Aberration of blastocyst microRNA expression is associated with human infertility

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Objective: To examine human blastocyst microRNA (miRNA) expression in correlation with human infertility. MicroRNAs are small, noncoding RNA molecules that regulate gene expression via mechanisms such as degradation and translational suppression of targeted messenger RNAs. Recent data has pointed to the importance of miRNAs in disease states and during mouse embryo development.
Design: Descriptive study.
Setting: Nonprofit research foundation.
Patient(s): Transferable quality human blastocysts donated with consent to research (n = 40).
Intervention(s): Quantitative real-time PCR.
Main Outcome Measure(s): MicroRNA expression profile.
Result(s): Morphologically similar blastocysts derived from patients with polycystic ovaries or male factor infertility exhibited a significant decrease in the expression of six miRNAs in comparison with donor fertile control blastocysts ($P < 0.05$). Annotation of predicted gene targets for these differentially expressed miRNAs included gene ontology (GO) biological processes involved in cell growth and maintenance and transcription as well as GO molecular functions implicated in nucleic acid binding and signal transducer activity. Three predicted miRNA target genes were selected for analysis and demonstrated significant altered expression consistent with aberrant miRNA profiles.
Conclusion(s): This study describes for the first time that transferable quality blastocysts derived from infertile patients (male factor infertility and polycystic ovaries) possess aberrant miRNA profiles. With growing evidence indicating the importance of miRNAs during development, an association may exist with human infertility. (Fertil Steril® 2010;93:2374–82. ©2010 by American Society for Reproductive Medicine.)

Key Words: MicroRNA, blastocyst, human infertility

MicroRNAs (miRNAs) are short (20–24 nucleotides), single-stranded noncoding RNA molecules that have been implicated in the normal functioning of eukaryotic cells from various organisms, including mammalian cells. They are highly conserved with close to 90% sequence homology between human, mouse, and rat (1). MicroRNAs are transcribed with a cap and poly-A tail before shortening by nuclear endonucleases and their dsRNA binding protein partners to 70-nucleotide stem-loop pre-miRNA structures. These pre-miRNA molecules are then processed into mature miRNA by interaction with the conserved ribonuclease III endonuclease, Dicer (2). MicroRNAs regulate gene expression either negatively by direct messenger RNA (mRNA) cleavage (3), mRNA decay by deadenylation (4) and/or translational repression by partial complementary mRNA annealing (5); or positively by targeting of gene promoters (6). Computational analyses predict the presence of up to 50,000 different miRNAs in a mammalian cell, each with hundreds to thousands of potential mRNA targets regulating approximately 30% of protein-coding genes (7). MicroRNAs have been associated with numerous biological processes including development (8), cell growth (9), and differentiation (10). They have also been connected with human disease including several types of cancers (11), viral infections (12), and heart disease (13).

The synthesis and degradation of miRNAs has been shown to play an important role during mouse oogenesis and embryonic development. Global miRNA expression profiling revealed that miRNAs are maternally inherited with the loss of approximately 60% between the one- and two-cell stages during the maternal zygotic transition (8). It appears that dynamic degradation and synthesis of miRNAs coexists during the development of the preimplantation mouse embryo with an overall increase in miRNAs toward the blastocyst stage (14). Specific stages of mouse embryonic prenatal development (E9.5, E10.5 and E11.5) also displayed distinct miRNA expression profiles with the discovery of novel mammalian miRNAs (15). The deletion of Dicer in somatic cells of the mouse female reproductive tract resulted in a decrease of

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specific miRNAs in Dicer conditional knockout females compared with wild-type. In addition, these females were considered infertile with compromised oocyte and embryo integrity (16). MicroRNA-mediated regulation of uterine gene expression during the time of implantation has also been demonstrated with the hypothesis that critical genes related to this process may be regulated by miRNAs. For example, cyclooxygenase-2 expression, which is critical for implantation in the mouse, is regulated following transcription by two miRNAs, hsa-miR-101a, and hsa-miR-199a (17). Indeed, implantation failure has been documented as a significant contributor to human infertility; thus the aberrant expression of critical miRNAs during the window of implantation may also be a contributor.

To obtain insight into miRNA expression during human embryonic development, quantitative real-time PCR (QPCR) was performed on individual human blastocysts for a set of 12 miRNAs. These miRNAs were specifically chosen from recent publications that demonstrated their expression in either mouse embryos (8, 14) or human embryonic stem cells (10): RNU48, hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7g, hsa-miR-19a, hsa-miR-19b, hsa-miR-21, hsa-miR-24, hsa-miR-34b, hsa-miR-92, and hsa-miR-93. Statistical analysis was performed to determine the relationship between miRNA expression and specific infertility diagnosis (male factor and polycystic ovaries) in addition to expression analysis of three predicted miRNA target genes.

MATERIALS AND METHODS

Blastocyst Culture

All gamete and embryo manipulations occurred in a pediatric isolette designed to control humidity, temperature, and pH fluctuations. Semen preparation was performed using a 50-70-95 discontinuous gradient of Pure Sperm (Nidacon, Gothenburg, Sweden). The resulting pellet was washed in fertilization medium with 100,000 sperm per milliliter added to each oocyte. If intracytoplasmic sperm injection (ICSI) was performed, all oocytes were first denuded using hyaluronidase. Intracytoplasmic sperm injection was performed on a Nikon inverted microscope (Nikon, Melville, NY) with Narashige micromanipulators (Narashige, East Meadow, NY). Assessment of fertilization took place 15–18 hours after insemination. Embryos with two pronuclei were cultured in groups of three or four in cleavage medium for 48 hours. At approximately noon on day 3, all embryos were transferred to blastocyst medium for an additional 48 hours of culture (day 5). On the morning of day 5, the percentage of blastocyst formation was determined and each blastocyst was assigned a score using the system of Gardner and Schoolcraft (18). Blastocysts that were not transferred, but were considered of transferable quality, were cryopreserved using a modified Menezo method (19) and donated at a later date with patient consent to research. The study was approved by the HCA-HealthONE Institutional Review Board.

Blastocyst miRNA Isolation

Cryopreserved morphologically similar individual human blastocysts (n = 22; including blastocysts from control fertile oocyte donor cycles with no known male factor infertility [DON; n = 10], blastocysts from male factor infertility alone [MF; n = 6], and blastocysts from women with polycystic ovaries [PCO; n = 6]) were thawed using a modified Menezo method (19). Following thawing, each individual blastocyst was evaluated to confirm survival after cryopreservation and morphological grade (Table 1). MicroRNA was isolated from each individual blastocyst, using the RNAqueous-Micro kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions with minor modifications. Briefly, thawed blastocysts were washed in phosphate-buffered saline (PBS) and placed in a 100-μL lysis solution before the addition of 100% ethanol and transfer to a silica-based filter where the RNA was bound. Samples were on-column deoxyribonuclease treated (Qiagen, Valencia, CA) and washed three times before elution in 20 μL. RNA was then concentrated down in a micro vacuum to ensure use of the entire template for reverse transcription.

Reverse Transcription and Quantitative Real-Time PCR

Samples were reverse transcribed using the Multiplex RT human pool 1 and the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems). Three microliters of a master mix containing 15 mM dNTPs, 50 U reverse transcriptase, and 3.8 U ribonuclease inhibitor, were combined with 9 μL of each sample and incubated using the following thermal cycling profile: 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. All resulting complementary DNA (cDNA) was stored at –20°C until analysis.

cDNA samples were diluted 1:10 to allow for the analysis of all 12 miRNA probe sets with duplicates in every individual blastocyst by QPCR. Standard curves were calculated for each probe set using 10-fold serial dilutions of input reference miRNA (Applied Biosystems). Five microliters of reference RNA (starting concentration of 50 ng/μL) was used for reverse transcription in a 15-μL reaction (final concentration of 16.7 ng/ μL). The reference cDNA was diluted 1:10 with 5 μL template added into each real time reaction (concentration of 8.35 ng). Tenfold serial dilutions in duplicates were used for the standard curve, at the following total concentrations: 8.35 ng, 0.835 ng, 0.0835 ng, and 0.00835 ng. Quantitative real-time PCR was performed using a 5-μL template, Taqman Universal PCR Master Mix, No AmpErase UNG on the ABI 7300 Real Time PCR System (Applied Biosystems) with the thermal cycling profile: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 minutes and 60°C for 1 minute. Expression of 11 miRNA probe sets (hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7g, hsa-miR-19a, hsa-miR-19b, hsa-miR-21, hsa-miR-24, hsa-miR-34b, hsa-miR-92, and hsa-miR-93) were analyzed relative to the expression of the miRNA control probe, RNU48 in each of the individual blastocyst samples. These miRNA probe sets were specifically chosen from recent publications that demonstrated their expression in either mouse embryos (8, 14) or human embryonic stem cells (10). All real-time PCR values were observed within the sensitive region of the standard curves. Statistical analysis was performed

TABLE 1

MicroRNA expression profile of 12 probe sets for individual human blastocysts, fertile donor oocyte controls, blastocysts derived from patients with MF, and blastocysts derived from patients with PCO.

Blastocyst no.	Sample group	Embryo grade	RNU48	hsa-let-7a[a]	hsa-let-7b	hsa-let-7c	hsa-let-7g	hsa-miR-19a[b]	hsa-miR-19b[b]	hsa-miR-21	hsa-miR-24[a]	hsa-miR-34b	hsa-miR-92[b]	hsa-miR-93[b]
1	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
2	DON	4AA	•	•	•	•	•	•	•	•	•	•	•	•
3	DON	4AA	•	•	•	•	•	•	•	•	•	•	•	•
4	DON	4AA	•	•	•	•	•	•	•	•	•	•	•	•
5	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
6	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
7	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
8	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
9	DON	4AB	•	•	•	•	•	•	•	•	•	•	•	•
10	DON	4BB	•	•	•	•	•	•	•	•	•	•	•	•
11	MF	4AA	↙	↓	↙	↙	↑	↙	↙	↙	↙	↙	↙	↙
12	MF	4AA	↙	↓	↙	↙	↓	↙	↙	↙	↓	↙	↙	↙
13	MF	4BA	↙	↙	↙	↙	↑	↙	↙	↙	↙	↙	↙	↙
14	MF	4BA	↙	↓	↙	↙	↑	↓	↓	↙	↓	↙	↙	↙
15	MF	4AA	↑	↓	↑	↑	↓	↙	↓	↙	↓	↑	↙	↙
16	MF	4AA	↙	↙	↑	↑	↓	↙	↓	↙	↙	↑	↙	↓
17	PCO	4AA	↙	↓	↓	↓	↓	↓	↓	↓	↙	↓	↓	↙
18	PCO	4AA	↙	↙	↙	↓	↑	↓	↓	↙	↓	↙	↓	↓
19	PCO	4AB	↙	↓	↙	↙	↓	↓	↓	↙	↓	↓	↓	↓
20	PCO	4BA	↙	↓	↙	↓	↑	↓	↓	↙	↓	↙	↙	↙
21	PCO	4AB	↙	↓	↑	↙	↑	↙	↙	↙	↓	↑	↙	↓
22	PCO	4AA	↙	↓	↑	↑	↙	↙	↓	↙	↓	↑	↙	↙

Note: DON = fertile donor oocyte control; • = expressed; ↙ = expressed, no change from control mean; ↓ = expressed, decrease; ↑ = expressed, increase

[a] Significant decrease in MF and PCO samples.

[b] Significant decrease in PCO samples.

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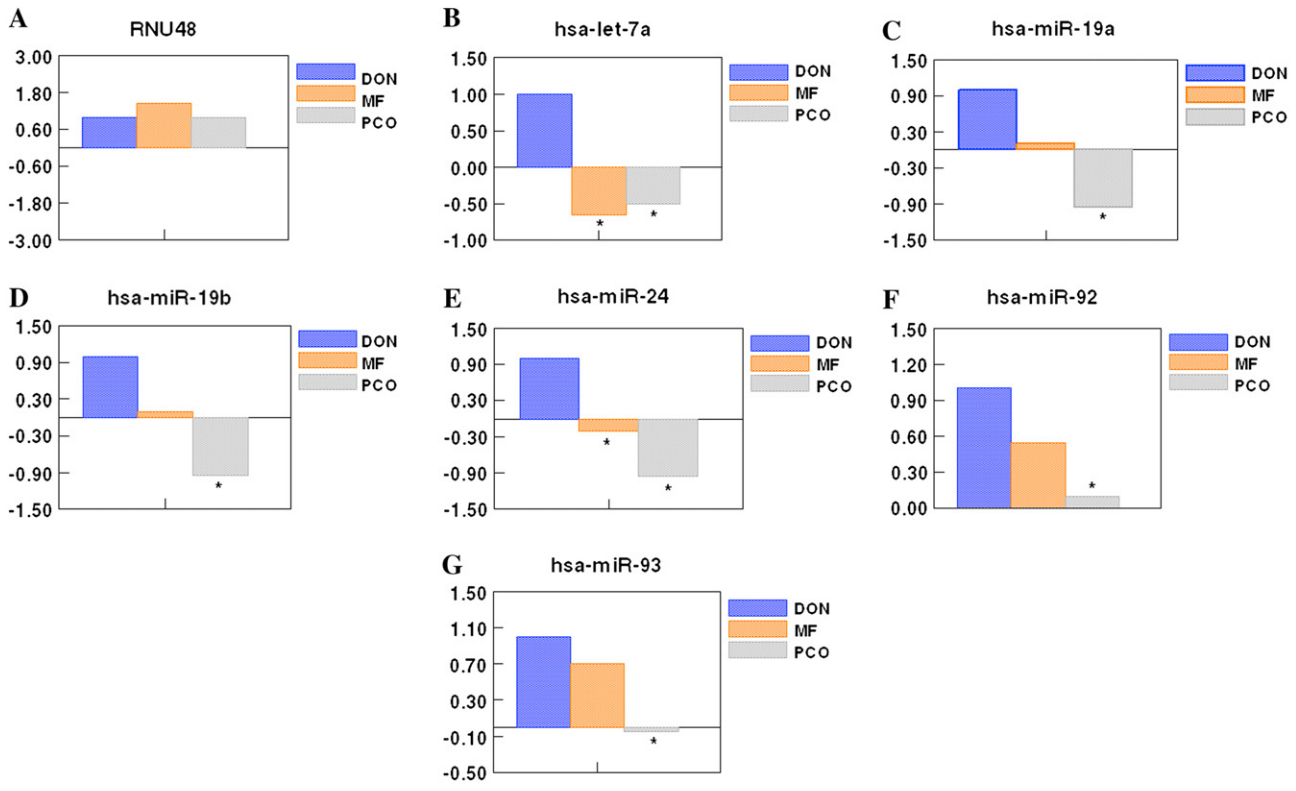
FIGURE 1

MicroRNA expression levels were quantified by QPCR relative to the miRNA control probe, RNU48 (**A**), which was consistently expressed in all blastocysts from each of the three groups; fertile donor oocyte controls (DON), blastocysts derived from patients with MF and blastocysts derived from patients with PCO. Each individual miRNA probe was analyzed on individual blastocysts with 6 miRNA profiles revealing significant differences in expression across the groups (**B**) hsa-let-7a (**C**) hsa-miR-19a (**D**) hsa-miR-19b (**E**) hsa-miR-24 (**F**) hsa-miR-93 and (**G**) hsa-miR-94 ($P < 0.05$).



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with REST-2005 software (www.gene-quatification.info) with significance at $P < 0.05$.

Quantitative Real-Time PCR for Gene Expression Analsis of miRNA Predicted Targets

Quantitative real-time PCR was performed using the Roche LightCycler (Roche, Indianapolis, IN) on cDNA generated from individual blastocysts (n = 18; including blastocysts from control fertile oocyte donor cycles with no known male factor infertility [n = 6], blastocysts from male factor infertility alone [n = 6], and blastocysts from women with polycystic ovaries [n = 6]) for the following genes: *ARIH2, KHSRP,* and *NFAT5* as described previously (20). The quantification of each gene was calculated relative to the housekeeping gene, *ACTB*. All QPCR reactions were performed in duplicates, and standard curves were calculated as described previously for each gene on 10-fold serial dilutions of input reference RNA. For amplification, the LightCycler FastStart DNA Master^{Plus} SYBR Green I kit (Roche) was used under the following thermal cycling conditions: 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, primer-dependant annealing temperatures ranging from 58°–60°C for 5 seconds, and extension ranging from 3–4 seconds at 72°C. All real-time PCR values were observed within the sensitive region of the standard curves. Statistical analysis was performed with REST-2005 software with significance at $P < 0.05$.

RESULTS

Blastocyst grading was documented after thawing and before miRNA analysis. This cohort of human blastocysts consisted of good quality, transferable grade, day 5 embryos of similar morphology (Table 1). A miRNA expression profile of all 12 probe sets (RNU48, hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7g, hsa-miR-19a, hsa-miR-19b, hsa-miR-21, hsa-miR-24, hsa-miR-34b, hsa-miR-92, and hsa-miR-93) was observed for each individual human blastocyst in all three groups; fertile donor oocyte controls, blastocysts derived from patients with MF and blastocysts derived from patients with polycystic ovaries (PCO; Table 1). The most abundant miRNA profile was observed for hsa-miR-92, whereas the least detectable

TABLE 2

Real-time PCR reaction information. The slope of the standard curve illustrates the PCR reaction efficiency (–3.32 ± 10%).[a] The intercept is the crossing point that theoretically represents one single copy of template. The R^2 value is the correlation coefficient, a measurement of how accurate the individual measurements are in defining the curve (>0.99).

Probe name	Slope	Intercept	R^2 value
RNU48	−3.48	23.8	0.999
Has-let-7a	−3.28	28.6	0.990
Hsa-miR-19a	−3.13	25.3	0.999
Hsa-miR-19b	−3.30	23.5	0.989
Hsa-miR-24	−3.46	28.5	0.990
Hsa-miR-92	−3.43	23.8	0.990
Hsa-miR-93	−3.15	27.5	0.990

[a] Mean ± SD.

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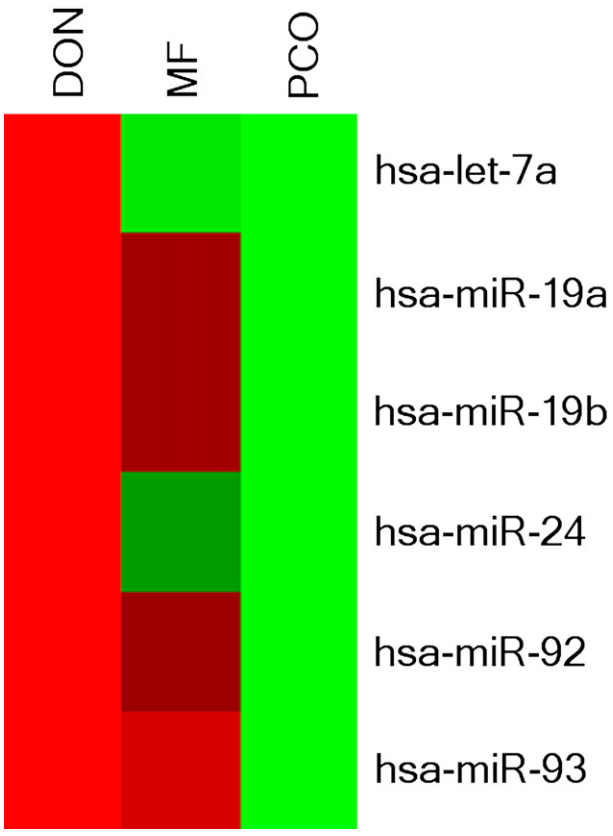
FIGURE 2

Heatmap displaying the differential expression of 6 miRNAs between blastocysts derived from infertile patients (MF and PCO), compared with blastocysts derived from fertile donor oocyte controls (DON; $P < 0.05$). The miRNA signature of MF blastocysts more closely resembled DON blastocysts. *Red* = higher expression; *green* = lower expression.



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miRNA profile was displayed by hsa-let-7g (data not shown). Quantitative real-time PCR analysis revealed that a subset of these miRNA probes was not comparable across the groups, even considering the similarity in blastocyst morphology.

Quantification was performed for each miRNA relative to the expression of the miRNA control probe, RNU48, which was consistently expressed in all blastocysts from each group (Figure 1). The miRNA hsa-miR-21 showed the most consistent expression profile across all three groups, whereas hsa-let-7b, hsa-let-7c, and hsa-miR-34b displayed some variability across individual blastocysts within both the MF and PCO groups, all without significance (Table 1). The most variable expression was observed for the miRNA hsa-let-7g with individual blastocysts displaying either a decrease or increase, without significance, within both the MF and PCO groups (Table 1).

A significant decrease in the expression of two miRNAs, hsa-let-7a and hsa-miR-24, was observed in blastocysts derived from patients with MF, in contrast with blastocysts derived from fertile donor oocyte controls ($P < 0.05$; Fig. 1B and E). Blastocysts derived from patients with polycystic ovaries showed a significant decrease in the expression of six miRNAs, including hsa-let-7a, hsa-miR-19a, hsa-miR-19b, hsa-miR-24, hsa-miR-92, and hsa-miR-93, compared with blastocysts derived from fertile donor oocyte controls ($P < 0.05$; Fig. 1B to G). Specific real-time PCR information for each of these six differentially expressed miRNAs is displayed in Table 2, including the slope of the standard curve and the reaction efficiency. The high efficiency of these reactions emphasizes the significance of the results.

A heat map was generated according to infertility indication with the MF group more closely resembling the fertile donor oocyte controls for these differentially expressed miRNAs. A clear decrease in expression for these six miRNA profiles is shown for morphologically similar blastocysts derived from patients with polycystic ovaries compared with either morphologically similar blastocysts from fertile donor oocyte controls or male factor infertility patients (Fig. 2).

Potential gene targets of these six differentially expressed miRNAs observed in blastocysts derived from patients with MFI and/or PCO ($P < 0.05$) were estimated by TargetsScan (http://www.targetscan.org). Table 3 displays the number of potential target genes for each individual miRNA as well as the detailed annotation including the most prominent GO biological processes, GO molecular functions, and total gene hits for each classification. Hsa-miR-19a revealed the largest number of gene targets with an estimated 626 genes, whereas hsa-miR-92 showed the lowest number of gene targets with only 49 genes. Annotation of these gene targets for each individual miRNA revealed some common GO

biological processes across these six differentially expressed miRNAs, including cell growth and maintenance, cell communication, and transcription, as well as nucleoside, nucleotide, and nucleic acid metabolism. Mutual GO molecular functions observed across these six differentially expressed miRNAs included, nucleic acid binding, signal transducer activity, and transcription regulator activity (Table 3).

From these lists of predicted miRNA gene targets (Table 3), three genes were chosen to demonstrate altered expression in association with aberrant miRNA profiles. Quantitative real-time PCR was performed on additional cryopreserved human blastocysts donated with consent for research; fertile donor oocyte control (n = 6), blastocysts derived from patients with MFI (n = 6), and blastocysts derived from patients with PCO (n = 6), generating an mRNA expression profile for the predicted target genes *ARIH2* (required for cell differentiation), *KHSRP* (mRNA decay promoting factor), and *NFAT5* (transcription factor) in all individual blastocysts. The PCR efficiency of each reaction was high (correlation coefficient >0.99; data not shown). Quantification was performed for each gene relative to the expression of the housekeeping gene, *ACTB*, which was consistently expressed in all blastocysts from each group.

ARIH2 is a predicted target gene for miRNA hsa-miR-19a. *ARIH2* exhibited significant altered gene expression only in blastocysts derived from patients with PCO ($P < 0.05$) (Fig. 3B), similar to the aberrant hsa-miR-19a miRNA expression profile ($P < 0.05$; Fig. 1C). The two genes *KHSRP* and *NFAT5* are both predicted target genes for miRNA hsa-miR-24. They displayed significant altered gene expression in both infertility groups; blastocysts derived from patients with either MF or PCO compared with blastocysts derived from fertile controls ($P < 0.05$; Fig. 3A and B), similar to the aberrant hsa-miR-24 miRNA expression profile ($P < 0.05$; Fig. 1E).

MicroRNAs regulate the expression of genes either negatively (3–5) or positively (6). A significant decrease in miRNA expression as observed in these blastocysts derived from infertile patients may therefore result in either an increase or decrease in the expression of a target gene, depending on the type of regulatory mechanism involved. The expression of the predicted target gene *ARIH2* was significantly increased in blastocysts derived from patients with PCO compared with blastocysts derived from fertile controls ($P < 0.05$; Fig. 3A and B). The expression of predicted target gene *KHSRP* was also significantly increased, whereas the expression of the predicted target gene *NFAT5* was significantly decreased in blastocysts derived from patients with either MF or PCO compared with blastocysts derived from fertile controls ($P < 0.05$; Fig. 3A and B).

DISCUSSION

MicroRNAs have been shown to broadly contribute to tissue specificity by either directly or indirectly altering mRNA transcript levels and protein synthesis (21). Recent reports suggest that an individual miRNA can fine tune protein production from hundreds to thousands of genes (22, 23). Numerous biological processes, including development, have been associated with miRNAs (8). MicroRNAs have also been linked with human disease, particularly cancer, with irregular miRNA expression being a common feature in malignancy and miRNAs modulating the expression of proteins critical to tumor genesis (11).

Highly specific and stage-dependent miRNA expression profiles have also been observed during mouse preimplantation and prenatal development (8, 14, 15). Indeed, levels of miRNA targets have been shown to be higher in mouse embryos than in mature mouse tissues, thereby supporting their role and importance during embryo development (24). This descriptive study characterizes an expression profile for a set of 12 miRNAs in morphologically similar, transferable quality human blastocysts. When analyzed in regard to infertility diagnosis, a potential association was observed with blastocysts derived from patients with male factor infertility or polycystic ovaries displaying specific aberrant miRNA profiles when compared with blastocysts derived from fertile donor oocyte control cycles. Clustering based on miRNA expression profiles grouped blastocysts derived from patients with MFI more closely with blastocysts derived from fertile donor oocyte controls. An assumption that morphologically similar, transferable quality blastocysts will have the same implantation potential is not always reflective of clinical outcome. Aberrant expression of blastocyst miRNAs could possibly be considered as an additional contributing factor to implantation failure in correlation with specific infertility diagnosis.

Hsa-let-7a and hsa-miR-24, were observed to be significantly decreased in blastocysts derived from both patients with MF and PCO compared with blastocysts derived from fertile donor oocyte controls ($P < 0.05$). Annotation of the gene targets for hsa-let-7a and hsa-miR-24 revealed mutual GO biological processes including cell growth and maintenance, transcription and protein metabolism, and mutual GO molecular functions including signal transducer activity and nucleic acid binding. Indeed, loss or decrease in expression of these miRNAs has been documented as a contributor to several different types of disease states. Hsa-let-7a is a founding member in the let-7 family with an estimate of at least 165 target genes. Decreased levels of hsa-let-7a have resulted in increased tumor genesis in several documented cancers, including the development and progression of malignant melanoma (25), gastric carcinoma (26), lymphoma (27) and chronic lymphatic leukemia (28). In addition, hsa-miR-24 has been shown to suppress *p16* (a tumor suppressor gene) expression in cervical carcinoma cells with decreased levels of hsa-miR-24, resulting in increased *p16* expression and replicative senescence (29).

An additional four miRNAs (hsa-miR-92, hsa-miR-93 hsa-miR-19a, and hsa-miR-19b) were significantly decreased in only the cohort of blastocysts from patients with PCO

TABLE 3

Gene ontology (GO) biological process and molecular function annotations associated with the gene targets of differentially expressed miRNAs ($P < 0.05$). The top six GO biological processes and molecular functions are described for each individual miRNA including the number of gene targets represented in each classification.

MicroRNA	$P < 0.05$	No. of target genes	GO biological processes	No. of genes	GO molecular function	No. genes
Hsa-let-7a	PCO	165	Cell growth and maintenance	30	Nucleic acid binding	26
	MF		Nucleoside, nucleotide and nucleic acid metabolism	23	Protein binding	18
			Protein metabolism	22	Hydrolase activity	18
			Cell communication	21	Signal transducer activity	17
			Transcription	15	Metal ion binding	12
			Transport	14	Transcription regulator activity	11
Hsa-miR-19a	PCO	626	Cell growth and maintenance	148	Nucleic acid binding	116
			Cell communication	127	Protein binding	74
			Nucleoside, nucleotide and nucleic acid metabolism	115	Transcription regulator activity	72
			Signal transduction	94	Signal transducer activity	63
			Transcription	92	Metal ion binding	60
			Development	70	Transcription factor activity	56
Hsa-miR-19b	PCO	60	Cell growth and maintenance	15	Signal transducer activity	9
			Cell communication	12	Nucleic acid binding	8
			Protein metabolism	11	Hydrolase activity	8
			Signal transduction	10	Transcription regulator activity	7
			Cell proliferation	8	Transferase activity	6
			Development	7	Adenosine triphosphate binding	6
Hsa-miR-24	PCO	349	Cell growth and maintenance	89	Nucleic acid binding	58
	MF		Cell communication	68	Signal transducer activity	46
			Protein metabolism	63	Adenosine triphosphate binding	35
			Nucleoside, nucleotide and nucleic acid metabolism	59	Transferase activity	34
			Signal transduction	57	Transcription regulator activity	34
			Transcription	46	Protein binding	33
Hsa-miR-92	PCO	49	Cell growth and maintenance	10	Signal transducer activity	7
			Cell communication	10	Nucleic acid binding	6
			Signal transduction	9	Transcription regulator activity	6
			Nucleoside, nucleotide and nucleic acid metabolism	8	Transporter acitvity	6
			Transcription	7	Protein binding	5
			Development	6	Hydrolase activity	4
Hsa-miR-93	PCO	102	Cell growth and maintenance	24	Nucleic acid binding	18
			Nucleoside, nucleotide and nucleic acid metabolism	17	Protein binding	13
			Transcription	14	Transcription regulator activity	11
			Protein metabolism	14	Adenosine triphosphate binding	11
			Cell proliferation	12	Transferase activity	10
			Signal transduction	12	Transcription factor activity	9

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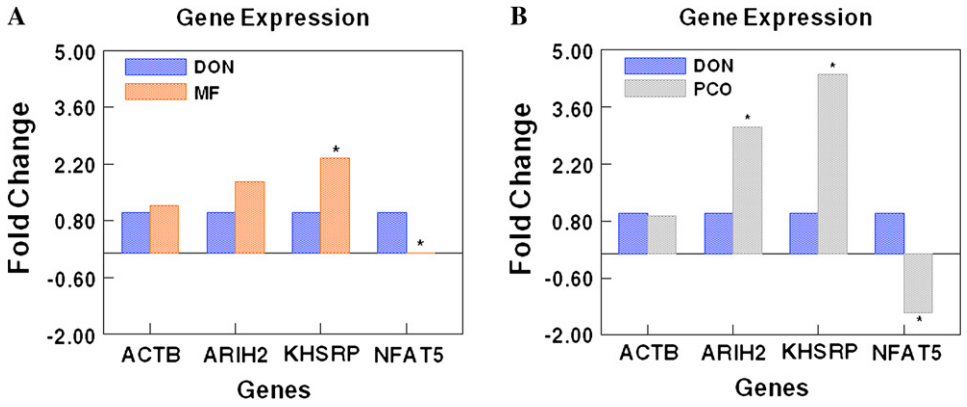
FIGURE 3

Expression of predicted target genes *ARIH2*, KHSRP, and *NFAT5*, was quantified by quantitative real-time PCR relative to the housekeeping gene *ACTB* on (**A**) blastocysts derived from patients with MF and (**B**) blastocysts derived from patients with PCO in comparison to blastocysts derived from donor fertile controls (DON).



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compared with fertile donor oocyte controls ($P < 0.05$). These four miRNAs belong to the miR-17-92 miRNA cluster located at 13q31.3, with gene target annotation revealing the most important GO biological processes to be cell growth and maintenance, and the primary GO molecular function to be nucleic acid binding. Chromosome 13 is acknowledged as a contributor to chromosomal aneuploidy in human conceptions. Infertility diagnoses such as severe MFI and advanced maternal age are risk factors for chromosomal aneuploidy, which could potentially alter the expression of miRNAs in human embryos.

Hsa-miR-92 was observed to be the most abundant miRNA from this set of 12 examined miRNAs. Both hsa-miR-92 and hsa-miR-93 have been associated with cancer pathogenesis with hsa-miR-92 expression being significantly deregulated in patients with chronic lymphatic leukemia (30) and during myeloid differentiation (31). Gene targets of hsa-miR-19a and hsa-miR-19b reveal negative regulation of *SOCS1*, which plays a critical role as inhibitor of IL-6 growth signaling (32). In addition, an hsa-miR-19a inhibitor induces strong growth reduction in anaplastic thyroid cancer cells (33).

Selection of three predicted gene targets—*ARIH2* (required for cell differentiation), *KHSRP* (mRNA decay promoting factor), and *NFAT5* (transcription factor)—from two aberrantly expressed miRNAs (hsa-miR-19a and hsa-miR-24) demonstrated that the expression of each of these predicted target genes was altered in blastocysts derived from infertile patients, compared with blastocysts derived from fertile patients in a similar pattern to the aberrant miRNA expression profile. These results highlight a potential association between miRNA aberration and the altered expression of predicted miRNA target genes.

In summary, this study describes differential profiles of miRNA expression in morphologically similar, transferable grade, human blastocysts. Such data are consistent with observations that human blastocysts with similar morphologies can vary in their metabolic (34) or proteomic fingerprints (35). Therefore, it would appear that the molecular signature of a human embryo is not always reflective of morphology. In addition, aberrant blastocyst miRNA expression was significantly correlated with a prognosis of MF or PCO. This finding suggests a possible role for embryonic miRNAs in the etiology of human infertility and potential subsequent implantation failure.

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