cancer, myocardial infarction, and drug-induced liver injury, respectively (3,6–9).

We hypothesized that human embryos could also secrete specific miRNAs in the extracellular environment as part of the blastocyst–endometrium interaction, which is necessary for implantation success. This interaction could determine the establishment of phase synchrony, ultimately aiming to create a hospitable environment for competent embryos at the implantation site, which is a prerequisite for successful implantation. Several studies have already illustrated dynamic changes in miRNA expression in gametes and during early embryonic development of mammalian species (10–14). Other studies have recently reported that the human endometrial epithelium releases exosomes into the uterine cavity. If these exosomes transfer their contents to either the blastocyst or the adjacent endometrium, they could influence implantation by affecting endometrial gene expression (15, 16). Furthermore, new communication systems mediating the cross-talk between the blastocyst and the endometrium based on secreted miRNAs have been recently discovered in animal models. In particular, LIF-dependent STAT activation and its impact on modulation of trophoblast functions during embryo implantation was described in several animal models (17–20) and recently direct evidence of the role of miR-181 secreted by endometrial cells in modulating embryo implantation through the regulation of LIF has been reported in mice (21).

However, to date no comprehensive data have been reported regarding blastocyst-derived miRNAs in the extracellular environment. In this context, growing embryos in vitro during IVF cycles offer a unique possibility to determine the presence of miRNAs in easily collectable embryonic culture media and identify noninvasive biomarkers associated with embryo reproductive competence. We comprehensively characterized the profile of miRNAs secreted by human preimplantation embryos in spent culture media and investigated whether secreted miRNAs can be used as predictive markers of clinical outcomes in in vitro fertilization (IVF) cycles.

MATERIALS AND METHODS

Study Design and Outcome Measures

In our prospective cohort study, the primary hypothesis was that human embryos secrete miRNAs in culture media, which can be used as biomarkers of embryo quality during IVF cycles. First, a protocol for miRNA purification and analysis based on quantitative polymerase chain reaction (qPCR) was set and validated on human embryonic stem cells (hESCs) of different concentrations and on spent blastocyst culture media (SBM) with and without biological variability. A miRNA profile of trophectoderm (TE) cells and their relative culture media was then generated and compared among these specimens. Next, SBM samples collected from embryos at different stages of preimplantation development (cleavage, morula, and blastocyst stages) were profiled for miRNAs to investigate when the secretion process begins in the preimplantation window. Finally, miRNAs profiles of SBMs prospectively collected from implanted versus unimplanted euploid blastocysts were compared to investigate the potential for the use of these biomarkers for embryo selection.

Embryos

The work described here was performed under a license from the United Kingdom Human Fertilization and Embryology Authority (research license numbers: R0075 and R0133) and also received local ethics approval (U.K. National Health Service Research Ethics Committee Reference: 06/Q0702/90). Informed consent was obtained from all participants, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the National Institutes of Health Belmont Report. No financial donations were offered. Cryopreserved embryos that were no longer needed for therapeutic use by patients were obtained from Guy's Assisted Conception Unit and external units through the Human Embryonic Stem Cell Coordinator's (hESCCO) network (22). To obtain a pure TE fraction, the inner cell mass (ICM) was biopsied from expanded blastocysts 12 hours after warming as described elsewhere (23). Whole TE samples and 25 μL of SBM from each embryo, collected immediately before TE isolation, were snap-frozen and later processed individually for miRNA analysis.

Human Embryonic Stem Cells

The hESC line KCL034 was cultured as previously described elsewhere (24–26). Smaller (approximately 10–50 cells) and larger (approximately 500–1,000 cells) clumps of hESCs were microdissected free of feeders, transferred directly to lysis buffer, and snap-frozen in liquid nitrogen.

Spent Culture Medium from Single-embryo Transfer Cycles

The spent culture medium was collected from embryos at the cleavage and morula stages or from expanded blastocysts of good quality and without signs of degenerating cells during in vitro fertilization (IVF) cycles. The ethics committee of Clinica Valle Giulia approved the study.

IVF Procedures and Spent Medium Collection

All embryos were obtained after controlled ovarian stimulation that was performed using a gonadotropin-releasing hormone-agonist long protocol and IVF. Fertilized oocytes were sequentially cultured in separate 25-μL microdrops (Sage) up to the blastocyst stage (days 5–6) in a humidified atmosphere containing 5% O_2 and 6% CO_2. On day 3 after fertilization, cleavage-stage embryos were transferred to a fresh individual 35-μL drop of Quinn's Advantage Blastocyst Medium (Cooper Surgical) with 5% Quinn's Advantage Human Serum Albumin (Cooper Surgical). The quality of the blastocysts was assessed immediately before cryopreservation and was defined according to the standard criteria (27).

The blastocyst vitrification and warming procedures were performed using a Vitrification and Warming Kit (Kitazato BioPharma) (28, 29). The entire vitrification and warming procedures were performed at room temperature as previously described elsewhere (30). Immediately after warming, the blastocysts were allocated to individually numbered 35-μL microdrops of blastocyst medium supplemented with 5% human serum albumin under mineral oil (Sage). The TE biopsy, 24-chromosome aneuploidy screening, and vitrification were performed as previously described elsewhere (31). Euploid blastocysts were selected for transfer based on the morphologic score and were warmed and cultured at 37°C (6% CO_2 and 5% O_2) until transfer. We transferred 25 μL of SBM to individually labeled PCR tubes containing 120 μL of lysis solution. The PCR tubes were then briefly centrifuged and stored at −20°C before miRNA isolation. Only cycles with single embryo transfers were included. The endometrial preparation and transfer procedures were performed as previously described elsewhere (32). Ongoing implantation was defined by the presence of a fetus with heart activity beyond 20 weeks of gestation (33).

MicroRNA Isolation, Retrotranscription, and Preamplification

The miRNA isolation and purification procedures were performed using the TaqMan miRNA Anti-miRNA Bead Capture (ABC) Purification Kit (Applied Biosystems), which was designed specifically for the rapid purification of miRNAs from small inputs of all human sample types including cell cultures. Human Panel A v3.0 beads are a type of super paramagnetic Dynabeads that are covalently bound to a unique set of 381 anti-miRNA oligonucleotides. The miRNA isolation relies on hybridization of endogenous miRNAs to the corresponding anti-miRNA oligonucleotides attached to the beads. The entire recoverable volume of sample was brought to a total volume of 150 μL with ABC buffer (and to a total volume of 200 μL with lysis buffer in the case of hESCs).

The purification protocol was followed according to the manufacturer's instructions. The bead hybridization and the miRNA elution steps were conducted on a Thermomixer (Eppendorf). All the washing steps were performed using the DynaMag-2 magnet (Applied Biosystems) to remove DNA, proteins, contaminants, and residual binding solution while keeping the miRNAs bound to the beads. The miRNAs were eluted in a final volume of 10 μL to concentrate them in the smallest possible volume (20 μL in the case of hESC). The cDNA was then generated using the specific Megaplex RT Primer Pools A v3.0 (Applied Biosystems) on a 7900 HT Real-Time PCR System (Applied Biosystems) according to the manufacturer's protocol.

The preamplification reaction was modified because two cDNA synthesis reactions were performed in parallel per sample to double the final sample volume. Specifically, two independent reactions were performed in two different tubes to create products that were joined at the end of the process; each tube contained 3.5 μL of retrotranscription product, 12.5 μL of TaqMan PreAmp Master Mix (2X), 2.5 μL of Megaplex PreAmp Primers (10X), and 6.5 μL of nuclease-free water. The thermal protocol used was the same as that reported by the manufacturer and consisted of eight cycles.

MicroRNA Analysis Using a TaqMan Low-Density Array

MicroRNA expression was evaluated using TaqMan Low-Density Array (TLDA) miRNA Cards (Panel A) (Applied Biosystems) with an Applied Biosystems Viia7 Real-Time PCR System. Card A contains 381 TaqMan miRNA assays, enabling the simultaneous quantitation of 377 human miRNAs plus three endogenous controls and a negative control. Fifty microliters of the preamplification product was added to 400 μL of nuclease-free water and mixed with 450 μL of TaqMan Universal Master Mix II, No UNG. The mixture was then dispensed into 384 wells by centrifugation on a Heraeus Megafuge 40 (Thermo Scientific) with the proper TLDA card adapters. The reactions were incubated in a 384-well plate at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on a ViiA 7 Real-Time PCR System (Applied Biosystems).

After a preliminary manual inspection of all amplification plots, the raw data were analyzed using SDS software (Applied Biosystems), and the cycle threshold (Ct) values were used as a readout. All Ct values below 35 were considered valid in order to capture all potential meaningful signals from such low-input samples where no comprehensive data about miRNAs spectra have been reported. False amplification curves were manually excluded from the downstream analysis. The data were processed using Real-Time StatMiner 5.0 software (Integromics).

Two blank media control drops (hereafter referred to as negative controls) were incubated in the same dishes as those with media drops containing embryos. They were processed for miRNA analysis as previously described.

Cross Validation of miRNAs Detected in TE and SBM Samples Using Single Assays

To cross-validate the results obtained through the protocol previously described, a completely different method for miRNA quantification was used, which instead involved column-based miRNA purification and analysis with no need of preamplification and using a SYBR green-based qPCR quantification. In particular, microRNAs isolation from TE and SBMs was conducted with miRCURY RNA Isolation Kit for Biofluids (EXIQON) according to manufacturer's instructions. The sample initial input was 25–30 μL, 1 μg carrier-RNA MS2 (Roche) was added to each sample before protein precipitation step, and elution was performed in 20 μL. Five different biologic replicates for both SBM and TE were analyzed.

Retrotranscription was conducted according to miRCURY LNA Universal RT individual microRNA assays protocol, modifying only the sample input volume. In particular, 2 μL of 5X reaction buffer, 1 μL of enzyme mix, and 0.5 μL of UniSP6 spike-in control (a synthetic template to monitor protocol efficiency) were added to 6.5 μL of sample. The thermal protocol respected the manufacturer's indications. Retrotranscription product was diluted 1:8 with nuclease-free water.

The qPCR reaction was performed according to the standard protocol on a Viia7 instrument (Applied Biosytems). In particular, the following assays selected as TE specific (miR-512-5p, miR-522-3p), two miRNAs significantly different between implanted and unimplanted blastocysts (miR-20a-5p, miR-30c-5p), five randomly selected from those present in SBM samples (miR-373-3p, miR-302b-3p, miR-146a, miR-512-3p, miR24-3p), and two cellular housekeeping (RNU44 and RNU48) were tested. We added 5 μL of ExiLENT SYBR Green Master Mix and 1 μL of specific assay to 4 μL of sample, thus obtaining a final dilution of the elute input volume of 1:31. A no-template control of real-time (RT) and qPCR were also included in the analysis, and also a blank culture media sample never exposed to embryo culture.

Statistical Analysis

After inspection of putative normalizers, a global expression-based normalization strategy was used, where the mean expression Ct values of all detectors with valid Ct were used as the normalization factor to normalize the data for a given sample. Whole-genome RT-qPCR based miRNA profiling in combination with a global mean normalization strategy has proven to be the most sensitive and accurate approach for high throughput miRNA profiling (34).

The mean and the range of the fold change for each miRNA were calculated as $2-\Delta\Delta$Ct, using the estimated $\Delta\Delta$Ct value $\pm$ standard error (SE) (35). Hierarchical clustering analysis based on normalized Ct values was performed through single linkage and Pearson's correlation as a similarity method to test the relationship between hESCs of different cell contents. The scale bar values represent the $\Delta\Delta$Ct values. The heat map was produced by median centering expression values for each miRNA and then performing hierarchical clustering.

To investigate the ability of the miRNA protocol to capture biological variation in SBM samples, the mean Ct value correlation coefficients between mixed and unrelated SBM samples were assessed and compared. The resulting P values were adjusted for multiplicity as described herein. The scale bar values represent the $\Delta\Delta$Ct values. The heat map was produced by median centering expression values for each miRNA and then performing hierarchical clustering. The differential expression analysis of SBM versus TE samples and of SBM samples from implanted versus unimplanted blastocysts was based on the nonparametric Wilcoxon test.

Each family of tests was made by several hypotheses tested simultaneously. Many false positives could arise by random chance if a single-inference significance level of 5% was used. We therefore collected all raw test statistics, assessed their dependence, and verified that [1] it was weak enough and [2] a permutation could be found leading to a decreasing pattern (36, 37). As both checks were passed, we deemed the Benjamini and Hochberg (38) method to be valid; we thus proceeded by collecting all raw P values, sorting them, and comparing the i-th ranked P value with the threshold $0.05i/m$, where m is the total number of P values. This would guarantee that the expected proportion of false positives over the number of rejections would be below 5%. A volcano plot was generated using statistical significance versus fold-change on the y-axis and x-axis, respectively.

Target Prediction and Pathway Analyses

The analyses were performed with DIANA miRPath v.2.0 software (http://diana.cslab.ece.ntua.gr) (39). The software exploits the miRNAs and pathway information provided by miRBase (http://www.mirbase.org) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) v58.1 (http://www.genome.jp/kegg). Both in silico-predicted and experimentally validated gene targets were selected in the present analysis. The software performs the prediction in silico using the algorithm DIANA-microTCDS, which detects and scores both miRNA binding sites in the 3′-UTR and coding sequences of the predicted targets. The default 0.8 threshold score was adopted.

The experimentally validated miRNA-gene interactions were instead provided by the DIANA TarBase v6.0 database (http://diana.cslab.ece.ntua.gr/tarbase). The "union of genes" clustering option was set, which selects unions of genes targeted by at least one of all the uploaded miRNAs, before performing the statistical calculation. The software then adopts the Fisher's combined probability method to calculate a merged P value for each single pathway. The false discovery rate was determined according to the Benjamini and Hochberg method (38) before displaying the results.

RESULTS

MicroRNAs in Spent Blastocyst Medium Are Expressed Also in Trophectoderm Cells

To determine whether sample low-input volume would influence the sensitivity and accuracy of the assay, we compared

FIGURE 1

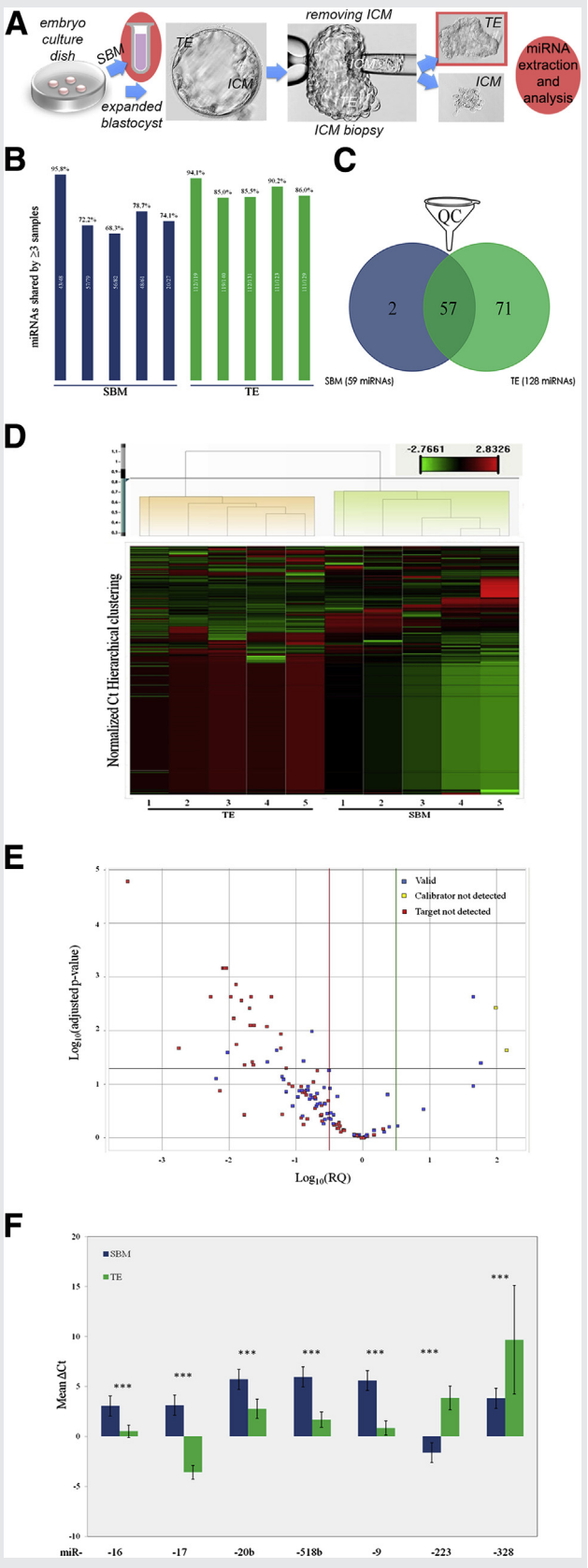

FIGURE 1 Continued

Comparison of the miRNA profiles between the trophectoderm (TE) and spent blastocyst culture media (SBM) samples. (**A**) Scheme illustrating the sample collection. Samples subjected to miRNA profiling are highlighted with a red frame. ICM = inner cell mass. (**B**) The majority of miRNAs detected in more than three TE and/or SBM samples were shared among all samples examined. (**C**) The Venn diagram shows that after a quality control (QC) filter, 57 of 59 miRNAs detected in SBM samples were also present in TE. (**D**) Correlation heat map among the miRNA expression profiles. (**E**) Volcano plot showing differential expression between the TE and SBM samples. Blue dots indicate assays detectable in both groups; yellow and red dots indicate assays detectable only in the SBM sample or TE sample, respectively. The Log10 of the relative quantitation is plotted on the *x*-axis, and the statistical significance (expressed as the negative Log10 of the adjusted *P* value) is plotted on the *y*-axis. The green line represents the cutoff to define the miRNAs that were more abundant in the SBM samples from implanted blastocysts, the red line represents the cutoff to define the miRNAs that were more abundant in the SBMs from unimplanted blastocysts, and the horizontal black line represents the cutoff for the statistical significance after a Benjamini-Hochberg false-detection rate correction. (**F**) Five miRNAs were statistically significantly more abundant in the TE samples compared with the SBM samples (miR-16, miR-17, miR-20b, miR-518b, and miR-9), whereas two miRNAs were statistically significantly more abundant in the SBM samples with respect to the TE samples (miR-223 and miR-328; $P<.01$).

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the miRNA profiles of small (approximately 10–50 cells, n = 3) and large (approximately 500–1,000 cells, n = 3) clusters of cells from the clinical grade hESC line KCL034. We found very high correlation coefficient between the small and large cell clusters, and none of the miRNAs were differentially expressed between the two groups (Supplemental Fig. 1, available online), suggesting that even if the starting sample input is scaled down approximately 10-fold, miRNA profiling using our established protocol is still representative and reliable.

To investigate the reliability of the protocol specifically for SBM samples, the mean correlation of Ct values among five mixed SBM samples (where the biologic variation was removed) were compared with the mean correlation of Ct values obtained from the analysis of five unrelated and biologically distinct SBM samples (Supplemental Fig. 2, available online). The mean correlation coefficient for the mixed samples (0.94 ± 0.2) was statistically significantly higher compared with the unrelated SBM samples (0.77 ± 0.06; $P<.01$), suggesting that the protocol is able to capture biologic variations between distinct SBM samples.

Next, we compared the miRNA profiles of five TE samples isolated from five different expanded human blastocysts of good quality at similar developmental stages and their SBM after conditioning for 12 hours (Fig. 1A). The minimum and maximum numbers of detectable miRNAs among all the TE biologic replicates were 123 and 140, respectively. Eighty miRNAs were detected in all five TE samples, 107 were detected in four out of five samples, and 128 were detected in three out of five samples. In the SBM samples, we detected a minimum of 27 and maximum of 82 different miRNAs.

Ten miRNAs were common in all SBM samples, 41 miRNAs were common in four out of five samples, and 59 were common in three out of five samples (see Fig. 1B and C).

The TaqMan Low Density Array (TLDA) cards included three assays (RNU-44, RNU-48 and U6-snRNA) for miRNAs that are commonly used as putative normalizers. The expression of all of these miRNAs was detected with high consistency in all the TE biologic replicates but only in four out of five SBM samples (Supplemental Fig. 3, available online). The nonhuman negative control miRNA, ath-miR-159a, was negative in all samples tested. In light of this evidence, a global normalization strategy was adopted in the subsequent analysis based on the geometric mean of all the Ct values obtained for all assays for each sample. The blank samples showed a consistent amplification for few miRNAs (miR-150, miR-891a, miR-155, miR-184, miR-212, miR-320, miR-486-3p, miR-126, miR-202, miR-370, miR-433, miR-518d, miR-548c, miR-520d) that were interpreted as false-positive signals and excluded from the subsequent analysis of test samples.

To optimize the selection of stably expressed miRNAs, only the miRNAs that were detected in at least three out of five biological replicates were included in the subsequent analysis. The majority of the detected miRNAs were shared by ≥ 3 samples (85.0%–94.1% for TE and 68.3%–95.8% for SBM; see Fig. 1B). After this filtering step, 128 different miRNAs were flagged as valid for the TE samples, and 59 were identified for the media samples, representing approximately 34% and 16% of the miRNAs on the arrays, respectively (see Fig. 1C). The mean Ct values of miRNAs included were 28.1 ± 3.5 and 29.9 ± 2.7 for TE and SBM samples, respectively.

As expected, the mean Ct values for TE was statistically significantly higher compared with the SBM samples ($P<.01$). Even though 7.9% and 15% of the miRNAs detected showed a Ct value above 33 for TE and SBM samples, respectively, we decided to include these targets for future analysis to describe the whole population of miRNAs that can be detected in this unique data set. A comparative analysis of the TE and SBM samples revealed that 96.6% (57 of 59; 95% CI, 88.3–99.6) of the miRNAs detected in the SBM were indeed expressed from TE cells, suggesting that the miRNAs detected in the media can be released from TE cells (see Fig. 1C). A bioinformatics analysis of the predicted and experimentally validated target genes for each miRNA detected in the SBM samples (Supplemental Table 1, available online) pointed to several pathways and biologic processes known to be involved in embryo implantation, including apoptosis, cell proliferation, and cell communication and differentiation (Supplemental Table 2, available online).

Hierarchical clustering analysis showed a sharp separation between TE and SBM samples based on miRNAs profiles (see Fig. 1D). A differential expression analysis showed that two miRNAs were statistically significantly more abundant in the SBM with respect to TE (miR-223, and miR-328, with fold changes of 44.7, and 58.0, respectively; $P<.01$), and five miRNAs were statistically significantly more abundant in the TE compared with the SBM samples (miR-16, miR-17, miR-20b, miR-518b, and miR-9, with fold-changes of 5.75, 105.7, 7.7, 19.4, and 26.7, respectively; $P<.02$) (see Fig. 1E and F). Moreover, two miRNAs were detected only in the SBM samples and not in the TEs. The absence of these miRNAs in TE cells can be assumed as a process of very high secretion from the TE, a higher stability into the extracellular environment, or an ICM origin that was not investigated here. Furthermore, 71 miRNAs were unique to the TE samples (see Fig. 1C and E). Although there may be several possible explanations for this difference between the cellular and extracellular miRNA populations, including cell lysis and preferential stability inside or outside of the cell, these results are consistent with the hypothesis that there is a specific packaging and export mechanism for extracellular miRNAs (4).

MicroRNAs in Conditioned Culture Media can Be Consistently Detected Only after Blastulation

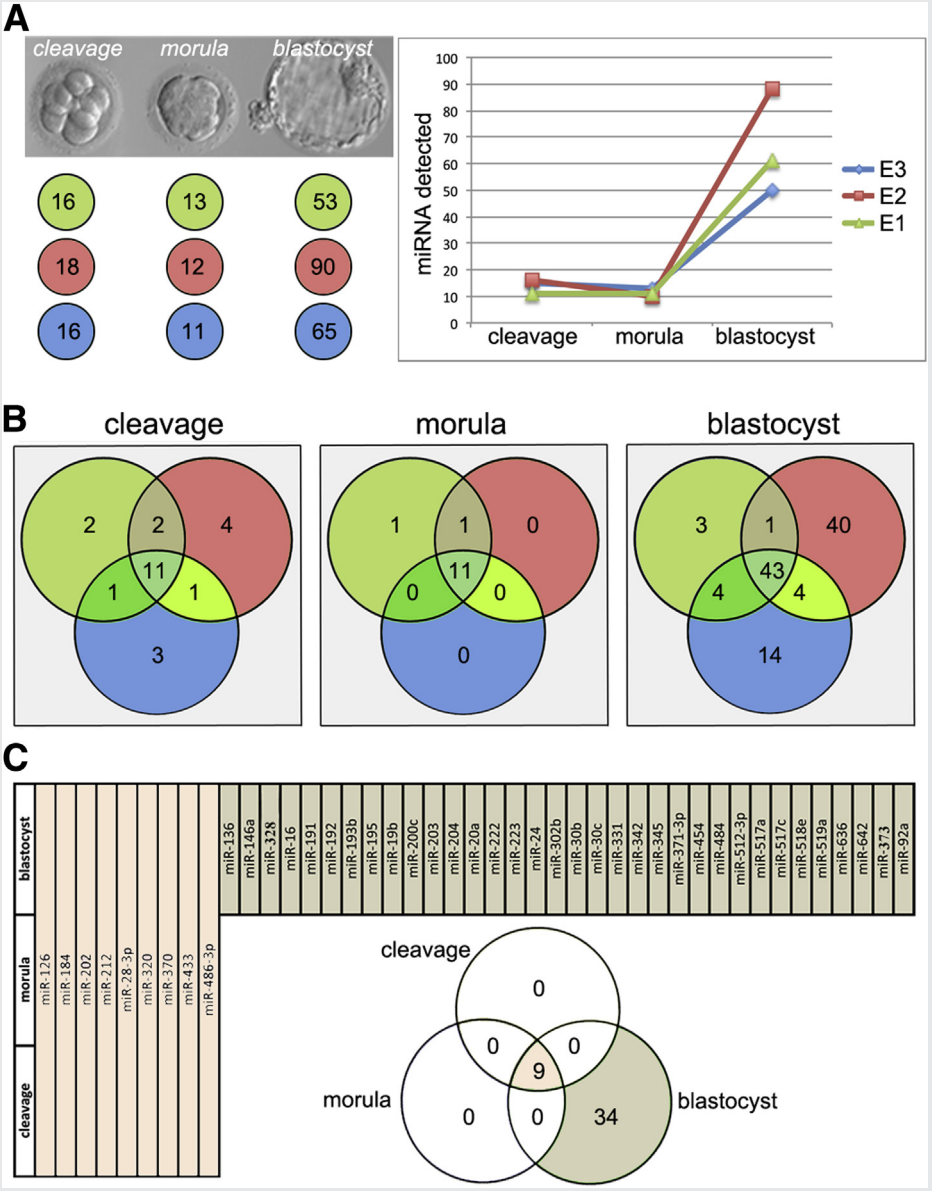
The miRNAs in the SBM may be secreted not only by TE but also by the embryo at earlier stages of development within a limited period of time (e.g., at the cleavage or morula stages). We therefore collected spent media samples from the same embryos ($n = 3$) at the cleavage, morula, and expanded blastocyst stages within the same IVF cycle (Fig. 2A). Analysis of miRNAs in the media collected from embryos at the cleavage and morula stages revealed a profile similar to that which was observed in the negative controls. In particular, the miRNAs detected in the spent culture media collected from embryos at the cleavage (11 miRNAs) and morula stages (11 miRNAs) were all previously detected in the negative controls (culture medium only), suggesting that this group of miRNAs is related to same intrinsic and systematic false-positive amplifications (see Fig. 2B and C). In the SBM samples, absolute numbers of 51, 88, and 65 different miRNAs were detected, with 43 expressed in all of the biologic replicates (see Fig. 2B). Excluding false positives, blastocyst SBMs showed 34 different miRNAs. This analysis suggests that the miRNAs can be consistently detected in culture media only after blastulation.

MicroRNAs that Are Differentially Expressed in Implanted versus Unimplanted Blastocysts Have Been Associated with Implantation Relevant Processes

Next, we investigated in a proof of principle analysis the potential of miRNAs detected in the SBM samples to serve as biomarkers for predicting the reproductive competence of blastocysts. The SBM samples from single vitrified-warmed embryo transfer of euploid blastocysts were compared among embryos that resulted in an ongoing implantation and embryos that failed to implant (Fig. 3A). To rule out the significant confounding factor of aneuploidies on implantation, only blastocysts shown to be chromosomally normal after TE biopsy and comprehensive chromosomal screening analyses were considered.

The SBM samples were prospectively collected from all embryos reaching the blastocyst stage from 44 consecutive patients attending a blastocyst-stage preimplantation genetic screening (PGS) cycle, with embryo cryopreservation and embryo transfer performed on the subsequent natural cycle.

FIGURE 2



MicroRNA (miRNA) detection in spent culture media collected at different stages of preimplantation embryo development. (**A**) Spent medium was collected at three different stages of preimplantation development (cleavage, morula, and blastocyst stages) from three embryos (E1, E2, and E3) cultured from the cleavage to the blastocyst stage. Marked increases in the number of miRNAs detected were observed simultaneously at the blastocyst stage in all three embryos. (**B**) Most of the miRNAs detected at each stage were shared among all the samples. (**C**) Only nine miRNAs were detected in all samples at all stages (miR-126, miR-184, miR202, miR-28-3p, miR-320, miR-212, miR-370, miR-433, and miR-486-3p). These same miRNAs were also detected in the negative controls.

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Eight patients transferred a euploid blastocyst twice. Fifty-three euploid blastocysts were transferred, 25 of which resulted in an ongoing implantation. The laboratory and clinical outcomes for these IVF-PGS cycles are reported in Supplemental Table 3 (available online).

After normalization and filtering steps (>60% detection rate across each biologic group), the total number of miRNAs detected in the SBM of implanted blastocysts was somewhat higher (n = 38) compared with that of unimplanted blastocysts (n = 33). The mean Ct values of miRNAs detected in SBM of implanted and unimplanted blastocysts were 28.5 ± 4.3 and 28.5 ± 4.5 (not statistically significant). The mean delta Ct values were also not statistically significantly different between the two groups; in particular, the values were 2.8 ± 5.6 for implanted blastocysts and 3.4 ± 4.9 for unimplanted blastocysts.

FIGURE 3

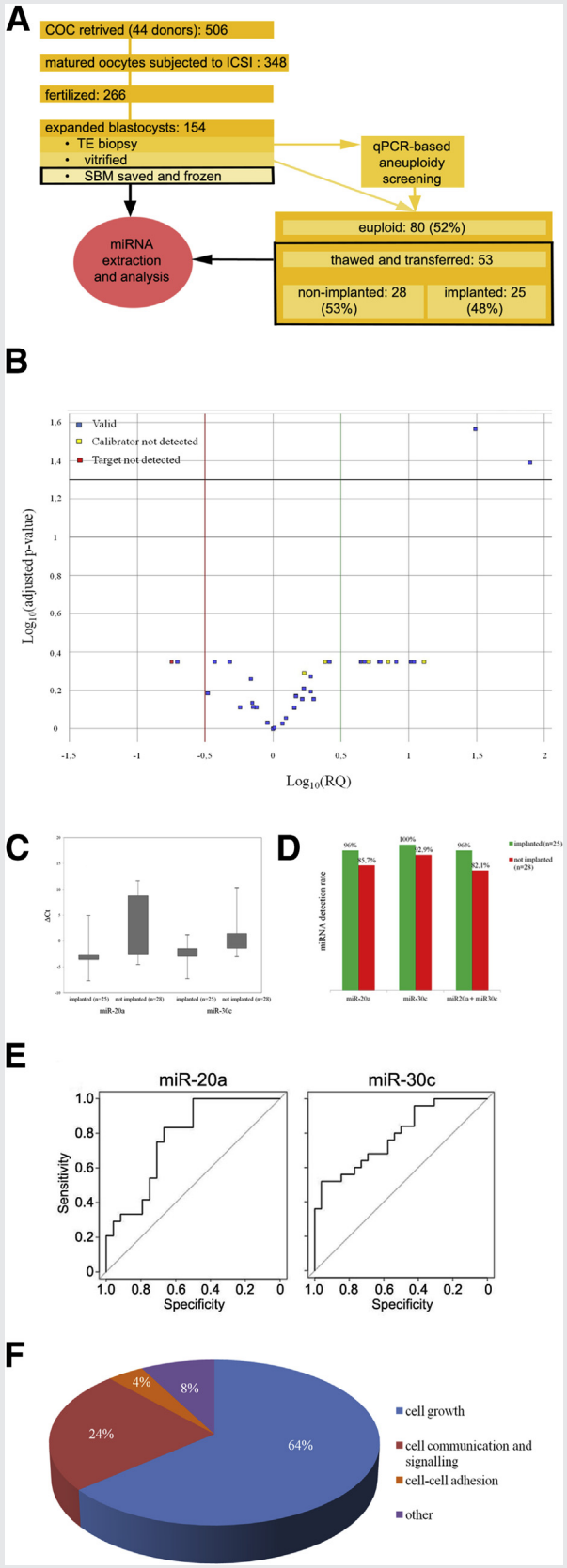

FIGURE 3 Continued

Differential expression of two miRNAs in the SBM of implanted embryos. (**A**) Scheme of the sample collection from embryos that were transferred into patients. Embryos were cultured to the blastocyst stage in vitro. Spent blastocyst culture media (SBM) samples were collected immediately before trophectoderm (TE) biopsy. Blastocysts were vitrified immediately after the biopsy. Quantitative polymerase chain reaction–based comprehensive chromosomal screening (CCS) was performed on the TE biopsies. Only euploid blastocysts were thawed for single-embryo transfer and SBM profiling. COC = cumulus oocyte complexes. (**B**) Volcano plot showing a comparison of the miRNAs in SBMs from implanted (n = 25) and unimplanted blastocysts (n = 28). Blue dots indicate assays detectable in SBMs from both groups; yellow dots indicate assays detectable preferentially in SBMs from implanted blastocysts; red dot indicates the assay preferentially detected in SBM of unimplanted blastocysts. The Log10 of the relative quantitation is plotted on the *x*-axis, and the statistical significance (expressed as the negative Log10 of the adjusted *P* value) is plotted on the *y*-axis. The green line represents the cutoff to define the miRNAs that were more abundant in the SBM from implanted blastocysts, the red line represents the cutoff to define the miRNAs that were more abundant in the SBM from unimplanted blastocysts, and the horizontal black line represents the cutoff for statistical significance after a Benjamin-Hochberg false-detection rate correction. Two miRNAs were identified as statistically significantly more abundant in the SBM from implanted blastocysts: miR-20a and miR-30c. (**C**) Box plot built on delta cycle threshold (Ct) values of miRNAs that were differentially expressed between SBM samples from implanted and unimplanted blastocysts. (**D**) Frequency of detection of miRNAs that were differentially expressed in the SBM of implanted and unimplanted blastocysts. (**E**) Receiver operating characteristic analysis showing the clinical predictivity of miR-20a and miR-30c on implantation potential of euploid blastocysts. (**F**) Pathway analysis for miR-30c and miR-20a. These miRNAs are predicted to regulate genes involved in 25 KEGG pathways, 23 of which may have a role in blastocyst–endometrial communication: cell-to-cell communication/cell signaling, cell-to-cell adhesion, and cell growth.

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A statistical analysis comparing the miRNA profiles between chromosomally normal implanted (n = 25) and unimplanted blastocysts (n = 28) revealed two differentially expressed miRNAs (miR-20a and miR-30c; $P<.05$) that showed increased concentrations in SBM from implanted blastocysts (see Fig. 3B). The mean Ct values for these miRNAs were 22.3 ± 3.0 versus 24.0 ± 2.1 (miR-20a) and 23.4 ± 3.4 versus 26.1 ± 2.7 (miR-30c) for implanted and unimplanted blastocysts, respectively. The normalized Ct values for these two miRNAs were statistically significantly lower for implanted blastocysts (see Fig. 3C). Furthermore, five miRNAs (miR-220, miR-146b-3p, miR-512-3p, miR-34c, miR-375) were preferentially detected in SBM samples of implanted blastocysts compared with blastocysts that failed to implant (see Fig. 3B). A combined secretion of these two miRNAs (miR-20a and miR-30c) was detected in the vast majority of implanted embryos (96%), and they were somewhat less prevalent in embryos that did not implant (see Fig. 3D). The receiver operating characteristic analysis performed on implantation with these two miRNAs showed that each single miRNA was predictive of implantation, with an area under the curve of 0.773 (95% CI, 0.637–0.908) and 0.786 (0.663–0.909) for miR20 and miR30c, respectively (see Fig. 3E).

Based on in silico-predicted and experimentally validated miRNA targets, miR-20a and miR-30c are involved in 25 different pathways and biologic processes (see Fig. 3F; Supplemental Table 4, available online). The pathways represented are mainly involved in cell-to-cell communication and signaling (6 of 25, 24%), cell-to-cell adhesion (1 of 25, 4%), and cell growth/cancer (16 of 25, 64%). It is interesting that, based on bioinformatics analysis, miR-20a has been experimentally validated to modulate five genes (*PTEN*, *NRAS*, *MAPK1*, *MYC*, and *CCND1*) and is predicted to regulate two additional transcripts (*SOS1*, *TCF7L1*) involved in endometrial cell proliferation and growth. Furthermore, miR-30c is predicted to regulate five distinct transcripts (*APC*, *KRAS*, *PIK3CD*, *SOS1*, and *FOXO3*) that are also involved in endometrial cell proliferation and growth.

Single Assay-based Cross-validation of Relevant miRNAs from TE and SBM Samples

To cross-validate the results obtained from the comparison of SBM samples from implanted and unimplanted blastocysts, a completely different method of miRNA analysis was used, which instead involved column-based miRNA purification and analysis with no need of preamplification and using a SYBR green-based qPCR quantification (Supplemental Table 5, available online). We assessed the expression of two distinct miRNAs that were exclusively detected in TE samples and never in SBM samples (miR-512-5p; miR-522-3p), confirming a stable expression in five different TE samples and the absence of detection in five unrelated SBM samples.

Next, we confirmed a consistent expression of the two miRNAs showed to be more abundant in SBM of implanted blastocysts (miR-20a and miR30c). Then another five miRNAs were randomly selected from the ones expressed in the SBM of euploid blastocysts and confirmed by single assay analysis on five unrelated SBM and five TE samples. Only one miRNA (miR-24-3p) was not confirmed in the majority of SBM replicates.

Finally, the two putative cellular normalizers, RNU44 and RNU 48, were also confirmed by single assays showing low stability and detection rate in SBM samples, as expected. One exogenous miRNA (UniSp6) was also run in parallel and showed a very stable and consistent detection across the different replicates, thus highlighting low technical variability across the samples analyzed. Blank samples, namely water and culture media never exposed to blastocyst culture, were run in parallel and showed no amplification for any of the tested miRNAs.

DISCUSSION

Here, for the first time, we have comprehensively characterized the population of miRNAs secreted from human blastocysts that are present in the spent culture media, where they may act to directly transfer information from the blastocyst to the surrounding endometrial cells, thus altering the potential for implantation success. These biological findings suggest the possibility of using miRNAs from spent culture media, which is easy to collect, as noninvasive biomarkers of embryo quality during IVF cycles.

First, one of the main challenges of the study was to develop and validate a reliable protocol for miRNAs quantification from low-input samples, such as 25 μL of culture media conditioned after 12 hours of blastocyst culture after warming. As preliminary validation step of the protocol, large and small clusters of hESCs were compared, showing the protocol to be highly robust even when scaled down on low-input samples. Furthermore, mixed SBM samples showed a statistically significantly higher correlation compared with unrelated samples, suggesting that the protocol was able to capture biologic variation between SBM samples.

Next, we characterized the miRNA signature of SBM samples showing that 96.6% of the miRNAs detected are expressed also from TE cells with few miRNAs being more abundant in the SBM or TE fraction. These results are consistent with a specific packaging and exporting hypothesis for extracellular miRNAs and in agreement with previous studies performed on cell lines where exosomes derived from myoblast or PC-prostate cancer cells contained differentially sorted miRNA (40, 41).

Intriguingly, the SBM samples were collected after 12 hours of blastocyst culture after warming, a time frame that was sufficient to allow a consistent quantification of many miRNAs. Several studies already claimed a consistent isolation of substantial amounts of miRNAs in the medium after culture of different cell types at variable timings. In agreement with our findings, a previous study by Wang et al. (4) that investigated the kinetic of miRNAs exportation from cultured cells after different biologic stimuli found that most of the miRNAs tested appeared to be actively exported in a short pulse lasting about an hour after serum deprivation (4).

Fourteen of the 59 miRNAs detected in the SBM samples were predicted to be involved in endometrial cell growth and proliferation, suggesting the existence of a previously unrecognized potential communication system between the embryo and the implantation site on the uterine wall. However, important pieces of the puzzle are still missing. Although there is evidence that vesicles containing miRNAs may be taken up by the cells (42), direct proof that endometrial cells are internalizing miRNA-containing complexes secreted by blastocysts is not yet available. Secreted miRNAs may target and convey information to neighboring TE cells and not to the uterus. Regardless, the elucidation of this novel communication system between an embryo and its implantation site will certainly be of the utmost importance in understanding the many biologic processes that govern implantation.

To our knowledge, only one study has attempted to profile miRNAs from blastocyst culture media so far (43). However, due to many specific differences in the protocol for miRNAs extraction and quantification, only 10 miRNAs were detected in culture media samples in that study, with eight of them shown to be false-positive signals.

Finally, to investigate the potential role of miRNAs analysis from SBM as embryonic biomarker, single-embryo transfer of euploid blastocysts was compared between implanted

and unimplanted embryos. This analysis revealed two differentially expressed miRNAs showing higher concentration in SBM samples of implanted blastocysts. Bioinformatics analysis of these miRNAs showed several biologic pathways and cellular function regulated from them, including cell proliferation, cell communication, and cell signalling. Importantly, these two miRNAs have been already reported as circulating in human body fluids (44–50). Even if further data are needed to confirm these preliminary findings, this is the first report highlighting the potential role of miRNAs quantification from the easily collectable SBM as a new biomarker to be further explored for embryo selection.

In the context of human embryo selection for IVF, the ideal embryonic biomarker would allow noninvasive assessment of the embryo, would be stable over time, specific to embryos, and easily measurable to allow rapid and accurate assessment of embryo quality, all features fitting this newly proposed approach. Indeed, a prominent feature of circulating miRNAs is their remarkable stability (51): they are protected by forming complexes with proteins such as Argonaute (52) and GW182 (53) or are encapsulated in exosomes that provide additional protection from degrading enzymes (54). Moreover, miRNAs are a specific product of the embryo that can be related to the quality of many differentiation processes that beset blastocyst differentiation and are not supposed to be already present in culture media. Furthermore, changing concentration of extracellular miRNAs has been already associated with a variety of pathologies (6, 8,55–57). Finally, real-time qPCR–based miRNA analysis can be performed in-house in a matter of hours with high accuracy, rendering fresh blastocyst transfers feasible with same-day media analysis.

It should be acknowledged as a limit of the study that due to statistical distribution there is always a high level of Ct variation when target quantities approach single copy (usually for Ct values >33 after the preamplification step), as in the case of conditioned culture media samples. In our analysis 15% of the miRNAs described in SBMs showed a Ct value >33. Therefore, miRNAs that yield Ct values in this range will unavoidably give rise to poorer precision and consequently less power to detect low-fold changes. However, we decided to include these miRNAs to capture all the biologic information from SBM samples, and differentially expressed miRNAs between implanted and unimplanted blastocysts showed a mean Ct value <30. Another issue is that the study relies on the resolution limits of the specific miRNAs panel used, including 381 targets of >1.000 discovered in human cells. Accordingly, untested miRNAs might still exist in SBM samples and may play a role in the blastocyst-endometrial dialogue. Furthermore, direct proof that endometrial cells are internalizing miRNA-containing complexes secreted by blastocysts is not yet available and is under investigation.

In conclusion, these biological and preliminary clinical findings open the possibility to further explore the analysis of miRNAs from the easily collectable spent culture media as a noninvasive biomarker of embryo quality during IVF cycles. In this study we provided a proof of principle that profiling miRNAs from SBM may enhance blastocyst assessment during IVF cycles or might be used together with TE-based aneuploidy screening to further improve selection among euploid blastocysts. This preliminary clinical investigation used a high-throughput approach to test for 381 different human miRNA sequences. A prospective multicentre study involving both private and academic IVF centers is currently ongoing where a custom panel of 46 selected miRNAs will be used to corroborate the present findings on a higher and defined sample size that will guarantee a two-sided 95% confidence interval of total length at most 6% to define the clinical power of embryo selection based on miRNAs profiling from SBM samples.

The use of this custom platform is also the first step toward the reduction of complexity and the development of a diagnostic assay based on the analysis of informative miRNAs only from SBM to select reproductively competent embryos at reasonable cost and with a low turnaround time of analysis. All these aspects will better fit with the clinical requirements of embryo selection IVF, providing a novel and practical tool for the noninvasive assessment of embryo quality.

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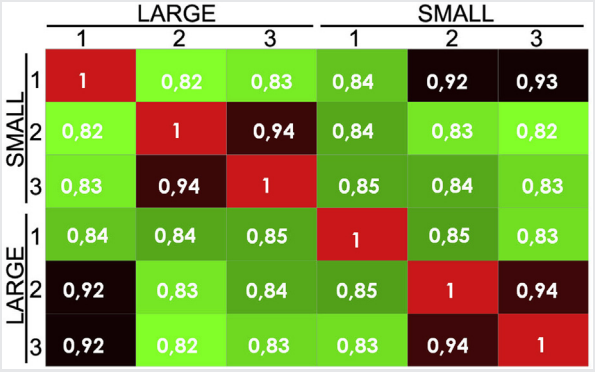
SUPPLEMENTAL FIGURE 1



Correlation matrix of microRNA (miRNA) profiles from large (n = 3) and small (n = 3) clusters of human embryonic stem cells (hESCs). Red represents the highest correlation, and green represents the lowest correlation. The numbers in squares express the exact Pearson's correlation values.

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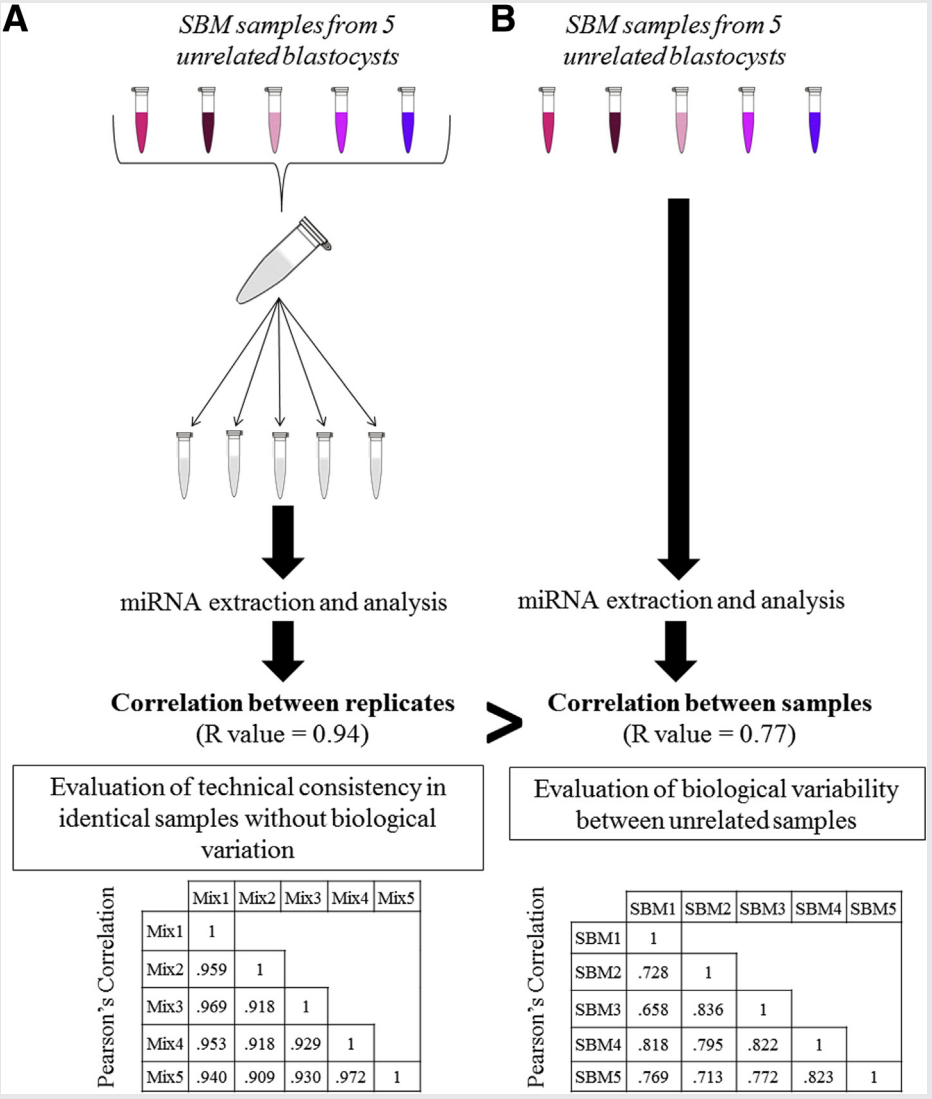
SUPPLEMENTAL FIGURE 2



To evaluate whether the protocol for microRNA (miRNA) quantification can capture biologic variations, five spent blastocyst culture media (SBM) samples were collected from different embryos, pooled together in a single tube, and divided again in five equal amounts to remove biologic variation between samples (**A**). Mean correlation coefficient of the cycle threshold (Ct) values for miRNAs detected in mixed samples (0.93 ± 0.2) was statistically significantly higher compared with unrelated SBM samples (0.77 ± 0.06; $P<.01$) (**B**), suggesting that the protocol is able to capture biologic variations between distinct SBM samples.

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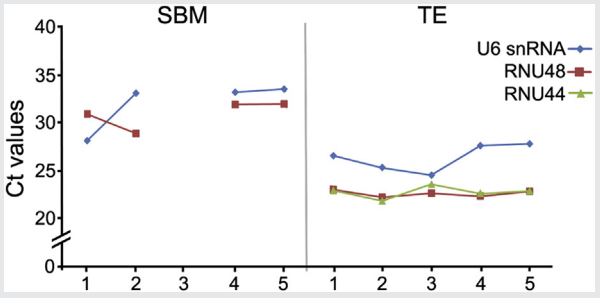
SUPPLEMENTAL FIGURE 3



Cycle threshold (Ct) values of microRNAs (miRNAs) commonly used as putative normalizers in the trophectoderm (TE) and related spent blastocyst culture media (SBM) samples. The expression of all these miRNAs was detected with high consistency in all the TE biologic replicates but not among the SBM samples. For SBM sample 3, no results could be detected. RNU44 was not detected in any of the media samples analyzed.

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