Reproductive Sciences
2019, Vol. 26(7) 879-890

DOI: 10.1177/1933719118792104
journals.sagepub.com/home/rsx
SAGE

Potential Marker Pathways in the Endometrium That May Cause Recurrent Implantation Failure

Ercan Bastu, MD[1], Irem Demiral, MD[2], Tuba Gunel, PhD[3], Ege Ulgen, MD[4], Ece Gumusoglu, PhD[3], Mohammad Kazem Hosseini, PhD[3], Ugur Sezerman, PhD[4], Faruk Buyru, MD[1], and John Yeh, MD[2]

Abstract

The aim of this prospective cohort study was to identify altered biologic processes in the endometrium that may be potential markers of receptive endometrium in patients with repeated implantation failure (RIF) as compared with fertile controls. The study was conducted in a university-affiliated in vitro fertilization (IVF) gynecology clinic and molecular biology and genetics laboratory. Healthy fertile controls (n = 24) and patients with RIF (n = 24) were recruited. Window of implantation gene profiling associated with RIF was performed. Six hundred forty-one differentially expressed genes were identified, and 44 pathways were found enriched. Upon clustering of the enriched pathways, 9 representative pathways were established. The important pathways that were identified included circadian rhythm, pathways in cancer, proteasome, complement and coagulation cascades, citrate cycle, adherens junction, immune system and inflammation, cell cycle, and renin–angiotensin system. The involvement of the circadian rhythm pathway and other related pathways may alter the endometrium's functioning to ultimately cause RIF. Furthermore, we found that the pathogenesis of RIF was multifaceted and that numerous processes were involved. We believe that a better understanding of the underlying mechanisms of RIF will ultimately give rise to better treatment opportunities and to better outcomes in IVF.

Keywords

recurrent implantation failure, global gene profiling, window of implantation, endometrium

Introduction

Several in vitro fertilization (IVF) treatment attempts resulting with failure raise the clinical diagnosis of repeated implantation failure (RIF). With no formal criteria defining the number of failed cycles or the total number of embryos transferred during these IVF attempts, taking into consideration the current success rate of IVF treatment and the mean number of embryos transferred in each cycle, the general recommendation for making this clinical diagnosis would be to wait for at least 3 consecutive IVF attempts with 1 to 2 high-quality embryos transferred in each.[1] There may be several reasons that cause RIF such as various female factors (ie, impaired endometrial factor, anatomic factors, thrombophilia, etc), male factor, immunologic factors, genetics, and embryo-related issues.

Success in IVF lies in the positive communication between the endometrium and embryo during a well-defined period in the secretory phase of the menstrual cycle, named the "window of implantation" (WOI). This communication appears to be based mainly on 2 factors: the quality of the embryo and the receptivity of the endometrium.[2] Thus, the endometrium is no longer seen as a passive member in this crosstalk, but an active player, and even the final decision-maker by allowing or preventing the implantation of the embryo.[3] When an infertile woman who has good quality embryos is undergoing IVF treatment and experiences RIF, an endometrial factor is a potential cause.

Endometrial receptivity is linked to the WOI. Navot et al found that not all embryos transferred between days 16 and 24 implanted.[4] The endometrium might be sensitive to the embryo for a specific period. Taking into consideration the numerous

[1] Department of Obstetrics and Gynecology, Acibadem University School of Medicine, Istanbul, Turkey
[2] Department of Obstetrics and Gynecology, Massachusetts General Hospital, Harvard University, Boston, MA, USA
[3] Department of Molecular Biology and Genetics, Istanbul University, Istanbul, Turkey
[4] Department of Biostatistics and Medical Informatics, Acibadem University School of Medicine, Istanbul, Turkey

Corresponding Author:
Ercan Bastu, Department of Obstetrics and Gynecology, Acibadem University School of Medicine, Ataşehir, Istanbul, Turkey.
Email: ercanbastu@gmail.com

studies, days 22 to 24 might be better defined as the WOI in normal regular menstrual cycle.[5]

The first step of any change in cell function or structure starts with transcription. Transcriptomics is the study of the entire set of gene expressions of certain cell types or tissues. Altered gene expression raises differences in functioning between cells. Therefore, gene expression analysis provides useful insight about the etiology of diseases. Microarray analysis enables the assessment of monumental numbers of genes in a very short amount of time. Even though many genes have been proposed as potential receptivity markers, there is no strong consistency between studies leading to one gene or a definite group of genes as receptivity marker(s).[6]

The importance of endometrial transcriptomics studies lies in the impact of any identification of one or more markers specific for receptivity. In addition to providing understanding to the very complicated process of human embryo implantation, these markers may help in staging the various phases of the endometrium (prereceptive, receptive, and postreceptive) and, more importantly, could lead to the development of targeted drug treatment for implantation-based infertility.[7] More importantly, in addition to the identification of differentially expressed genes (DEGs), identifying the enriched pathways is crucial for uncovering molecular processes altered in RIF and understanding the aberrant mechanisms underlying the disease.

In the current study, global gene profiling of the endometrium during the WOI was performed in patients with RIF using microarray technology to identify altered biologic processes in the endometrium that might be potential markers of receptive endometrium, and the results were compared with fertile controls.

Materials and Methods

Study Design, Size, and Duration

Twenty-four patients with recurrent implantation failure treated at the IVF clinic and 24 fertile control patients recruited from the gynecology clinic of Istanbul University School of Medicine during 2014 to 2015 were involved in this prospective cohort study. Repeated implantation failure was determined as failure of pregnancy in $\geq$3 consecutive IVF cycles with $\geq$1 transfer(s) of good quality embryo in each cycle. The exclusion criteria for this group were active pelvic infections, undiagnosed vaginal bleeding, uterine anomalies, endometriosis, and karyotype anomalies in one or both partners. The fertile control patients had a history of at least 1 live birth with no associated comorbidities. They were selected among patients who presented to our gynecology clinic for well woman examinations. Patients with any other gynecologic disorders or under any medications were excluded.

Messenger RNA fractions were extracted from 48 endometrial biopsies obtained using a Pipelle endometrial suction curette (Laboratoire CCD, Paris, France) during the WOI (LH + 7 to LH + 10) and between 12:00 to 15:00 at the gynecology and infertility clinics of Istanbul University School of Medicine by a single clinician (I.D.). Samples were put in RNAlater solution (Thermo Fisher Scientific, Waltham, Massachusetts) and kept in a 4°C refrigerator for 1 night and then transferred to liquid nitrogen in a −80°C refrigerator the following day. Once all the samples were obtained, they were transferred in liquid nitrogen to the Department of Molecular Biology and Genetics for analysis. The study was approved by the ethics committee of Istanbul University School of Medicine (Istanbul, Turkey).

RNA Isolation and Microarray Hybridization

Total RNA was extracted from the endometrium tissues samples, as 24 patients with RIF and 24 controls, under cold chain conditions using a QIAGEN RNeasy Plus Mini Isolation Kit (Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of obtained total RNA were first measured using a NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA) and secondly with an Agilent RNA Nano Chip kit on Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, California). High-quality (RNA integrity up to 8) isolated total RNA samples were selected for microarray analysis. Sample preparation and hybridization were adapted from the Agilent technical manual (1 color). In short, first-strand complementary DNAs were transcribed from 100 ng total RNA with the use of T7-Oligo (dT) promoter primers. Samples were transcribed in vitro and Cy-3 labeled, all with a Low Input Quick Amp Labeling kit (Agilent Technologies,Santa Clara, CA, USA). The labeling reaction typically yielded 120 to 200 ng/μL of complementary RNA (cRNA) with a specific activity >10. Fragmented cRNA samples were hybridized on an Agilent Sure Print G3 8×60 K Version 2 Microarray slide by incubation at 65°C for 17 hours with constant rotation. Six Agilent Sure Print G3 8×60 K Version 2 Microarray slides were used for 48 samples. Each slide contained 8 identical arrays containing 50 599 biologic probes, representing over 24 500 RefSeq genes and over 10 000 lincRNAs. The microarray slides were then washed in 2 steps of 1 minute in 2 washing buffers (Agilent Technologies). In order to enhance reproducibility across the different chemical surfaces used, a reference sample was spotted in duplicate on each type of surface to calculate experimental intra- and inter-coefficient of variation and to optimize the array reading parameters (such as laser intensity, focus mass, and mass range). The reference sample was taken from a pool (5 μL) of tissue samples (500 μL per patient) from 5 women with RIF and from 5 controls without RIF selected at random. The slides were scanned using an Agilent SureScan Microarray Scanner (model G2600D) and hybridization signals were extracted using the Agilent Feature Extraction software, version 11.0.1.1.

Microarray Differential Expression Analysis

The data obtained from Agilent Feature Extraction software were analyzed with the R package limma.[8] Quality assessment of raw and processed data was performed using the R package

array QualityMetrics.[9] After median intensity values were read into R, the data were background-corrected using the normexp method. The background-corrected data were then normalized through quantile normalization. After removing the control probes and averaging over replicate probes, the preprocessed data were used for differential expression and pathway enrichment analyses.

The data were first used for differential expression analysis.[10] The MA plot (Supplemental Figure 1) and the locally-weighted scatterplot smoothing fitted curve showed no bias toward any of the 2 groups in the comparison. The Benjamini-Hochberg method was used to obtain adjusted P values. The differentially expressed probes that met the criteria $|\log_2(\text{Fold Change})| \geq 1$ and adjusted P value $\leq .05$ were accepted as significant.

Next, the probes were mapped to their corresponding HGNC gene symbols using the GEO annotation GPL17077. There were probes with missing gene symbols. Thus, some of the missing gene symbols were annotated using the chromosomal locations via the R package biomaRt.[11]

The list of DEGs (Supplemental Table 1) was established using the set of genes targeted by the differentially expressed probes. The adjusted P values for these genes were used in the enrichment analyses. In cases where multiple probes targeted the same gene, adjusted P values corresponding to the probe with the lowest adjusted P value were used. To prevent false-positive DEGs, we applied a strict filter ($|\log_2 FC| \geq 1$ and adjusted $P \leq .05$). Furthermore, we focused on pathways identified using subnetworks, only keeping DEGs that were in strong interactions with other DEGs. This resulted in further elimination of false-positive DEGs.

Pathway Enrichment Analysis

Pathway enrichment analysis of the DEGs was conducted using the functional enrichment tool PANOGA.[12] PANOGA incorporates protein–protein interaction (PPI) information while extracting significant pathways. It helps to identify disease-related genes and functionally relevant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways through the identification of genes within the pathways. Kyoto Encyclopedia of Genes and Genomes is a database collection obtained from genomes, metagenomes, metabolomes, personal genomes, and pathogen genomes.[13] Kyoto Encyclopedia of Genes and Genomes pathways are grouped under 7 main categories: metabolism, genetic information processing, environmental information processing, cellular process, organismal systems, human diseases, and drug development.

The PANOGA procedure can be summarized as follows: DEGs were first mapped onto a PPI network. In this study, Goh et al's human PPI network was used.[14] Next, active subnetworks containing the majority of the disease-affected genes in the human PPI network were investigated. The active modules algorithm was employed to identify the subnetworks taking into account the P values of each DEG to extract active subnetworks that overlapped at most 50% with each other.[15]

Following the identification of subnetworks, their biologic importance was evaluated. For each subnetwork, the number of genes that were found in a specific KEGG pathway was calculated and compared with the overall number of genes described for that pathway. For this functional enrichment step, a 2-sided hypergeometric test was used. Bonferroni correction was used for multiple testing of P values. If a KEGG pathway was determined to be significantly enriched (Bonferroni-adjusted $P < .05$) for at least one of the active sub-networks, this pathway was added to the final list of significant KEGG pathways associated with the disease. If a pathway was found enriched in more than 1 subnetwork, only the most significant one (ie, the one with the lowest Bonferroni-adjusted P value) was reported.

PANOGA was run 20 times, the lowest Bonferroni-adjusted P value (here on, referred to as P value) over these 20 iterations was reported. Finally, the enriched pathways were ranked per their respective P values.

Clustering of Enriched Pathways

In order to establish representative pathways among highly similar groups of enriched pathways, clustering was performed as described previously by Chen et al.[16] In brief, an overlap index matrix (OI) containing overlap indices between all pairs of pathways was calculated. For each pathway P_i in the data set, let G_i be the set of all genes in P_i. For a pair of pathways P_i and P_j, $OI_{i,j}$ was defined as below:

$$OI_{i,j} = \frac{|G_i \cap G_j|}{\min(|G_i|, |G_j|)}$$

Afterward, defining each row o_i of the matrix OI as the gene overlap profile of pathway P_i, the Pearson correlation coefficient R was calculated for each pair of o_i and o_j. This was then transformed into pairwise distances (PD) as $\text{PD} = 1 - \text{R}$. Lastly, using this distance metric, pathways were clustered via average-linkage hierarchical clustering. From the 715 differentially expressed probes, 641 DEGs were identified and 44 KEGG pathways were found affected. Overlap between pathways is very common; therefore, for a better and cleaner understanding of the affected pathways, clustering was performed. The dendrogram was partitioned into clusters at the manually selected cutoff value of 0.72. This cutoff value was chosen in order to group biologically similar pathways together while obtaining distinct pathways best reflecting the mechanisms underlying RIF. Examining clusters of related pathways, the dendrogram was partitioned at the manually selected PD cutoff value of 0.72. The representative pathway for each cluster was decided as the one with the lowest P value, as determined via PANOGA.

Results

Demographic data and hormonal levels of RIF patients and fertile controls are presented in Table 1, and there is no statistical difference between the 2 groups ($P > .05$). In the differential expression analysis, 715 differentially expressed probes

Table 1. Demographics and Hormone Levels of RIF Patients and Fertile Controls.

	Fertile Controls (n = 24)	RIF Patients (n = 24)	*P* Value
Age, years	31.13 ± 3.860	32.76 ± 2.33	NS
BMI, kg/m^2	23.15 ± 1.65	25.36 ± 1.43	NS
Parity	1.77 ± 0.45	None	<.05
Day 3 FSH, mIU/mL	7.55 ± 1.67	6.52 ± 3.18	NS
Day 3 E2, mIU/mL	61.86 ± 25.56	56.41 ± 65.10	NS
Day 3 prolactin, ng/mL	14.69 ± 5.66	12.45 ± 4.17	NS
Day 3 TSH, μIU/mL	2.16 ± 1.42	2.28 ± 1.04	NS

Abbreviations: BMI, body mass index; RIF, repeated implantation failure; NS, nonsignificant.

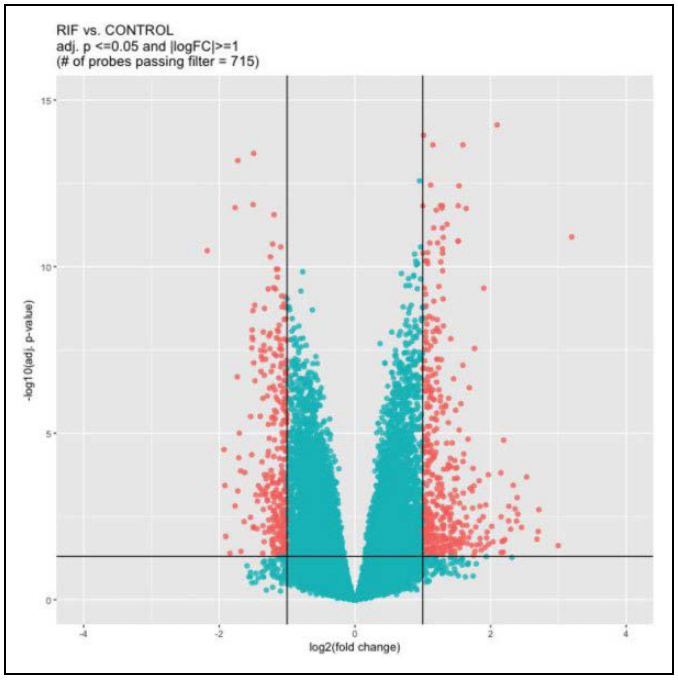

Figure 1. Volcano plot of the probes, repeated implantation failure (RIF) versus control. The horizontal axis represents the $\log_2$ fold change of the probes, and the vertical axis represents the $-\log_{10}$-transformed adjusted *P* values of the individual probes. The horizontal black line represents the adjusted *P* value cutoff value of .05, while the 2 vertical black lines represent the $\log_2$ fold change absolute value cutoff value of 1. The red points represent the statistically significant probes, while the blue ones are not deemed significant.

that met the abovementioned criteria were identified. Figure 1 represents the differential expression data in the form of a Volcano plot. These differentially expressed probes corresponded to 641 DEGs (Supplemental Table 1).

In the top 25 DEGs presented in Table 2, it is observed that 21 out of the top 25 DEGs were upregulated (PHF8, MARK2, SEPT9, RBM47, FAM21C, SMG5, LATS1, FAM107B, PAPOLA, ROCK2, HOOK3, MLL5, NCOA4, MAP4, CIZ1, AGAP1, ACTB, MOB1A, USP33, TOP1, KIAA1429) and 4 out of the top 25 DEGs (XLOC_l2_015397, LOC100130557, EEF1A1, C1orf229) were downregulated in patients with RIF in comparison with fertile control patients.

In the pathway enrichment analysis, 44 KEGG pathways were found enriched via PANOGA. Supplemental Figure 2 shows a bubble chart of these 44 enriched KEGG pathways, grouped by KEGG subcategories. The top 10 significantly enriched pathways were pathways in cancer, proteasome, ErbB signaling pathway, complement and coagulation cascades, citrate cycle (TCA cycle), adherens junction, transforming growth factor β signaling pathway, chemokine signaling pathway, proteoglycans in cancer, and colorectal cancer.

As observed in Supplemental Figure 2, some of these 44 enriched KEGG pathways belonged to the same KEGG subcategories, such as the signal transduction pathways, the immune system pathways, and the cancer-specific type pathways. As implied by this grouping of pathways by KEGG subcategories, many of the enriched pathways were biologically related to each other. Therefore, hierarchical clustering of the enriched pathways via the approach described above was used. To obtain biologically coherent clusters, the dendrogram was manually inspected and the height at which the tree should be partitioned was determined on an ad hoc basis. The representative pathways for each cluster were determined as those with the lowest *P* values within each cluster. Figure 2 illustrates the cluster dendrogram, cut at the PD value of 0.72, resulting in 12 clusters. Table 3 lists the 9 representative pathways for these clusters along with the associated DEGs. The representative pathways were pathways in cancer, proteasome, complement and coagulation cascades, TCA cycle, adherens junction, circadian rhythm, immune system and inflammation, cell cycle and renin–angiotensin system (RAS).

Discussion

In the present study, the aim was to discover whether RIF patients have a different gene profile during the WOI and subsequently explain the different gene expression patterns compared to fertile women. In other words, we aimed to uncover different mechanisms that play a role in the pathogenesis of RIF. Via enrichment analysis of the DEGs, circadian rhythm, pathways in cancer, proteasome, complement and coagulation cascades, TCA, adherens junction, immune system and inflammation, cell cycle, and RAS pathways were found to be significantly dysregulated. In addition to previously reported mechanisms implicated in the pathogenesis of RIF, we uncovered novel potential mechanisms including circadian rhythm, proteasome, TCA cycle, and adherens junction pathways. These may potentially be mechanisms that induce the nonreceptive endometrium in RIF.

The transcriptomic era in the endometrium and its receptivity started in 2002. The first publication regarding transcriptomics in the endometrium was by Carson et al, who compared the early luteal phase with the mid-luteal phase in samples taken from fertile patients.[17] The significance of DEGs is better understood when affected pathways are revealed. It should be emphasized that genes, through up- or downregulation,

Table 2. The Top 25 Most Significantly Differentially Expressed Genes.

Probe ID	Gene Symbol	Description	Fold Change	Adjusted *P* Value	Pathway(s)
A_24_P130865	PHF8	PHD finger protein 8	9.17	1.29E-11	
A_24_P914495	MARK2	MAP/microtubule affinity-regulating kinase 2	4.27	5.60E-15	
A_33_P3368188	SEPT9	Septin 9	3.12	1.80E-12	Bacterial invasion of epithelial cells
A_24_P226108	RBM47	RNA binding motif protein 47	3.02	2.21E-14	
A_33_P3256894	FAM21C	Family with sequence similarity 21, member C	2.88	1.70E-11	
A_33_P3326349	SMG5	smg-5 homolog, nonsense-mediated mRNA decay factor (*C. elegans*)	2.87	1.48E-12	
A_23_P366230	LATS1	LATS, large tumor suppressor, homolog 1 (drosophila)	2.87	1.72E-11	Hippo signaling pathway
A_33_P3288942	FAM107B	Family with sequence similarity 107, member B	2.57	5.34E-12	
A_23_P376239	PAPOLA	Poly(A) polymerase α	2.47	1.33E-11	
A_33_P3257140	ROCK2	Rho-associated, coiled-coil containing protein kinase 2	2.44	1.48E-12	Pathways in cancer, chemokine signaling pathway, proteoglycans in cancer, focal adhesion, pathogenic *Escherichia coli* infection, shigellosis, salmonella infection
A_23_P216080	HOOK3	hook homolog 3 (Drosophila)	2.44	6.91E-12	
A_33_P3277407	MLL5	Myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, *Drosophila*)	2.43	1.78E-12	
A_33_P3319463	NCOA4	Nuclear receptor coactivator 4	2.4	1.47E-12	Pathways in cancer, thyroid cancer
A_33_P3346573	MAP4	Microtubule-associated protein 4	2.33	1.96E-11	
A_33_P3247237	CIZ1	CDKN1A interacting zinc finger protein 1	2.29	2.03E-12	
A_33_P3317305	AGAP1	ArfGAP with GTPase domain, ankyrin repeat, and PH domain 1	2.24	6.87E-12	
A_23_P135769	ACTB	β-actin	2.22	2.21E-14	Adherens junction, proteoglycans in cancer, bacterial invasion of epithelial cells, focal adhesion, Hippo signaling pathway, apoptosis, pathogenic *E coli* infection, shigellosis, salmonella infection
A_23_P108922	MOB1A	MOB kinase activator 1A	2.17	3.57E-13	Hippo signaling pathway
A_24_P405190	USP33	Ubiquitin-specific peptidase 33	2.15	1.96E-11	
A_33_P3371493	TOP1	Topoisomerase (DNA) 1	2.02	1.14E-14	
A_23_P215980	KIAA1429	KIAA1429	2	1.48E-12	
A_19_P00807615	XLOC_l2_015397	Noncoding RNA	−2.29	2.75E-12	
A_24_P315500	LOC100130557	Uncharacterized LOC100130557	−2.32	2.12E-11	
A_19_P00808320	EEF1A1	Eukaryotic translation elongation factor 1 α 1	−2.81	3.97E-14	
A_24_P831309	C1orf229	Chromosome 1 open reading frame 229	−2.83	1.37E-12	

ultimately alter pathway functioning. We discovered novel pathways that were shown to have a potential role in the pathogenesis of RIF. Representative pathways were significantly different compared with the control group, which clearly demonstrates the multifaceted and interconnected mechanisms underlying RIF (Table 3). We deliberately chose to interpret the pathway findings instead of genes. The top 25 genes give an idea on RIF, whereas interpretation of single genes is difficult

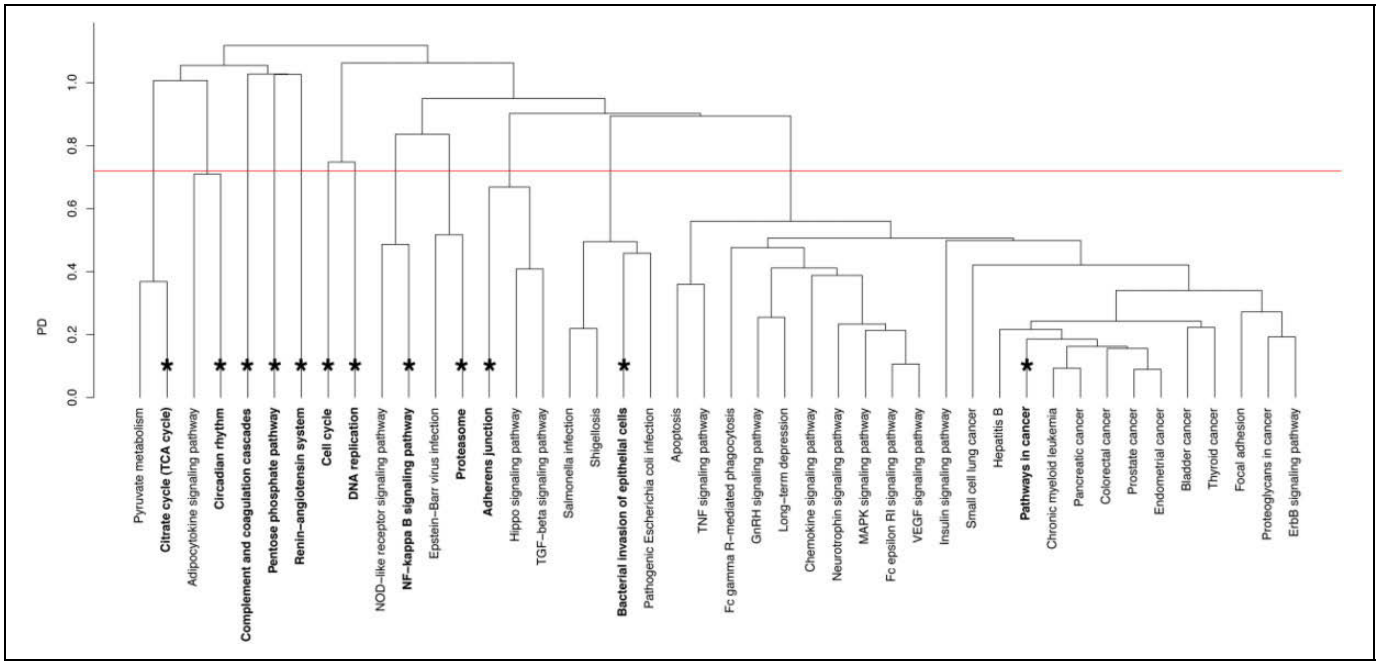

Figure 2. Clustering dendrogram of enriched pathways. The distance measure is pairwise distance (PD) calculated as described in the methods section. The dendrogram is cut at the height of 0.72, resulting in 12 clusters that are biologically relevant. All 44 KEGG pathways enriched via PANOGA form the base of the dendrogram. Pathways relevant to each other are grouped in each cluster. One pathway, per the lowest *P* value, was chosen to be the representative pathway in each cluster. Hence, from 44 KEGG pathways enriched via PANOGA, 12 representative pathways were obtained.

Table 3. Representative Pathways for the Abovementioned Clusters Along With Lists of the Involved Upregulated and Downregulated DEGs.[a]

KEGGID	Pathways	*P* Value	Up-/ Downregulated[b]	Unchanged[c]	Upregulated	Downregulated
hsa05200	Pathways in cancer	6.95E-16	13/4	378	CBLC, AXIN2, NCOA4, RALBP1, TCF7L1, MECOM, JUP, PTGS2, BIRC3, DAPK2, LPAR3, ROCK2, PLCB1	BDKRB1, EDNRB, GNG4, CXCL12
hsa03050	Proteasome	1.77E-14	0/1	43		PSMD14
hsa04610	Complement and coagulation cascades	2.16E-12	1/2	76	SERPINA5	F13A1, BDKRB1
hsa00020	Citrate cycle (TCA cycle)	1.23E-10	0/1	29		SDHC
hsa04520	Adherens junction	2.70E-10	4/1	67	TCF7L1, WASF1, BAIAP2, ACTB	SORBS1
hsa04710	Circadian rhythm	1.17E-08	1/1	29	PRKAG2	PRKAG1
hsa04064	Immune system and inflammation	1.33E-07	3/2	90	BIRC3, TNFRSF13C, PTGS2	CXCL12, VCAM1
hsa04110	Cell cycle	2.18E-07	3/1	120	ANAPC1, CDC14B, CDC14A	CDC25B
hsa04614	Renin/angiotensin system	1.49E-06	1/1	21	MME	AGT

[a]The pathways are ordered by *P* values. *P* value corresponds to the *P* values associated with the given pathway. Upregulated and downregulated list the up- and downregulated differentially expressed genes (DEGs) in the given pathway, respectively.
[b]Upregulated and downregulated indicates the number of upregulated and downregulated DEGs, respectively.
[c]Unchanged corresponds to the number of neither up- nor downregulated genes in the given pathway.

and obscure in meaning. Below, the role of these clinically relevant representative pathways in the pathogenesis of RIF is discussed.

Circadian Rhythm

This pathway controls several key cellular processes and thus seems to be a crucial regulator of the pathogenesis of RIF.

Previous studies suggested that normal circadian rhythmicity was important for the immune system[18] and supported reproductive function.[19,20] An important role of Circadian rhythm in the pathogenesis of RIF might be its regulation of the immune system. This hypothesis is supported by Yu et al's study.[18] It was shown that the transcription factor NFIL3, which plays a role in the regulation of circadian rhythm, suppresses T-helper (Th) cells that produce interleukin (IL) 17. Disruption of the immune system and inflammation appears to be a key component in the pathogenesis of RIF.

In addition to the well-established role of the circadian clock in the hypothalamus and pituitary gland, there is also strong evidence that the uterus also expresses clock genes rhythmically.[21] In our study, the gene CLOCK, which plays a central role in the regulation of circadian rhythms, was found downregulated. It can be concluded that the circadian rhythmicity of the endometrium in patients with RIF is slowed down and that this leads to the disruption of several cellular processes, including cell metabolism, immune and inflammatory responses, hypoxia/hyperoxia response pathways, endoplasmic reticular stress, and autophagy, which might be crucial for implantation.

A potential treatment option arises considering the involvement of circadian rhythm in the pathogenesis of RIF: melatonin, a pineal hormone that regulates circadian rhythms. Some important studies investigated melatonin expression levels related to implantation rates.[19,20] In addition, clinical studies suggested that melatonin supplementation in IVF might lead to better pregnancy rates by affecting implantation processes.[20,22] These findings imply that melatonin treatment may improve implantation in patients with RIF. Circadian rhythm is also a noteworthy pathway because it is the therapeutic target of melatonin, which may potentially improve implantation in patients with RIF.

Pathways in Cancer

Pathways in cancer contains pathways involved in cancer processes such as cell signaling, proliferation, angiogenesis, and adhesion. Cancer and pregnancy share similarities. They are both perceived as "nonself" by the host, they both cause inflammation, and they both exhibit invasions despite the normal host reaction.[23] Tolerance to "nonself" products is warranted for a pregnancy, whereas it is feared in cancer. There are some genes that are differently expressed compared with fertile controls.

The inflammation caused by early pregnancy and tumor cells may act as a stimulator for angiogenesis. Some molecules, such as TGF-β, transform immune system cells into supporters, which reinforce angiogenesis.[24,25] One may conclude that the immunologic reaction toward a tumor or a blastocyst can be transformed to an angiogenetic impulse.

CBLC, one of the upregulated genes, plays a role in the epidermal growth factor receptor 1 (EGFR1) signaling pathway, which induces growth, differentiation, migration, adhesion, and cell survival through various interacting signaling pathways.[26] The EGFR1 signaling pathway is in the cluster represented by pathways in cancer.

AXIN2 and transcription factor 7-like 1 are other upregulated and cancer-related genes in patients with RIF. Gambadauro and Gudmundsson hypothesized that a relation might exist between endometrial cancer and RIF.[27] In another publication, it was shown that thin endometrium, which is one of the etiologies in RIF, was associated with dysregulation of 10 cancer-related genes, consistent with the current findings.[28]

Our results showed that pathways in cancer was significantly altered in patients with RIF compared with the fertile control group. This supports the idea that the necessary inflammatory response needed in an implantation may be lacking; therefore, the blastocyst (if otherwise capable of implantation) may be digested by immune cells.

Proteasome

The proteasome pathway is another significantly altered pathway in patients with RIF compared with fertile participants. This pathway is important for implantation. Inhibition of proteasome was shown to significantly inhibit mouse blastocyst implantation.[29] Moreover, Manohar et al investigated proteasome subunit α type-5 and found that it was significantly downregulated in mid-secretory phase endometrium of infertile women as compared with early secretory phase endometrium of infertile women and suggested that it might be one of the pathogenic causes of unexplained infertility.[30] The DEG involved in the proteasome pathway is downregulated in patients with RIF and may have a role in the pathogenesis of RIF.

Complement and Coagulation Cascades

The complement system is active throughout the menstrual cycle with increased expression during the secretory phase.[31,32] Present at the embryo implantation site, some complement system molecules influence the communication between the endometrium and embryo.[33] For instance, the complement protein C1q is found to have an important role in the endometrium, starting from the implantation process to playing as a contributing factor in the etiology of preeclampsiaç.[34,35] Savaris et al revealed the complement and coagulation cascades pathway as one of the most significantly enriched pathways in pregnant endometria.[36]

An active complement system, in addition to protecting the endometrium by securing epithelial integrity, raises the concern that a misdirected reaction toward a blastocyst could happen when the embryo is perceived as foreign.[37] Therefore, many complement regulatory proteins such as C4BPA, membrane cofactor protein (MCP), DAF, and CD59 control a very sensitive balance in the endometrium at a microcellular level.[37-39] Importantly, *CD46*, the gene that encodes MCP, was found to be significantly upregulated in this study ($\log_2$FC = 0.58, adj. $P < .001$). This implies that complement regulation is also altered.

Tapia et al combined data sets reported in studies that were performed in normal cycling women, in which microarray analysis for endometrial receptivity was used.[40] They then developed a consensus endometrial receptivity transcript list with 40 up- and 21 downregulated transcripts. In parallel to our findings, they observed that these genes were mostly involved in immune response, complement activation, and cell cycle regulation.[40] Our results demonstrate that the complement and coagulation cascades pathway was altered with 1 upregulated and 2 downregulated genes. This pathway in the etiology of RIF is also supported by several other studies.[41-43] This pathway has the major function of regulating the immune environment in the endometrium. All foreign molecules are processed in this multistep program. Exaggerated response is controlled via multiple mechanisms. It might be hypothesized that a misdirected reaction toward an embryo perceived as nonself could lead to implantation failure. This hypothesis may help to develop therapies targeting immunologic pioneers.

Citrate Cycle

In the glycolysis reaction, glucose is metabolized through pyruvate, which then enters the tri-carboxylic acid (TCA) cycle and is oxidized to produce ATP.[44] The activity of aerobic respiration in oogenesis, fertilization, and preimplantation development could be evidence of the use of aerobic respiration during these processes.[44,45] The cluster that is represented by TCA cycle also contains the pathway pyruvate metabolism. Together, these enriched pathways imply that carbon metabolism is altered in the endometrium of patients with RIF. Dysregulation of this pathway may cause energy depletion. Without sufficient energy, blastocyst implantation and progress to healthy pregnancy may be affected negatively.

Adherens Junction

Adherens junction is a hallmark of epithelial cells.[46] Regulation of cell shape, maintenance of tissue integrity, and translation of the forces arising from actomyosin throughout the tissue can be highlighted as the main functions of adherens junction.[47]

Thie et al observed atypical adherens junction in a human uterine epithelial cell line that showed apical adhesiveness, thus were receptive trophoblast implantation.[48] This disrupted adherens junction was thought to be correlated with a lack of polar organization. Subsequently, they reinforced their findings by showing rudimentary adherens junction, lack of tight junction, and nonpolar organization of the actin cytoskeleton in this receptive cell line.[49] Buck et al observed that the subapical location of adherens junction was redistributed along the entire lateral membrane at the onset of the WOI.[50] In a subsequent study, they reported increased invasiveness in cells showing this redistribution pattern.[51] Koler et al, using microarray analysis, reported an altered adherens junction pathway in RIF.[52] Revel et al showed that the microRNA miR145 was upregulated in RIF.[53] In our study, 5 DEGs in the adherens junction pathway were identified: 4 up- and 1 downregulated. It could be hypothesized that a too "sticky" endometrium might behave like a barrier to an embryo willing to implant. All of the aforementioned studies, in addition to our study's results, support the idea of an altered adherens junction pathway in the endometrium leading to refractory behavior to an upcoming blastocyst implantation.

Involvement of the Innate Immune System and Inflammation (NF-κB Signaling Pathway)

Innate immunity has a major representation at the maternal–fetal interface, most immune cells (70%) being CD56 + natural killer (NK) cells followed by 20% of monocytes.[54] Diminution of immune cells, instead of helping the pregnancy, terminates the pregnancy. Studies showed that NK cells were critical for trophoblast invasion in the uterus and diminution of its level prevented blastocyst implantation and decidual formation. Natural killer cells in human decidua have a role in regulating trophoblast invasion by the production of IL-8 and interferon-inducible protein-10 chemokines.[55] The early implantation stage is characterized by high levels of proinflammatory cytokines (IL-6, IL-8, and TNF-α) and Th-1 activity. These cytokines can be secreted by the cells of the immune system, as well as the endometrial cells that are recruited to the site of implantation.[56,57]

The representative pathways NF-κB signaling pathway as well as the pathways TNF signaling pathway, chemokine signaling pathway, and complement and coagulation cascades all reflect aberrant activity of the immune system in the endometria of patients with RIF compared with fertile controls. It might be concluded that this altered immune and inflammatory response in RIF disrupts the implantation process. Investigating and treating this immune system dysregulation with immunotherapy may help embryo implantation and continuation of pregnancy.

Cell Cycle

As three quarters of the DEGs in the cell cycle pathway are upregulated, the dysregulation of this pathway implies activation of proliferation and inhibition of apoptosis. Importantly, it was reported that cell cycle regulation played an important role in uterine stromal cell decidualization in implantation.[58] In addition, Koler et al demonstrated that many cell cycle genes had altered expression levels in patients with RIF.[52] The expression of a number of cell cycle genes in secretory phase endometrium has also been found to be dysregulated.[59,60] These findings all underline the importance of the cell cycle pathway in the etiology of RIF. In our study, we found a significantly altered cell cycle pathway in the RIF group. Treatment alternatives such as melatonin, increasing progesterone dosage, or steroid replacement may regulate expression in secretory phase. After regulation, in this nonreceptive phase of endometrium, embryo implantation may be enhanced by these treatments.

Renin–Angiotensin System

The RAS is known to be an important regulator of blood pressure and sodium and fluid homeostasis. Angiotensinogen, renin, angiotensin converting enzyme, Ang I, Ang II, and the AT1 and AT2 receptors, which are RAS components, are found in the placenta, uterus (endometrium and myometrium), fetal membranes, and amniotic fluid.[61,62] It was shown that in pregnancy, there can be a direct role of angiotensin II in terms of embryo implantation and uterine blood flow.[63,64] This may lead to difficulty in embryo implantation. In our study, 2 dysregulated RAS genes were found as potential factors contributing to RIF compared with fertile controls: MME, which was upregulated, and AGT which was downregulated. Previous studies also support the idea of an important role of RAS in the pathogenesis of RIF.

Comparison With Previous Publications

The findings of our study were compared with other RIF endometrial microarray studies dating back to 2002 when the first publication by Carson et al on microarray analysis in the endometrium was released.[17] The observation that alterations of different sets of genes give rise to the same phenotype suggests that the underlying pathways could be, for the most part, the same. Therefore, pathways shared with previous publications were investigated. Supplemental Table 2 lists the pathways identified in this study that were also identified in other studies, along with the involved DEGs in our study. The pathways that were found to be common emphasize the importance and effectiveness of a pathway-based approach in identifying the underlying causes.

The most recent transcriptomics study in RIF by Koot et al demonstrated a gene expression signature containing 303 genes accurately predicted RIF.[63] The authors also performed functional analysis using Gene Set Enrichment Analysis (GSEA) of the expression profiles using Gene Ontology (GO) slim categories. The GSEA approach uses only the list of genes provided to perform enrichment statistics. However, the approach we used, PANOGA, initially finds interactions of the provided genes and then performs enrichment analysis on the distinct sets of interacting genes. Making full use of the interaction information enabled us to identify novel mechanisms in addition to the previously determined mechanisms. Additionally, we used KEGG pathways rather than GO terms because we believe that curated pathway information is more reliable and easier to interpret in conjunction with the literature. Further to the abovementioned advantages of performing enrichment analysis after active subnetwork identification instead of performing enrichment analysis directly on the list of DEGs, another strong aspect of our approach was to cluster the enriched pathways. Clustering allowed grouping of similar pathways with a large number of common genes and prevented us from overemphasizing them. More importantly, clustering reduced the complexity of analysis and enabled the identification of processes that participated in RIF formation.

Strength and Limitations

The main strength of our study lies in the analysis of enriched pathways and the identification of multiple interconnected processes that contribute to the pathogenesis of RIF. We did not limit our view by only looking for a single gene or group of genes responsible for implantation failure. Elucidating pathways that are interrupted in this disease provided a more accurate understanding of implantation failure because different genes may alter one pathway at various points. In other words, implantation failure could be explained better by aberrations in specific pathways, but not specific genes. We believe this is why we could not find a consistent set of genes among published studies.

Through strict filtering of the differential expression analysis results, we obtained DEGs that changed by 2-fold overall in the RIF group compared with the fertile control group. That is, the mean expression level of any DEG in the RIF group was at least double the mean expression level in the control group. Furthermore, before pathway enrichment analysis, we identified active subnetworks, thereby ensuring that the subnetwork consisted of genes that had extensive interactions with the DEGs. Finally, to obtain biologically coherent concise mechanisms, we clustered the enriched pathways and identified representative mechanisms. The resulting representative pathways were therefore highly unlikely to have occurred by chance.

There were a few limitations to this study. First, the DEGs were not validated with qPCR analysis or immunohistochemical staining. The emphasis was instead put on significantly altered pathways because it is widely accepted that molecular mechanisms, that is, pathways, have more relevance to the etiology of a disease than sets of DEGs.

Secondly, endometrial samples were taken in unstimulated cycles. Therefore, the samples do not mimic the state of the endometrium of patients with RIF undergoing stimulation. This is a problem because ovarian stimulation is known to affect the endometrial gene expression pattern.[65] However, the purpose of this study was to discover pathways that interfered with recurrent implantation failure without the extra burden of ovarian stimulation on the endometrium.

Conclusion

In conclusion, our study aimed to provide an understanding of the mechanisms behind implantation failure encountered in patients who experience negative outcomes during IVF. Following differential expression analysis, pathway enrichment analysis was performed, and the enriched pathways were clustered to identify distinct processes.

With a comparison of 24 patients with RIF with 24 fertile controls, we found common mechanisms that should theoretically be independent of any cycle-to-cycle variability in gene expression levels in the endometrium. We propose that these dysregulated mechanisms shared by all patients might be fundamental to the pathogenesis of RIF.

More importantly, the approach of our study enabled the identification of novel mechanisms that provide novel insight into the formation of RIF, including disruption of circadian rhythm and altered immune system. Disruptions of these pathways turn the endometrium into a field where an optimal blastocyst cannot invade and subsequently start a pregnancy. We hope that our findings will provide a better understanding of the underlying molecular causes of RIF, which will ultimately give rise to better treatment opportunities.

Authors' Note

An earlier version of this research was presented at the American Society for Reproductive Medicine (ASRM) Annual Meeting (San Antonio, Texas, USA) in 2017.

Acknowledgment

The authors would like to thank David F. Chapman, BSc, for editing the language of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research received funding from the Scientific Research Projects Coordination Unit of Istanbul University (Grant no. 31161).

Supplemental Material

Supplemental material for this article is available online.

References

1. Simon A, Laufer N. Repeated implantation failure: clinical approach. *Fertil Steril*. 2012;97(5):1039-1043.
2. Brosens JJ, Salker MS, Teklenburg G, et al. Uterine selection of human embryos at implantation. *Sci Rep*. 2014;4:3894.
3. Weimar CH, Kavelaars A, Brosens JJ, et al. Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos. *Plos One*. 2012;7(7):e41424.
4. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, Rosenwaks Z. The window of embryo transfer and the efficiency of human conception in vitro. *Fertil Steril*. 1991;55(1):114-118.
5. Casper RF, Yanushpolsky EH. Optimal endometrial preparation for frozen embryo transfer cycles: window of implantation and progesterone support. *Fertil Steril*. 2016;105(4):867-872.
6. Horcajadas JA, Pellicer A, Simon C. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update*. 2007;13(1):77-86.
7. Diaz-Gimeno P, Ruiz-Alonso M, Blesa D, Simon C. Transcriptomics of the human endometrium. *Int J Dev Biol*. 2014;58(2-4):127-137.
8. Ritchie ME, Phipson B, Wu D, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
9. Kauffmann A, Gentleman R, Huber W. ArrayQualityMetrics—a bioconductor package for quality assessment of microarray data. *Bioinformatics*. 2009;25(3):415-416.
10. Phipson B, Lee S, Majewski IJ, Alexander WS, Smyth GK. Robust hyperparameter estimation protects against hypervariable genes and improves power to detect differential expression. *Ann Appl Stat*. 2016;10(2):946-963.
11. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat Protoc*. 2009;4(8):1184-1191.
12. Bakir-Gungor B, Egemen E, Sezerman OU. PANOGA: a web server for identification of SNP-targeted pathways from genome-wide association study data. *Bioinformatics*. 2014;30(9):1287-1289.
13. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases, and drugs. *Nucleic Acids Res*. 2017;45(D1):D353-D361.
14. Goh KI, Cusick ME, Valle D, Childs B, Vidal M, Barabasi AL. The human disease network. *Proc Natl Acad Sci U S A*. 2007;104(21):8685-8690.
15. Ideker T, Ozier O, Schwikowski B, Siegel AF. Discovering regulatory and signalling circuits in molecular interaction networks. *Bioinformatics*. 2002;18(suppl 1):S233-S240.
16. Chen YA, Tripathi LP, Dessailly BH, Nystrom-Persson J, Ahmad S, Mizuguchi K. Integrated pathway clusters with coherent biological themes for target prioritisation. *Plos One*. 2014;9(6):e99030.
17. Carson DD, Lagow E, Thathiah A, et al. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod*. 2002;8(9):871-879.
18. Yu X, Rollins D, Ruhn KA, et al. TH17 cell differentiation is regulated by the circadian clock. *Science*. 2013;342(6159):727-730.
19. Kim MK, Park EA, Kim HJ, et al. Does supplementation of in vitro culture medium with melatonin improve IVF outcome in PCOS? *Reprod Biomed Online*. 2013;26(1):22-29.
20. Rizzo P, Raffone E, Benedetto V. Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy outcome in IVF cycles. A prospective, clinical trial. *Eur Rev Med Pharmacol Sci*. 2010;14(6):555-561.
21. Kennaway DJ, Boden MJ, Varcoe TJ. Circadian rhythms and fertility. *Mol Cell Endocrinol*. 2012;349(1):56-61.
22. Fernando S, Rombauts L. Melatonin: shedding light on infertility?—A review of the recent literature. *J Ovarian Res*. 2014;7:98.
23. Holtan SG, Creedon DJ. Mother knows best: lessons from fetomaternal tolerance applied to cancer immunity. *Front Biosci (Schol Ed)*. 2011;3:1533-1540.
24. Keskin DB, Allan DS, Rybalov B, et al. TGFbeta promotes conversion of CD16+ peripheral blood NK cells into CD16-NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci U S A*. 2007;104(9):3378-3383.
25. Koopman LA, Kopcow HD, Rybalov B, et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med*. 2003;198(8):1201-1212.

26. Kandasamy K, Mohan SS, Raju R, et al. NetPath: a public resource of curated signal transduction pathways. *Genome Biol*. 2010;11(1):R3.
27. Gambadauro P, Gudmundsson J. Endometrial cancer in a woman undergoing hysteroscopy for recurrent IVF failure. *Gynecol Surg*. 2017;14(1):4.
28. Maekawa R, Taketani T, Mihara Y, et al. Thin endometrium transcriptome analysis reveals a potential mechanism of implantation failure. *Reprod Med Biol*. 2017;16(2):206-227.
29. Wang HM, Zhang X, Qian D, et al. Effect of ubiquitin-proteasome pathway on mouse blastocyst implantation and expression of matrix metalloproteinases-2 and -9. *Biol Reprod*. 2004;70(2):481-487.
30. Manohar M, Khan H, Sirohi VK, et al. Alteration in endometrial proteins during early- and mid-secretory phases of the cycle in women with unexplained infertility. *Plos One*. 2014;9(11): e111687.
31. Hasty LA, Lambris JD, Lessey BA, Pruksananonda K, Lyttle CR. Hormonal regulation of complement components and receptors throughout the menstrual cycle. *Am J Obstet Gynecol*. 1994;170(1 pt 1):168-175.
32. Nogawa Fonzar-Marana RR, Ferriani RA, Soares SG, Cavalcante-Neto FF, Teixeira JE, Barbosa JE. Expression of complement system regulatory molecules in the endometrium of normal ovulatory and hyperstimulated women correlate with menstrual cycle phase. *Fertil Steril*. 2006;86(3):758-761.
33. Bulla R, Bossi F, Tedesco F. The complement system at the embryo implantation site: friend or foe? *Front Immunol*. 2012; 3:55.
34. Agostinis C, Tedesco F, Bulla R. Alternative functions of the complement protein C1q at embryo implantation site. *J Reprod Immunol*. 2017;119:74-80.
35. Madhukaran SP, Alhamlan FS, Kale K, Vatish M, Madan T, Kishore U. Role of collectins and complement protein C1q in pregnancy and parturition. *Immunobiology*. 2016;221(11): 1273-1288.
36. Savaris RF, Hamilton AE, Lessey BA, Giudice LC. Endometrial gene expression in early pregnancy: lessons from human ectopic pregnancy. *Reprod Sci*. 2008;15(8):797-816.
37. Tapia-Pizarro A, Figueroa P, Brito J, Marin JC, Munroe DJ, Croxatto HB. Endometrial gene expression reveals compromised progesterone signaling in women refractory to embryo implantation. *Reprod Biol Endocrinol*. 2014;12:92.
38. Jensen TS, Bjorge L, Wollen AL, Ulstein M. Identification of the complement regulatory proteins CD46, CD55, and CD59 in human fallopian tube, endometrium, and cervical mucosa and secretion. *Am J Reprod Immunol*. 1995;34(1):1-9.
39. Lobo SC, Huang ST, Germeyer A, et al. The immune environment in human endometrium during the window of implantation. *Am J Reprod Immunol*. 2004;52(4):244-251.
40. Tapia A, Vilos C, Marin JC, Croxatto HB, Devoto L. Bioinformatic detection of E47, E2F1 and SREBP1 transcription factors as potential regulators of genes associated to acquisition of endometrial receptivity. *Reprod Biol Endocrinol*. 2011;9:14.
41. Huang J, Qin H, Yang Y, et al. A comparison of transcriptomic profiles in endometrium during window of implantation between women with unexplained recurrent implantation failure and recurrent miscarriage. *Reproduction*. 2017;153(6):749-758.
42. Shi C, Han HJ, Fan LJ, et al. Diverse endometrial mRNA signatures during the window of implantation in patients with repeated implantation failure. *Hum Fertil (Camb)*. 2017:1-12.
43. Tapia A, Gangi LM, Zegers-Hochschild F, et al. Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy. *Hum Reprod*. 2008;23(2): 340-351.
44. Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Mol Reprod Dev*. 2012;79(5): 311-320.
45. Wilding M, Coppola G, Dale B, Di Matteo L. Mitochondria and human preimplantation embryo development. *Reproduction*. 2009;137(4):619-624.
46. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta*. 2008;1778(3):660-669.
47. Niessen CM, Leckband D, Yap AS. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. *Physiol Rev*. 2011; 91(2):691-731.
48. Thie M, Fuchs P, Butz S, et al. Adhesiveness of the apical surface of uterine epithelial cells: the role of junctional complex integrity. *Eur J Cell Biol*. 1996;70(3):221-232.
49. Thie M, Denker HW. In vitro studies on endometrial adhesiveness for trophoblast: cellular dynamics in uterine epithelial cells. *Cells Tissues Organs*. 2002;172(3):237-252.
50. Buck VU, Windoffer R, Leube RE, Classen-Linke I. Redistribution of adhering junctions in human endometrial epithelial cells during the implantation window of the menstrual cycle. *Histochem Cell Biol*. 2012;137(6):777-790.
51. Buck VU, Gellersen B, Leube RE, Classen-Linke I. Interaction of human trophoblast cells with gland-like endometrial spheroids: a model system for trophoblast invasion. *Hum Reprod*. 2015;30(4): 906-916.
52. Koler M, Achache H, Tsafrir A, Smith Y, Revel A, Reich R. Disrupted gene pattern in patients with repeated in vitro fertilization (IVF) failure. *Hum Reprod*. 2009;24(10):2541-2548.
53. Revel A, Achache H, Stevens J, Smith Y, Reich R. MicroRNAs are associated with human embryo implantation defects. *Hum Reprod*. 2011;26(10):2830-2840.
54. Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol*. 2002;2(9):656-663.
55. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci*. 2011;1221:80-87.
56. Koga K, Mor G. Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy disorders. *Am J Reprod Immunol*. 2010;63(6):587-600.
57. Yoshinaga K. Review of factors essential for blastocyst implantation for their modulating effects on the maternal immune system. *Semin Cell Dev Biol*. 2008;19(2):161-169.

58. Das SK. Cell cycle regulatory control for uterine stromal cell decidualization in implantation. *Reproduction*. 2009;137(6): 889-899.
59. Kao LC, Germeyer A, Tulac S, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology*. 2003;144(7):2870-2881.
60. Koot YE, van Hooff SR, Boomsma CM, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. *Sci Rep*. 2016;6:19411.
61. Anton L, Brosnihan KB. Systemic and uteroplacental renin–angiotensin system in normal and pre-eclamptic pregnancies. *Ther Adv Cardiovasc Dis*. 2008;2(5):349-362.
62. Hagemann A, Nielsen AH, Poulsen K. The uteroplacental renin-angiotensin system: a review. *Exp Clin Endocrinol*. 1994;102(3): 252-261.
63. Botelho Lourenco EL, Muller JC, Boareto AC, et al. Effects of angiotensin-converting enzyme inhibitor derived from tropaeolum majus L. in rat preimplantation embryos: evidence for the dehydroepiandrosterone and estradiol role. *Evid Based Complement Alternat Med*. 2014;2014:209207.
64. Speth RC, Daubert DL, Grove KL. Angiotensin II: a reproductive hormone too? *Regul Pept*. 1999;79(1):25-40.
65. Martinez-Conejero JA, Simon C, Pellicer A, Horcajadas JA. Is ovarian stimulation detrimental to the endometrium? *Reprod Biomed Online*. 2007;15(1):45-50.