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ORIGINAL ARTICLE *Infertility*

Uterine fluid microRNAs are dysregulated in women with recurrent implantation failure

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STUDY QUESTION: Is the composition of microRNAs (miRNAs) in uterine fluid (UF) of women with recurrent implantation failure (RIF) different from that of healthy fertile women?

SUMMARY ANSWER: The composition of miRNAs in UF of women with RIF is different from that of healthy fertile women and the dysregulated miRNAs are associated with impaired endometrial receptivity and embryo implantation.

WHAT IS KNOWN ALREADY: It has previously been demonstrated that the miRNAs secreted from endometrial cells into the UF contribute to the achievement of endometrial receptivity. Endometrial miRNAs are dysregulated in women with RIF.

STUDY DESIGN, SIZE, DURATION: In this descriptive laboratory case–control study, miRNA abundancy was compared between UF collected during implantation phase from healthy fertile women (n = 17) and women with RIF (n = 34), which was defined as three failed IVF cycles with high-quality embryos.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Recruitment of study subjects and sampling of UF were performed at two university clinics in Stockholm, Sweden and Tartu, Estonia. The study participants monitored their menstrual cycles using an LH test kit. The UF samples were collected on Day LH + 7–9 by flushing with saline. Samples were processed for small RNA sequencing and mapped for miRNAs. The differential abundance of miRNAs in UF was compared between the two groups using differential expression analysis (DESeq2). Further downstream analyses, including miRNA target gene prediction (miRTarBase), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (g:Profiler) and external validation using relevant published data, were performed on the dysregulated miRNAs. Two miRNAs were technically validated with quantitative real-time PCR (RT-PCR).

MAIN RESULTS AND THE ROLE OF CHANCE: After processing of the sequencing data, there were 15 samples in the healthy fertile group and 33 samples in the RIF group. We found 61 differentially abundant UF miRNAs (34 upregulated and 27 downregulated) in RIF compared to healthy women with a false discovery rate of <0.05 and a fold change (FC) of ≤ -2 or ≥ 2. When analyzed with published literature, we found that several of the differentially abundant miRNAs are expressed in endometrial epithelial cells and have been reported in endometrial extracellular vesicles and in association with endometrial receptivity and RIF. Their predicted target genes were further expressed both in the trophectodermal cells of blastocyst-stage embryos and endometrial mid-secretory epithelial cells, as assessed by publicly available single-cell transcriptome-sequencing studies. Pathway analysis further revealed that 25 pathways, having key roles in endometrial receptivity and implantation, were significantly enriched. Hsa-miR-486-5p (FC −20.32; *P*-value = 0.004) and hsa-miR-92b-3p (FC −9.72; *P*-value = 0.004) were successfully technically validated with RT-PCR.

[†]The authors consider that the first two authors should be regarded as Joint First Authors.

LARGE SCALE DATA: The data are available in Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/ with GEO accession number: GSE173289.

LIMITATIONS, REASONS FOR CAUTION: This is a descriptive study with a limited number of study participants. Moreover, the identified differentially abundant miRNAs should be validated in a larger study cohort, and the predicted miRNA target genes and enriched pathways in RIF need to be confirmed and further explored *in vitro*.

WIDER IMPLICATIONS OF THE FINDINGS: RIF is a major challenge in the current IVF setting with no diagnostic markers nor effective treatment options at hand. For the first time, total miRNAs have been extensively mapped in receptive phase UF of both healthy women with proven fertility and women diagnosed with RIF. Our observations shed further light on the molecular mechanisms behind RIF, with possible implications in future biomarker and clinical treatment studies.

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Key words: endometrial receptivity / recurrent implantation failure / infertility / uterine fluid / microRNAs / in vitro fertilization / embryo-endometrial crosstalk

Introduction

Embryo implantation is a delicate and tightly regulated process. It can only occur during a very short time frame in the luteal phase of the menstrual cycle known as the window of implantation (WOI). The WOI opens around 6 days after the LH surge (Day LH + 6) and closes around LH + 10 (Harper, 1992).

Much research has been put into understanding the molecular specifics of the WOI. Most studies have focused on the tissue and cellular level of the endometrium while a perhaps less explored area, but one that is gaining in attention, is the uterine cavity microenvironment and the composition of the uterine fluid (UF). Tubal and peritoneal fluid as well as plasma exudate and endometrial secretions all contribute to the UF mix and molecular components include lipids, carbohydrates, amino acids, prostaglandins and proteins (Berlanga *et al.*, 2011), with marked changes in composition during the WOI (Gemzell-Danielsson and Hamberg, 1994). Nucleic acids have also been identified in UF, and more specifically, in microRNA (miRNA)-containing extracellular vesicles (EVs), suggesting a role for miRNAs in embryo-endometrial communication (Ng *et al.*, 2013; Li *et al.*, 2020). miRNAs are important short regulatory RNA molecules that negatively affect gene expression by either translational repression or degradation of mRNA (Krol *et al.*, 2010). All in all, UF constitutes the optimal microenvironment to facilitate implantation and is an important factor to understand in the WOI context.

In the ART setting, women with recurrent implantation failure (RIF) present a special challenge to accurately diagnose and treat. Defined by three failed implantations despite good-quality embryos, RIF forms an umbrella term that might include several different underlying conditions and pathologies (Simon and Laufer, 2012). Efforts have been made to understand the molecular mechanisms in RIF. The endometrial receptivity array (ERA) was developed based on bulk endometrial tissue gene expression of mRNAs and uses endometrial biopsies to pinpoint the individual WOI in women for personalized embryo transfer (pET) in IVF treatment (Díaz-Gimeno *et al.*, 2011). The ERA test can thus detect a 'non-receptive' or 'out-of-phase' endometrium in RIF patients and provide a treatment option with pET (Ruiz-Alonso *et al.*, 2013). Using biopsy material and modern transcriptomics, it has also been shown that miRNAs are dysregulated in the endometrium in women with RIF (Rekker *et al.*, 2018). The use of biopsies has however its disadvantages and there is a need for a less invasive method to sample the endometrium. UF has been suggested as a possibility to replace the need for biopsies. Recent protein analysis of UF has indeed shown that the UF composition differs in RIF patients and can be used to assess endometrial receptivity in the IVF setting (Kasvandik *et al.*, 2020).

In the present study, we set out to investigate the composition of total miRNAs in receptive phase UF from healthy fertile women as well as from RIF patients. We utilized a high throughput small RNA sequencing technique developed for detection of miRNAs in minute concentrations (Hagemann-Jensen *et al.*, 2018). Our aim was to draw a detailed map of UF miRNAs that are involved in the implantation process and may be affected in women with disturbed implantation.

Materials and methods

Ethics

This study was approved by the regional ethics committees at Karolinska Institutet (approval no. 2016/795-31/4) and University of Tartu (276/M-15). Study subjects were included only after written informed consent was obtained.

Study population

Two study groups were included: healthy fertile women and women diagnosed with RIF. UF samples were collected from 17 healthy fertile women during the time period of March 2017 to September 2019 at the Karolinska University Hospital, Stockholm, Sweden. All the women were recruited as healthy study volunteers through advertisements on university campus, on social media and the website studentkaninen.se. The study participants met the following inclusion criteria: age 18–40 years, proven fertile (at least one spontaneous pregnancy that had not ended in a miscarriage), regular menstrual cycles (21–35 days) and no use of hormonal or intrauterine contraception for at least three months prior to sample collection. Exclusion criteria included known

systemic, endocrinological or gynecological diseases, infertility, regular use or use prior to sample collection, of non-steroidal anti-inflammatory drugs, ongoing pregnancy or breast-feeding, ectopic pregnancy and more than two miscarriages.

UF samples were collected from 34 women diagnosed with RIF during the time period of April 2013 to June 2015 at Nova Vita Clinic, Tallinn, Estonia. RIF was defined as at least three failed IVF/ICSI cycles despite using high-quality embryos according to standard clinical routine. The following inclusion criteria had to be full-filled: minimum three failed IVF/ICSI cycles after either fresh or frozen embryo transfers, age 18–42 and no use of hormonal preparations for 3 months prior to sample collection and an endometrial thickness of minimum 7 mm at the time of sample collection. Women with congenital uterine abnormalities, known systemic or endocrinological comorbidities and/or ongoing pregnancy were excluded from the study.

All women at both study sites were checked for absence of *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Neisseria gonorrhoeae* vaginally and in urine, and for hepatitis B and HIV in the blood.

Collection of uterine fluid

Study participants were instructed to monitor LH levels in urine using a self-test kit (Clearblue, SPD Swiss Precision Diagnostics GmbH, Switzerland or BabyTime® hLH urine cassette, Pharmanova, Beit Shemesh, Israel) and to contact the study coordinator at the local research clinic when the LH surge was detected (Day LH + 0). Samples were collected on Day LH + 7 to 9 from both healthy fertile women and from RIF patients. UF samples were collected vaginally using a neonatal feeding tube, 8 French (Vygon, France) or an intrauterine insemination catheter (Cooper Surgical, Trumbull, CT, USA) depending on availability, placed close to the inner opening of the cervical canal. Then 1–2 ml of sterile clinical grade saline was flushed into the uterine cavity using a syringe, incubated for 30 s and then aspirated and collected in a 1.5 ml Eppendorf tube. Samples were centrifuged at 2000 rpm/400×*g* for 5 min at 4°C to remove cells and debris and then the supernatant was transferred to a new tube and stored at −80°C until further processing.

RNA extraction

Total RNA was extracted from 50 µl of UF mixed with 300 µl of Guanidium-based RLT plus buffer (Qiagen, Hilden, Germany), washed twice with ethanol, re-suspended in RNase free water and stored at −80°C until further processing. RNA quantity was checked using the Qubit RNA High Sensitivity Assay Kit (Invitrogen, Oregon, USA) on a Qubit 4 Fluorometer (Invitrogen, Singapore).

Small RNA library preparation and next-generation sequencing

Small RNA library preparation for sequencing was adapted from Hagemann-Jensen *et al.* (2018). cDNA libraries were prepared on a 96-well-plate using 3 µl of input RNA from each sample. A thermal cycler from BIOER Life Touch (Techtum, China) was used in all steps. After the second PCR, the cDNA libraries were purified individually with AMPure XP beads (Beckman Coulter Inc., USA) in a 1:1 bead to sample ratio. The quality of the purified cDNA product was checked on a High Sensitivity DNA chip (Agilent Technologies, USA) using an Agilent 2100 Bioanalyzer System (Agilent Technologies, USA) and the quantity was measured with a Qubit 4 Fluorometer (Invitrogen, Singapore) using the Qubit 1X dsDNA High Sensitivity Kit (Invitrogen, Oregon, USA). Samples were indexed using customized barcodes (IDT Technologies, USA). From each library, 5 ng of cDNA was pooled and small RNA sequencing was performed on an Illumina NextSeq 550 platform at the SciLifeLab, National Genomics Infrastructure, Karolinska Institutet.

Technical validation of microRNAs with real-time PCR

Technical validation using quantitative real-time PCR (RT-PCR) was done in the same cohort of samples used for sequencing. A couple of miRNAs were selected based on published literature, novelty and availability of TaqMan primers. First, 5 ng of RNA was reverse transcribed to cDNA using the TaqMan Advanced microRNA cDNA synthesis Kit (Applied Biosystems, USA), and this was followed by RT-PCR using TaqMan advanced microRNA assays (Applied Biosystems, USA) on a One Step Plus Real-time PCR system (Applied Biosystems, Singapore) according to the manufactures protocol. Each sample was run in triplicates to allow for assessment of technical variability. Fold change (FC) was calculated using the comparative Ct-method. An endogenous control miRNA was selected by performing RT-PCR for 30 commonly expressed miRNAs using TaqMan™ Advanced miRNA Human Endogenous Controls 96-well Plate (Thermo Fischer, Catalogue No: A34643). Based on a high and stable abundance in UF, hsa-miR-423-5p was chosen as endogenous control. The abundancy pattern of the reference miRNA in the sequencing data is shown in Supplementary Fig. S1A. One sample in the healthy group was excluded since it was considered as an extreme outlier with CT values >39 for the housekeeping gene in all PCR runs. RT-PCR primer assay IDs are available in Supplementary Table SI.

Statistical analysis

Differences in miRNA abundance between the two groups after validation with RT-PCR were analyzed with Mann–Whitney *U*-test. Boxplot figures of the miRNA abundancy are presented as relative quantification of miRNA abundancy. A CT value <35 was used as a cutoff for sample inclusion in the statistical analysis. A *P*-value <0.05 was considered statistically significant. Data were analyzed using SPSS version 27 software (IBM).

Bioinformatic analysis

Pre-processing

The small RNA sequencing data have been deposited into the Gene Expression Omnibus (GEO) with accession number: GSE173289. The initial quality of the FASTQ files was assessed using the software FastQC (version 0.11.9). All samples passed the initial quality control. The subsequent processing of the data was performed according to a previously published pipeline (Hagemann-Jensen *et al.*, 2018). First, the unique molecular identifier (UMI) sequences were removed from the FASTQ files and appended to their respective headers. The sequencing data were then quality checked for overrepresented sequences and guanine-cytosine and adapter content. The Illumina small RNA 3′-

adapter and the two cytosine-adenine bases linked to the UMI were finally removed with Cutadapt (version 1.7.1).

Read alignment, annotation and quantification

The trimmed reads were aligned to hg38 using STAR (version 2.7.5b). Samples with an overall mapping percentage <60% were excluded from the analysis. Aligned reads were filtered for a read length of 18–40 nucleotides. Next, PCR amplicons were collapsed based on UMIs. The filtered, aligned molecules were annotated using miRBase (miRNAs), GtRNAdb (transfer RNAs (tRNAs)) and GENCODE transcripts (small nucleolar RNAS (snoRNAs), small nuclear RNAs (snRNAs), Y RNAs and small Cajal body-specific RNA (scaRNA)). PCR duplicates and precursor molecules were removed from the annotated reads. Quantification of the aligned and annotated molecules were performed according to the custom-made published pipeline (Hagemann-Jensen *et al.*, 2018).

Differential expression analysis

To identify differentially abundant miRNAs in our data, we used the Bioconductor software DESeq2 (1.30.0). Differentially abundant miRNAs with a false discovery rate (FDR) <0.05 and a FC of ≤ -2 or ≥ 2 were considered statistically significant.

microRNA target gene prediction and functional enrichment analyses

Target genes were retrieved for the miRNAs with an FDR <0.05 and an FC ≤ -2 or ≥ 2 from miRTarBase (Release 8.0) (Chou *et al.*, 2018). MiRTarBase is a database with experimentally validated miRNA target genes. Only target genes with strong experimental evidence were included in the downstream analysis. Biological Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene ontology molecular functions and cellular components enriched for the predicted miRNA target genes were identified using the web tool g:Profiler (version e101_eg48_p14_baf17f0) (Raudvere *et al.*, 2019) by utilizing the default g:SCS algorithm. Protein–protein interaction (PPI) clusters were also predicted based on predicted miRNA target genes using the Metascape MCODE enrichment analysis (version v3.5.20211101) (Zhou *et al.*, 2019). An adjusted *P*-value of <0.05 was considered statistically significant.

Validation in external datasets

Dysregulated miRNAs were compared to relevant previously published endometrial miRNA data (Ng *et al.*, 2013; Logan *et al.*, 2018; Rekker *et al.*, 2018). Target genes of dysregulated miRNAs were matched with genes expressed in trophectoderm of Days 5–7 human blastocyst-stage embryos reported in the single-cell dataset published by Petropoulos *et al.* (2016). 10× Genomics single-cell sequencing data from endometrial tissue published by Wang *et al.* (2020) was downloaded from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111976. R (version 3.6.1) and the Seurat package (version 3.1.1) were used in R-Studio (version 1.2.1335) to subset mid-secretory cells and find marker genes expressed in epithelial cells.

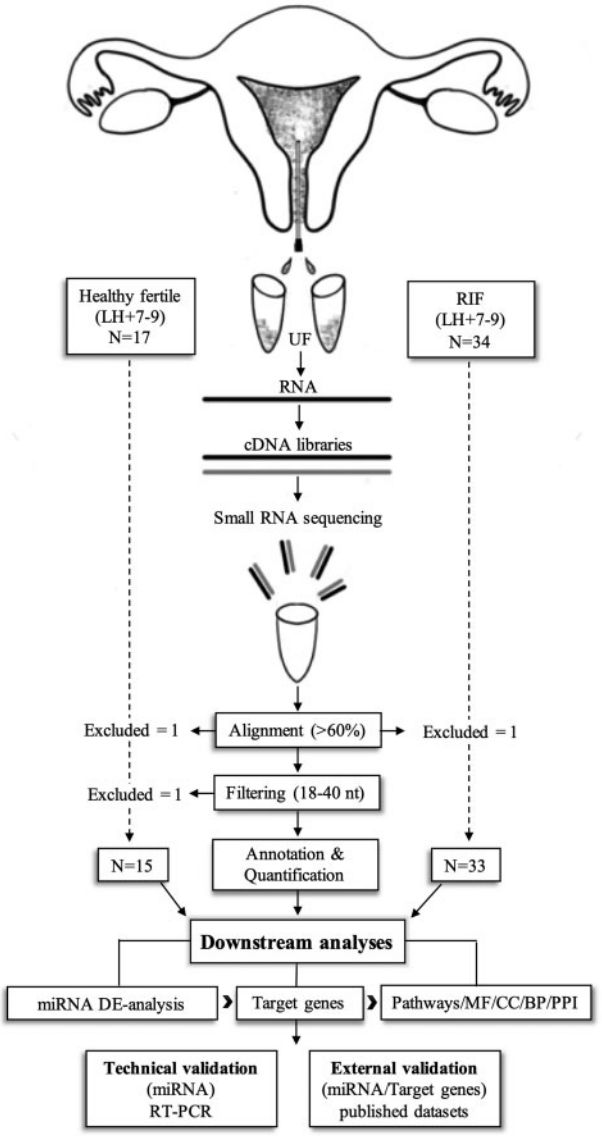

Figure 1. Overview of the study design. BP, biological processes; CC, cellular component; DE, differential expression; MF, molecular functions; miRNA, microRNA; nt, nucleotides; PPI, protein-protein interactions; RIF, recurrent implantation failure; UF, uterine fluid.

Results

Small RNA molecules in uterine fluid

To explore the content of small RNAs in UF from healthy fertile women and women with RIF, we utilized next-generation small RNA sequencing and subsequent bioinformatic analyses (Fig. 1). Two samples, one in each group, did not meet the alignment criterion and one sample in the healthy group did not pass the read-length filter and were subsequently excluded. In total, 15 healthy and 33 RIF samples remained after preprocessing of the

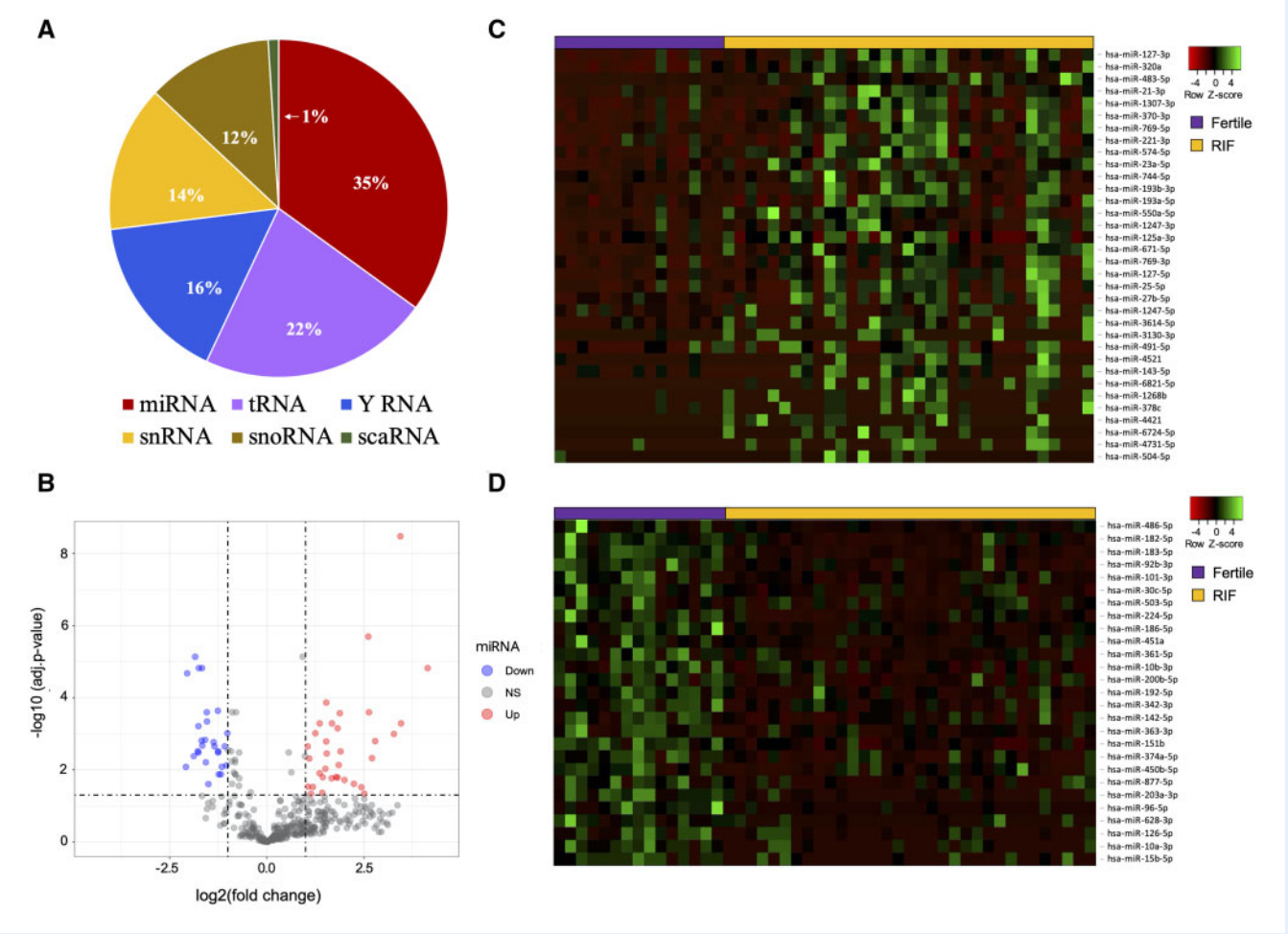

Figure 2. Overview of the small RNA sequencing data. (**A**) Pie chart showing the distribution, in percentage, of small RNA types in uterine fluid from healthy fertile women and women with RIF. (**B**) Volcano plot of upregulated (red) and downregulated (blue) miRNAs in uterine fluid from women with RIF. (**C** and **D**) Heatmaps based on z-scores showing the abundancy pattern of the upregulated (C) and downregulated (D) miRNAs. Each column in the heatmap represents a sample. The following cutoffs for significance were used for the differentially abundant miRNAs presented in B–D: FDR <0.05 and FC ≤ -2 or ≥ 2. FC, fold change; FDR, false discovery rate; miRNA, microRNA; NS, not significant; RIF, recurrent implantation failure; scaRNA, small Cajal body-specific RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; tRNA, transfer RNA; UF, uterine fluid.

data (Fig. 1). After initial filtering and annotation, we identified 1155 miRNAs, 706 tRNA fragments, 538 Y RNA-derived small RNAs, 465 snRNA-derived small RNAs, 393 snoRNA-derived small RNAs and 24 scaRNA-derived small RNAs (Fig. 2A). Hierarchical clustering of samples was performed based on normalized miRNA counts and showed clustering of samples according to group (Supplementary Fig. S1B). The clinical characteristics for the groups are shown in Table I.

Differentially abundant UF miRNAs in women with RIF

To further investigate the role of miRNAs in RIF, the differential abundance of miRNAs in UF between the two groups was analyzed using DESeq2. We identified 61 miRNAs that were dysregulated in women with RIF compared to healthy fertile women (FDR < 0.05 and FC ≤ -2 or ≥ 2) (Table II and Fig. 2B–D). Out of these miRNAs, 34 were upregulated and 27 downregulated in RIF. The downregulated miRNAs showed a more stable and uniform abundancy pattern (Fig. 2C and D).

Target genes of differentially abundant miRNAs in RIF

As miRNAs function by targeting mRNA transcripts and negatively regulating their expression (Carthew and Sontheimer, 2009), we explored the target genes for the dysregulated miRNAs in UF from RIF patients. We utilized the web-based tool miRTarBase (Chou *et al.*, 2018) and filtered for experimentally validated targets with strong evidence. This

Table I Clinical characteristics of the healthy fertile and RIF group.

Parameter	Healthy fertile (n = 15)	RIF (n = 33)
Age (years) median (range)	32 (22–39)	35 (26–42)
BMI (kg/m^2) median (range)	24 (20–33)	23 (18–39)
Menstrual cycle length (days) median (range)	28 (26–30)	28 (24–32)
Number of pregnancies median (range)	1 (1–4)	1 (0–4)
Live birth (≥ 1) %	58[a]	18
Miscarriage (≥ 1) %	1[b]	36
Ectopic pregnancy (≥ 1) %	0	21
Abortion (≥ 1) %	55[b]	12
Number of fresh embryo transfers median (range)	–	3 (0–10)
Number of frozen embryo transfers median (range)	–	1 (0–4)
Number of IVF failures median (range)	–	4 (3–10)
Duration of infertility (years) median (range)	–	6 (2–14)

Values are presented as median and range or percentage.
n, number; RIF, recurrent implantation failure.
[a]Three missing values.
[b]Four missing values.

yielded a list with 108 potential target genes for our upregulated miRNAs and 357 potential target genes for our downregulated miRNAs (Supplementary Table SII).

Biological processes and pathways critical for endometrial receptivity and implantation are enriched for the identified miRNA target genes

In order to put the target genes of the dysregulated miRNAs in a biological context, we utilized the web-based tool g:Profiler (Raudvere *et al.*, 2019) to perform functional enrichment analysis on our target gene lists. When looking at enriched KEGG (Kanehisa and Goto, 2000) pathways, we found 69 significantly enriched pathways for the target genes of upregulated miRNAs and 110 significantly enriched pathways for the target genes of downregulated miRNAs (Supplementary Table SIII). Among the significantly enriched pathways, we could identify 25 unique pathways that have been previously reported to be involved in endometrial receptivity and implantation (Fig. 3A and B, references listed in Supplementary Table SIV). The top 10 statistically most significant pathways out of those associated with endometrial receptivity and implantation and their associated target genes overlapping between the pathways for the up- and downregulated miRNAs, are shown in Fig. 3C and 3D, respectively.

Target genes enriched in the top 10 pathways associated with RIF and implantation for up- and downregulated miRNAs (presented in Fig. 3C and D) were further analyzed for gene ontology molecular functions, cellular components and biological processes as well as Metascape MCODE PPI clusters. Protein, enzyme and transcription factor binding were among the most significant molecular functions predicted to be related to the target gene products of both up- and downregulated miRNAs (Supplementary Fig. S2A and B). Examples of the most significant cellular components in which the target gene product may exert its function were cytoplasm, membrane microdomain and membrane raft for the upregulated miRNA target genes (Supplementary Fig. S3A) and transferase complex, protein kinase complex and cytosol for the downregulated miRNA target genes (Supplementary Fig. S3B). Some of the enriched biological processes were negative regulation of developmental processes and regulation of cell population proliferation for the upregulated miRNAs (Supplementary Fig. S4A) and phosphorylation and regulation of cell death for the downregulated miRNAs (Supplementary Fig. S4B). PPI network analysis identified three clusters of densely connected protein products of our upregulated miRNA target genes (Supplementary Fig. S5) and four clusters for the downregulated miRNA target genes (Supplementary Fig. S6). Individual PPI cluster scores are presented in Supplementary Table SV.

Differentially abundant UF miRNAs are found in endometrial tissue and extracellular vesicles during the WOI

Next, we wanted to explore our results in the endometrial context. Logan *et al.* (2018) have profiled miRNAs in endometrial epithelial cells of healthy women using microarray technology. Out of 61 of our dysregulated miRNAs, 55 overlapped with miRNAs reported to be expressed in endometrial epithelial cells of that study (Fig. 4A, Supplementary Table SVI) (Logan *et al.*, 2018). Ng *et al.* (2013) studied EVs (exosomes and microvesicles) in UF and the miRNA content of EVs derived from the endometrial epithelial cell-line (ECC-1) *in vitro*. We found that 19 of our dysregulated miRNAs were among the 229 miRNAs present in ECC-1 EVs reported by Ng *et al.* (2013) (Fig. 4B), suggesting that our results could reflect a dysregulation of the endometrial epithelial secretome.

Rekker *et al.* (2018) studied miRNA expression in endometrial tissue at the time of the WOI in RIF patients and reported 21 dysregulated miRNAs compared to healthy women. Three out of these were found similarly dysregulated in our UF-derived miRNAs (Fig. 4C). In the same study, they also reported differentially expressed miRNAs in endometrial tissue in healthy women between the pre-receptive phase and the WOI, thereby describing normal miRNA regulation during implantation. When we compared our up- and downregulated miRNAs to the ones reported by Rekker *et al.* (2018), we found nine miRNAs that were present in both datasets (Fig. 4D). We also noted that some had opposite expressional direction between the two studies, which further supports dysregulation of these miRNAs during the WOI in RIF.

Differentially abundant miRNAs' targets exist in both endometrial and embryonic cells

UF miRNAs have the potential to act on both the endometrium and on the embryo. To investigate the possible location of our targets of

Table II Differentially abundant miRNAs in receptive phase uterine fluid from women with recurrent implantation failure compared to healthy fertile women (FC ≤ -2 or ≥ 2 and FDR < 0.05).

Upregulated miRNA			Downregulated miRNA		
miRNA ID	**FC**	**FDR**	**miRNA ID**	**FC**	**FDR**
hsa-miR-6821-5p	17.7	<0.001	hsa-miR-96-5p	−4.2	0.008
hsa-miR-4521	11.0	<0.001	hsa-miR-186-5p	−4.1	<0.001
hsa-miR-483-5p	10.9	<0.001	hsa-miR-628-3p	−3.7	0.004
hsa-miR-4421	9.7	0.001	hsa-miR-183-5p	−3.6	<0.001
hsa-miR-1268b	6.9	0.002	hsa-miR-126-5p	−3.4	0.003
hsa-miR-3130-3p	6.6	0.005	hsa-miR-451a	−3.4	0.003
hsa-miR-127-5p	6.2	<0.001	hsa-miR-374a-5p	−3.4	<0.001
hsa-miR-671-5p	6.1	<0.001	hsa-miR-224-5p	−3.4	<0.001
hsa-miR-504-5p	5.7	0.046	hsa-miR-203a-3p	−3.2	0.002
hsa-miR-6724-5p	5.4	0.030	hsa-miR-361-5p	−3.2	<0.001
hsa-miR-4731-5p	4.7	0.024	hsa-miR-877-5p	−3.2	0.002
hsa-miR-378c	4.0	0.019	hsa-miR-363-3p	−3.0	0.002
hsa-miR-193b-3p	3.7	0.003	hsa-miR-15b-5p	−3.0	0.006
hsa-miR-23a-5p	3.7	<0.001	hsa-miR-486-5p	−2.9	<0.001
hsa-miR-3614-5p	3.6	0.007	hsa-miR-92b-3p	−2.9	<0.001
hsa-miR-1247-3p	3.6	0.016	hsa-miR-10a-3p	−2.8	0.025
hsa-miR-769-5p	3.6	<0.001	hsa-miR-200b-5p	−2.6	0.002
hsa-miR-143-5p	3.4	0.016	hsa-miR-192-5p	−2.5	0.002
hsa-miR-370-3p	3.2	<0.001	hsa-miR-151b	−2.4	<0.001
hsa-miR-1247-5p	3.2	0.017	hsa-miR-101-3p	−2.4	0.003
hsa-miR-1307-3p	2.9	0.004	hsa-miR-142-5p	−2.4	0.003
hsa-miR-127-3p	2.9	<0.001	hsa-miR-450b-5p	−2.4	0.013
hsa-miR-550a-5p	2.9	0.002	hsa-miR-503-5p	−2.3	0.013
hsa-miR-25-5p	2.9	0.009	hsa-miR-342-3p	−2.2	0.008
hsa-miR-27b-5p	2.7	0.016	hsa-miR-30c-5p	−2.1	0.002
hsa-miR-491-5p	2.7	0.043	hsa-miR-10b-3p	−2.1	0.008
hsa-miR-574-5p	2.6	<0.001	hsa-miR-182-5p	−2.0	0.001
hsa-miR-769-3p	2.6	0.012			
hsa-miR-193a-5p	2.4	0.001			
hsa-miR-21-3p	2.3	0.030			
hsa-miR-221-3p	2.2	0.046			
hsa-miR-125a-3p	2.1	0.005			
hsa-miR-744-5p	2.1	0.029			
hsa-miR-320a	2.1	0.002			

Small RNA sequencing was used to detect miRNAs in uterine fluid. Differential expression analysis was performed with DESeq2.
FC, fold change; FDR, false discovery rate; miRNAs, microRNAs.

the dysregulated miRNAs, we searched for the presence of our target genes in relevant cell types utilizing publicly available single-cell transcriptome sequencing data.

The extensive mapping of human endometrial cell types by Wang *et al.* (2020) was used to look at the specific gene expression of endometrial epithelial cells during the WOI. We found that several of the target genes for the dysregulated miRNAs in RIF were active in human endometrial epithelial cells during the WOI, indicating that our dysregulated miRNAs may target genes in the epithelial cells of the endometrium during implantation (Fig. 5A).

Using the comprehensive single-cell RNA sequencing study on human embryos by Petropoulos *et al.* (2016), we looked at the specific gene expression (P-value < 0.05) in Days 5–7 trophoblasts of blastocyst-stage embryos and compared to our miRNAs target genes (Fig. 5B). We found that some of the target genes were indeed active in peri-implantation trophoblasts,

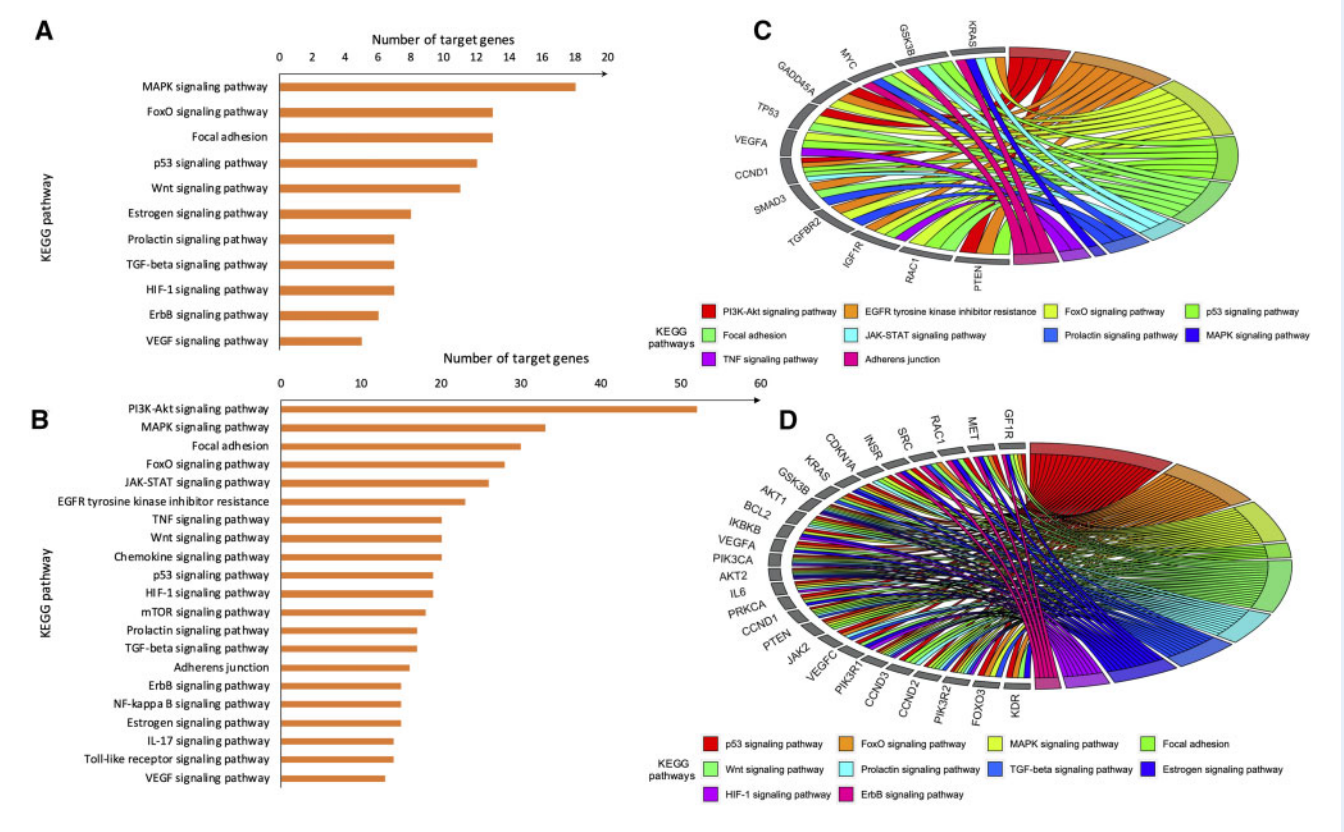

Figure 3. Biological pathways related to endometrial receptivity and implantation. (**A** and **B**) Bar charts showing the number of target genes per pathway for all significant KEGG pathways (adjusted *P*-value < 0.05) related to endometrial receptivity and/or implantation for up- (A) and downregulated (B) UF miRNAs in RIF. (**C** and **D**) The top 10 most significant KEGG pathways (adjusted *P*-value < 0.05) related to endometrial receptivity and/or implantation and their associated target genes for up- (C) and downregulated (D) UF miRNAs in RIF. Each target gene presented in the figure was present in a minimum of three pathways for the up and downregulated miRNAs respectively. miRNA, microRNA; RIF, recurrent implantation failure; UF, uterine fluid.

suggesting that they may be affected by the dysregulation of miRNAs in RIF.

Selected UF miRNAs validated by qPCR

To technically validate our results of the small RNA sequencing data, the relative abundance of two dysregulated miRNAs (hsa-miR-486-5p and hsa-miR-92b-3p) were examined with RT-PCR. We chose to validate one miRNA, hsa-miR-486-5p, that has been previously linked to implantation failure (Yang *et al.*, 2018), and one miRNA, hsa-miR-92b-3p, which has not been studied in the context of RIF. In addition, both miRNAs were among the most abundant miRNAs and showed a stable abundancy pattern in both groups (Fig. 2C and D). In line with the sequencing results, both miRNAs were downregulated in the RIF group with a FC of −20.32 (*P*-value = 0.004) for hsa-miR-486-5p and a FC of −9.72 (*P*-value = 0.004) for hsa-miR-92b-3p (Supplementary Fig. S7, Fig. 2D).

Discussion

RIF is a major challenge in the current IVF setting with no diagnostic markers nor effective treatment options at hand. For the first time, total miRNAs have been extensively mapped in receptive phase UF of both healthy women with proven fertility and women diagnosed with RIF. We show the presence of several dysregulated miRNAs in the UF of women with RIF and validate their significance and potential role in implantation using external datasets and pathway enrichment analysis.

A receptive endometrium is a pre-requisite for a successful implantation and poor endometrial receptivity has been suggested as a putative cause of RIF (Ruiz-Alonso *et al.*, 2013). In a recent study, Li *et al.* (2020) compared the abundancy of miRNAs specifically in EVs from UF collected from healthy women in natural cycles and from women undergoing IVF after ovarian stimulation. Several of our miRNAs overlap with the reported dysregulated miRNAs in receptive phase of natural and ovarian stimulation cycles of that study (Li *et al.*, 2020). Furthermore, hsa-miR-320a was reported as downregulated in women with a successful IVF pregnancy, which is in line with our results where this miRNA was reported as upregulated in RIF (Li *et al.*, 2020). In another study on UF and endometrial receptivity, Vilella *et al.* (2015) reported 27 WOI-specific miRNAs in UF of healthy women utilizing microarray technology. Two of our dysregulated miRNAs, miR-183-5p

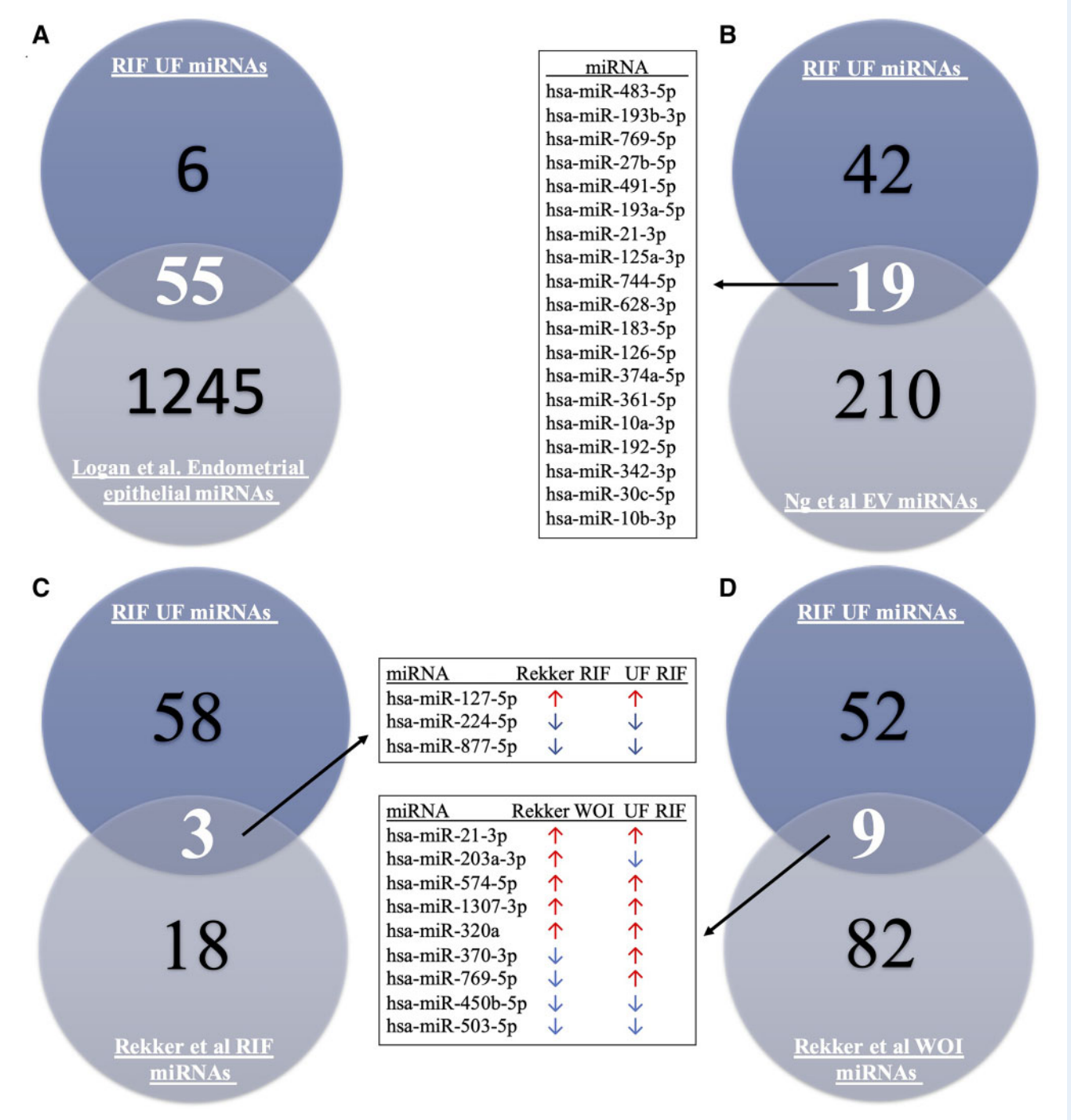

Figure 4. External validation of dysregulated miRNAs. (**A**–**D**) Venn diagrams showing overlap with our dysregulated UF miRNAs in RIF and microRNAs that are: (A) expressed in endometrial epithelial cells (Logan *et al.*, 2018), (B) present in extracellular vesicles from endometrial epithelial cells (Ng *et al.*, 2013), (C and D) differentially expressed in the endometrium of (C) women with RIF and (D) healthy fertile women during the WOI (Rekker *et al.*, 2018). EV, extracellular vesicle; miRNA, microRNA; RIF, recurrent implantation failure; UF, uterine fluid; WOI, window of implantation.

and miR-320a, overlapped with the results of that study, implicating a role for them in endometrial receptivity (Vilella *et al.*, 2015). Indeed, downregulation of miR-183-5p, along with miR-182-5p and miR-96-5p, as we show in RIF in our study, have been linked to poor endometrial receptivity in a functional *in vitro* study by Akbar *et al.* (2020). Notably, they demonstrated that reduced expression of miR-183-5p both *in vitro* in a human endometrial cell line and *in vivo* in the endometrium of mice reduced embryo

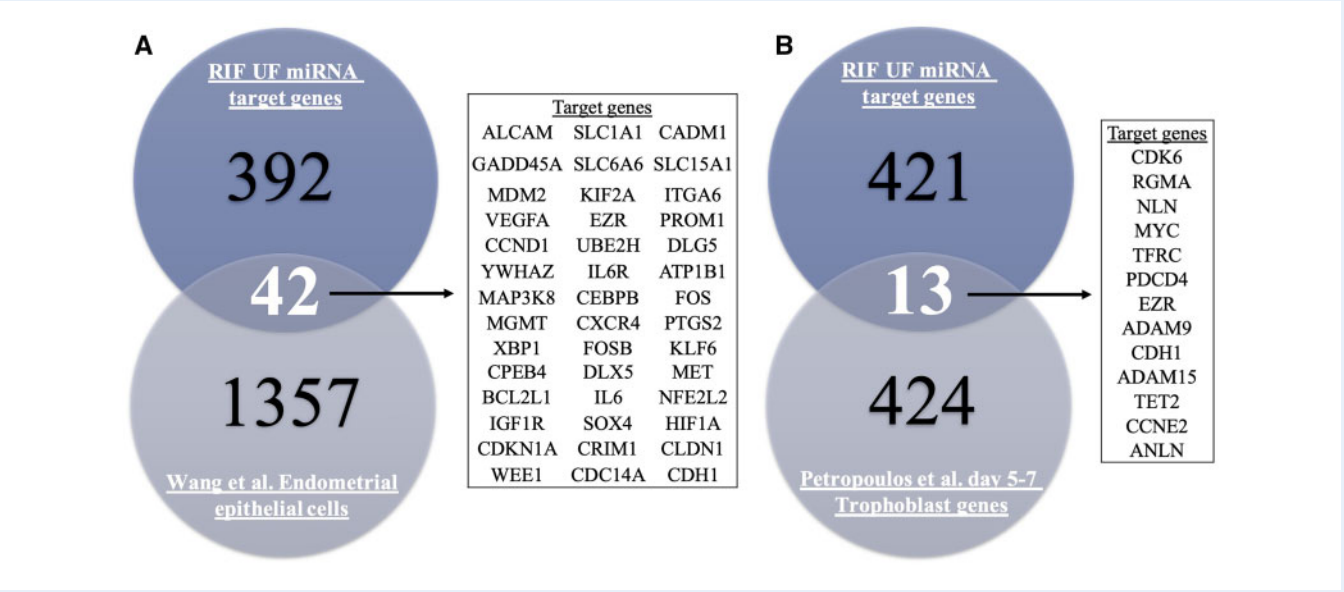

Figure 5. External validation of predicted target genes of dysregulated miRNAs. (**A** and **B**) Venn diagrams showing overlap with the predicted target genes of our dysregulated miRNAs and (A) genes expressed in the endometrial epithelial cells (Wang *et al.*, 2020) and (B) lineage-specific genes in Days 5–7 trophectoderm (Petropoulos *et al.*, 2016) during the WOI. miRNA, microRNA; RIF, recurrent implantation failure; UF, uterine fluid.

attachment whereas upregulation promoted it, suggesting that downregulation of miR-183-5p could potentially contribute to poor endometrial receptivity and thus implantation failure in women with RIF (Akbar *et al.*, 2020).

Further supporting a role of our dysregulated miRNAs in RIF, external validation of our findings with a study by Rekker *et al.* (2018) demonstrated an overlap with differentially expressed miRNAs in the endometrial tissue of women with RIF. Interestingly, two of the overlapping miRNAs, miR-224-5p and miR-127-5p, have previously been shown to target genes of the implantation associated pathway PI3K/AKT, which was one of the most significantly enriched pathways in our study (Grewal *et al.*, 2008; Fabi *et al.*, 2017; Kuang *et al.*, 2019; Li *et al.*, 2019; Qiao *et al.*, 2020). In addition, aberrant expression of miR-127-3p (sharing the same precursor miRNA as miR-127-5p) that was upregulated in our study as well as one of our downregulated miRNAs that we also technically validated, miR-486-5p, have been associated with poor embryo-transfer outcome and recurrent miscarriage in a study by Yang *et al.* (2018). *BCL6*, a well-known endometrial receptivity gene, is also one of the target genes of miR-127-3p, which further support a role of this miRNA in endometrial receptivity and subsequently in RIF (Chen *et al.*, 2013).

In line with previous published literature outlined above, we show that the predicted target genes of our dysregulated miRNAs are significantly enriched in numerous pathways having key roles in implantation, including pathways associated with endometrial receptivity such as JAK/STAT, vascular endothelial growth factor (VEGF) and estrogen-mediated signaling (Young, 2013; Rosario and Stewart, 2016; Guo *et al.*, 2021). These results provide clues about the biological contexts of the dysregulated miRNAs in RIF and indeed, similar to our study, VEGF, P53, JAK/STAT, HIF-1, IL-17 and TGF-beta signaling have previously been associated with RIF (Rekker *et al.*, 2018; Shi *et al.*, 2018; Wang *et al.*, 2019; Yu *et al.*, 2019). Further studies are however needed to investigate how the pathways are affected. We also show that target genes of our miRNAs overlap with genes expressed in the endometrial mid-secretory epithelium (Wang *et al.*, 2020). Hence, we speculate that miRNAs in UF could potentially regulate genes crucial for endometrial receptivity through paracrine signaling. In RIF, the implications of this could be impaired endometrial receptivity and implantation, for example, by deregulation of *VEGFA* (Guo *et al.*, 2021), which is a predicted target gene for several of the dysregulated UF miRNAs.

Embryo endometrial cross-talk is crucial for a successful implantation. It has previously been reported that miRNAs secreted from the endometrium in EVs can be taken up by the embryo and subsequently affect embryo adhesion during implantation (Vilella *et al.*, 2015). Furthermore, Liu *et al.* (2020) recently demonstrated that EVs specifically secreted from endometrial cells of RIF patients negatively affects growth and invasion of the embryo during implantation. The overlap of our data with miRNAs identified in EVs secreted from ECC-1 cells reported by Ng *et al.* (2013), suggest that some of the miRNAs identified in our study could be transported in EVs from the endometrium to the embryo. In line with this observation, we showed that some of the predicted target genes of our UF miRNAs are indeed expressed in the blastocyst, including *CDH1*, encoding the cell-to-cell adhesion protein E-cadherin forming adherens junctions between cells (Petropoulos *et al.*, 2016). E-cadherin is known to be involved in embryo adhesion during implantation and it also indirectly affects Wnt-signaling, one of the important pathways regulating embryo-endometrium crosstalk (Shih *et al.*, 2002; Nelson and Nusse, 2004; Chen *et al.*, 2009; Kokkinos *et al.*, 2010). Indeed, Wnt-signaling and adherens junctions were two of the significantly enriched pathways in our study. Accordingly, our data

together with previously published literature, collectively implies that an abnormal content of miRNAs in UF of women with RIF may not only affect endometrial receptivity but also crosstalk between the embryo and the endometrium during implantation. Aside from EVs, it is likely that some of the identified miRNAs may exist in UF as part of apoptotic bodies, free or bound to proteins that originate from endometrial secretions, plasma exudates or peritoneal fluid, which all contribute to the mix of components in UF (Berlanga *et al.*, 2011; Bhusane *et al.*, 2016).

Our results present an explorative overview of differentially abundant miRNAs in RIF and suggest possible target genes and cells of these miRNAs and thus lays out direction for future investigations into the functional role of miRNAs in RIF. Further *in vitro* studies are needed to validate the predicted target genes and to understand how identified miRNAs might act on cells to affect the implantation process. Another key aspect to further address is what possible mechanisms underly the dysregulation of miRNAs in RIF UF. Possible explanations that require further investigation range from genetic to epigenetic mechanisms to defects in exocytosis or exosomal pathways.

Our findings also constitute a pilot investigation for the use of UF miRNAs as non-invasive biomarkers for the RIF condition. Thus, they could in the future potentially assist in clinical decisions and timing of embryo transfer if the risk of implantation failure is predicted as high. We show here that robust and biologically relevant differences in miRNA abundancy, some of which have not been reported in this context before, can be detected between healthy and pathological samples of UF that was collected with a minimally invasive procedure. Of course, the possible implications of this need to be further studied in larger clinical settings but it is a promising starting point. As we could see a very stable abundancy pattern in several of the downregulated miRNAs, including the ones chosen for technical validation (hsa-miR-486-5p and hsa-miR-92b-3p), we suggest future investigations looking into these as potential biomarkers. In the clinical setting, a diagnostic marker analyzed by PCR or a panel of markers analyzed by sequencing would be convenient, cheap and readily available tools.

Some limitations should be considered when interpreting the data of this study. Recent studies suggest that the endometrium is a limited issue in implantation failure when applying preimplantation testing for aneuploidy (PGT-A) on the embryos (Pirtea *et al.*, 2021, 2021). However, the usefulness of PGT-A is still debatable as some studies have shown no benefit of PGT-A to live-birth rates (Sarkar *et al.*, 2021) or have demonstrated improved live-birth rates only for women over the age of 35 (Simopoulou *et al.*, 2021), suggesting that currently available evidence is insufficient to support PGT-A in routine clinical practice (Madero *et al.*, 2021). Therefore, we used the most common definition of RIF which only includes morphological scoring for embryo quality assessment. Although collection of UF is a minimally invasive procedure, minor amounts of blood contamination may still occur due to accidental scratching of the catheter to the wall of the uterine cavity or cervical canal. However, to avoid this we used the smallest diameter possible of the catheter, placed it close to the inner opening of the cervical canal during UF collection, and only included the supernatant of the UF after centrifugation. In order to avoid contamination of cervical mucus, extra care was taken to not aspirate during the passage through the cervical canal. Furthermore, a batch effect due to different UF collection sites cannot be excluded. We did, however, use the same protocol at both collection sites and carefully normalized the cDNA input concentrations when submitting the cDNA libraries for sequencing, which was performed in the same laboratory for all samples. Finally, although we have applied strict clinical inclusion and exclusion criteria for all the study participants and collected the samples in the same phase of the menstrual cycle in order to make the study population of the two groups as homogenous as possible, the sample size is limited. Furthermore, we used previously published data from endometrial samples, ECC-1 cells *in vitro* and embryos to validate our results (Ng *et al.*, 2013; Petropoulos *et al.*, 2016; Logan *et al.*, 2018; Rekker *et al.*, 2018; Wang *et al.*, 2020). Although we found several interesting overlaps with our data, the heterogeneity of the bio-specimens and *in vivo*/*in vitro* settings should be considered when interpreting the results.

Conclusions

In conclusion, we show that miRNAs can be extensively mapped in receptive phase UF and that several miRNAs are differentially abundant in UF from women with RIF compared to healthy fertile women. Based on external validation and pathway analysis, we speculate that an aberrant composition of miRNAs in UF may contribute to RIF by affecting endometrial receptivity and/or embryo-endometrial crosstalk during implantation. However, functional *in vitro* and *in vivo* studies are needed to establish their role in the pathogenesis of RIF. In addition, the identified UF miRNAs constitute a promising panel of non-invasive biomarkers for RIF that could be further studied in larger clinical trials to evaluate their clinical utility.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in Gene Expression Omnibus (GEO) at https://www.ncbi.nlm.nih.gov/geo/ and can be accessed with GEO accession number: GSE173289.

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Authors' roles

K.G.-D. was responsible for the study conception, regulatory permits and overall study conduct. K.G.-D. and A.S. were responsible for study funding. P.G.L. contributed with the original research idea. K.G.-D., P.G.L., A.S., N.R.B., C.V.G. and C.F. designed and planned different subparts of the study. C.F. and C.V.G. recruited the study subjects in Sweden. C.F. coordinated and collected the cohort of UF samples from healthy fertile women in Sweden. A.S. and M.P. were responsible for the UF RIF sample collection in Estonia. O.R.F. developed the small

RNA sequencing and the RNA extraction protocol. C.V.G. and N.R.B. optimized the small RNA sequencing protocol. C.V.G. performed the RNA extraction and small RNA library construction. N.R.B., C.V.G. and C.F. performed the RT-PCR validation. N.R.B. supervised all the laboratory work. V.M. set-up the bioinformatic pipeline and performed the processing of raw data and downstream analyses. C.F. and C.V.G. analyzed and interpreted the data. A.S., M.P., P.G.L. and N.R.B. provided continuous scientific input during the data analyses. C.F. and C.V.G. wrote the manuscript. K.G.-D., N.R.B., A.S., V.M., P.G.L., M.P. and O.R.F. critically reviewed the manuscript. All authors approved on the final version of the manuscript.

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Conflict of interest

There are no conflicts of relevance for the current study.

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