

Endometrial changes in estrogen and progesterone receptor expression during implantation in an oocyte donation program

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Abstract. Implantation is the final and most important stage of embryogenesis and is of paramount importance in achieving a successful pregnancy. Progesterone and estrogen are steroid hormones responsible for the regulation of the implantation window and the current study hypothesized that their receptors may be implicated in women undergoing oocyte donation. A total of 15 women aged 25-32 years old (mean \pm SD, 28.9 \pm 2.89) undergoing oocyte donation were recruited into the present study. Participants underwent ovarian stimulation with gonadotrophin-releasing hormone antagonist and recombinant follicle-stimulating hormone. Endometrial aspiration biopsy was performed on the day of oocyte retrieval and after 5 days (on days 0 and 5, respectively). Endometrial histology and evaluation of estrogen receptor (ER) α and progesterone receptor (PR)-B were performed on days 0 and 5. The ER nodal staining percentage on day 0 was age-associated, with patients aged <30 years demonstrating 100% staining and those aged >30 years exhibiting 90% staining. Pathological staining revealed statistically significant differences between days 0 and 5 following all staining procedures. Wilcoxon signed-rank test resulted in the following P-values, for ER (nodes % and stromal %) day 0/5, P=0.0001; for PR (nodes % and stromal %) day 0/5, P=0.0001 and P=0.035, respectively; for ER (grade nodes and stromal %) day 0/5, P=0.0001; and PR (grade nodes and stromal %) day 0/5 P=0.0001 and P=0.016, respectively. Synchronization between blastocyst development and the acquisition of endometrial receptivity is a prerequisite for the success of *in vitro* fertilisation (IVF). Aside from the recent discovery of molecules that are considered crucial for successful embryo implantation, assessing the functional

characteristics of the endometrium may offer unique insights into this process, thus improving IVF results.

Introduction

Implantation is defined as the organized process through which the blastocyst attaches to the endometrium and invades the epithelium to form the placenta. Implantation is directly dependent on the synchronization of the fertilized egg's progression into a blastocyst and the specific differentiation of the endometrium through molecular and cellular changes regulated by agents with an endocrine, paracrine or autocrine activity (1,2). This synchronization occurs over a certain period of time, called the 'window of implantation', and requires a molecular dialogue of sorts between the secretory activity of the endometrium and that of the blastocyst (1-3). Numerous studies have implicated adhesion molecules, extracellular matrix proteins, growth factors, extracellular substrate degradation proteins and pro-inflammatory agents in the implantation process of the blastocyst into the endometrium (4). Collectively these data point towards a complex molecular process, whose underlying mechanisms have not been fully elucidated.

Progesterone and estrogen are the steroid hormones responsible for the regulation of the implantation window. Progesterone induces changes in signalling pathways that lead to the establishment of a receptive endometrium (5). Estrogen receptors (ER) α and β and progesterone receptors (PR)-A and -B are expressed in the epithelium and stroma of the human endometrium. ER mediates most of the biological effect of estrogens by interacting with its site-specific DNA and with other coregulatory proteins, while ER and PR signalling during implantation is carried out through paracrine and autocrine factors mediated by growth factors, as well as cytokines (6). Progesterone exerts its effects by activating the canonical PRs to act in a genomic fashion to regulate transcriptional responses of implantation-related genes (5). For example, progesterone drives an increase in the gene expression of integrin α v β 3 in epithelial cells (7). Integrins are a family of transmembrane binding glycoproteins consisting of two protein subunits (α and β). Integrins function as receptors for extracellular matrix molecules, glycoproteins and other cells, and their concentration in adhesion points leads to the creation of a network of cytoskeletal proteins

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and intracellular signalling (8,9). The importance of these adhesion molecules has been widely studied in mice with blastocysts lacking the $\beta 1$ subunit that fail to implant (10,11).

Other adhesion molecules also play a crucial role in this dialogue underlying the adhesion and attachment of the blastocyst in the adequately prepared endometrium (12). E-cadherin, for example, is critical to the creation and maintenance of blastocyst adhesion ligands (12-14). Since E-cadherin has been found in the trophoblast and endometrium, it has been suggested to participate in the initial adhesion and attachment of the blastocyst during implantation (15).

On the other hand, estrogens exert their effects by activating primarily the nuclear steroid hormone receptor. ER α appears to be upregulated during the proliferative phase and downregulated during the implantation window, an event driven primarily by progesterone (16). Of note, elevated levels of ER α during implantation were associated with a decrease in $\beta 3$ integrin expression in patients with polycystic ovarian syndrome and endometriosis (17). It has been suggested that the disappearance of ER α at the time of implantation may disturb the expression pattern of proteins that regulate endometrial receptivity.

Despite the existence of a plethora of studies on the role of these steroid hormones and their receptors at the endometrial level, knowledge around their protein expression and tissue distribution during the implantation window (days 0-5) in humans remains limited. One of the main reasons for this lack of knowledge is that participants in such studies are required to undergo endometrial biopsy, which obviously affects endometrial receptivity and subsequently the success of *in vitro* fertilisation (IVF). Therefore, it is not feasible to carry out a study in women undergoing IVF, since the procedure will have an adverse effect on the outcome. The patient population of the present study consisted of women undergoing ovarian stimulation for egg donation.

In this study the following question was addressed: Can morphological and functional markers be used to evaluate the changes in the endometrium during implantation in women undergoing IVF?

In this study we investigated whether morphological and functional markers (i.e. ER and PR) can be used to evaluate the changes in the endometrium during implantation in women undergoing IVF.

Materials and methods

Study population and design. The study was conducted at the 1st Dept. of OB-GYN, Centre for Human Reproduction of the Aristotle University of Thessaloniki, 'Papageorgiou' General Hospital and the 'Biogenesis' Assisted Reproduction Centre, (both in Thessaloniki, Greece). The participants recruited for this prospective study included 15 oocyte donors (age range, 25-32 years; mean age, 28.9 \pm 2.89) undergoing IVF treatment. The inclusion criteria were white race, no uterine-ovarian pathology, age <35 years and no prior known medical pathology. All donors had undergone extensive preoperative work-up, which included common blood tests, karyotyping, specific test for cystic fibrosis and pap smear. All donors were non-smokers and had given their informed consent (NP: A 13032 15/7/10). Informed consent was written, and patients agreed to the use of their samples in scientific research.

Participants underwent ovarian stimulation with gonadotrophin-releasing hormone antagonist and recombinant follicle-stimulating hormone (18-20). Endometrial aspiration biopsy was performed using a Z-Sampler (Gynetics) on the day of oocyte retrieval and 5 days later (Fig. 1A).

Immunohistochemistry. Endometrial histology was evaluated blindly, using the Noyes criteria by a single specialized pathologist (20). The tissue was fixed in neutral buffered formalin 10% and followed the usual technical procedure for histological samples and embedding in paraffin blocks. Sections (3 μ m thickness) were sliced from the blocks placed on slides and then stained with hematoxylin and eosin for the evaluation of histologic characteristic. Immunohistochemistry was used to evaluate expression of ER α and PR-B, using monoclonal antibodies (ER: Clone 4f11, PR: clone 16+SAN27; Leica). Immunohistochemistry was performed on Ventana Benchmark XT automatic immunostainer (Ventana Medical Systems Inc.) using OptiView DAB IHC detection kit (Roche) as a detection method. As part of the automated service positive and negative controls (immunoglobulin G control) were tested simultaneously with the test slides (Fig. S1). The percentage of epithelial cell nuclei positive for ER α and PR-B receptors was recorded on days 0 and 5. Slide photos were captured using a x10-magnification lens on a Leica DMi1 Inverted Microscope (Leica Microsystems, Inc.), with all scale bars set to 0.25 mm.

Bioinformatic analysis. GeneMANIA (<http://genemania.org/>), a user-friendly web interface that provides large datasets to perform analyses for gene and protein interactions and prioritise genes for functional assays. Using this software, the relationship between ER and PR with 43 previously identified genes (21) involved with unexplained infertility (UIF) and recurrent implantation failure (RIF) were used to generate PPI networks using GeneMANIA.

Statistical analysis. Statistical analysis was performed using the SPSS version 23 (IBM Corp.). The data were non-parametric (Shapiro-Wilk normality test $P < 0.005$), and as such, the Wilcoxon signed-rank, Mann-Whitney U and Fisher's exact tests were used for analysis. A two-tailed $P < 0.05$ was considered to indicate a statistically significant difference. Sample stratification (for ER staining only) was performed for ages (± 30 years old) and to determine whether there was differential gene expression between the two days (yes, no).

Results

Differential expression of steroid hormone receptors during the implantation window. Both ER α and PR-B were expressed abundantly on both days (0 and 5; Fig. 1B). The ER α nodal staining percentage on day 0 was age-related, with patients aged <30 years showing 100% staining and those aged >30 years showing 90% staining (Mann-Whitney U test; $P = 0.014$; Fig. 2A).

Both steroid hormone receptors showed significant variation between days 0 and 5, both in the nodal and stromal preparations. According to Wilcoxon signed-rank test; for ER (nodes % and stromal %) Day 0/5, $P = 0.0001$; for PR (nodes % and stromal %) Day 0/5, $P = 0.0001$ and $P = 0.035$, respectively;

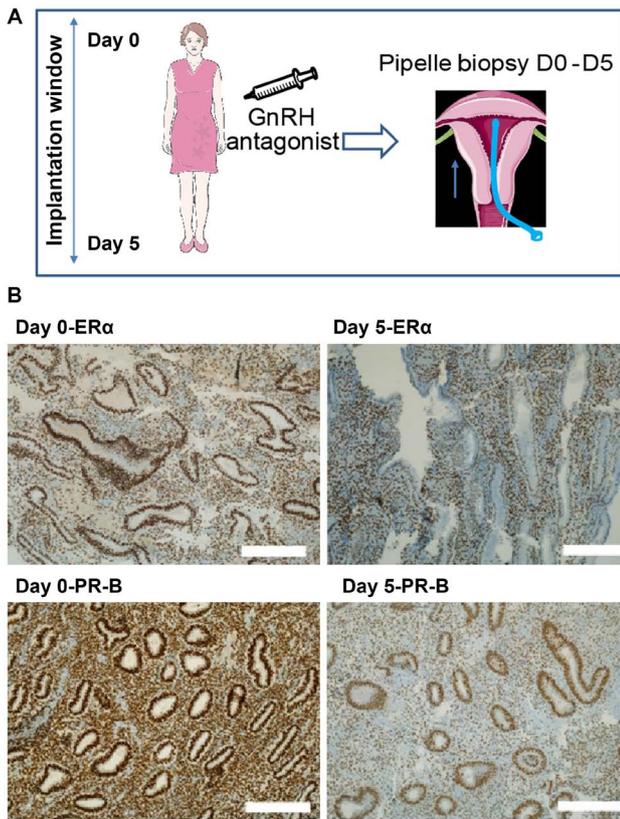


Figure 1. Biopsy removal and immunohistochemistry for ER α and PR-B. (A) Schematic diagram of the biopsy procedure. (B) Examples of immunohistochemistry for ER α and PR-B. Scale bar, 0.25 mm. ER α , estrogen receptor α ; PR-B, progesterone receptor-B; D, day; GnRH, gonadotropin releasing hormone.

for ER (Grade nodes and stromal) Day 0/5, $P=0.0001$; and for PR (Grade nodes and stromal) Day 0/5, $P=0.0001$ and $P=0.016$, respectively (Fig. 2B and C; Table I).

Involvement of ER and PR-B in endometrial gene networks: An in-silico analysis. In a recent meritorious study, microarray datasets collected during the time of human uterine receptivity and implantation were compared to the transcriptome signature of women with unexplained infertility (UIF) and recurrent implantation failure (RIF) (21). In that study, the authors have identified 24 and 21 shared differentially expressed genes (DEGs) between the transcriptome of women with UIF and RIF and those of normal endometrial receptivity samples (Table II) (21).

In view of the differential expression of ER and PR-B during the implantation window, their potential involvement was investigated further by studying potential interactions between the common genes for UIF and RIF using GeneMANIA software. The previously identified UIF genes (21) that potentially interact with ER are as follows: Thyrotropin releasing hormone (TRH), TRH receptor (TRHR), GLI family zinc finger 1, matrix metalloproteinase 26 (MMP26), retinol binding protein 4, serpin family A member 1, MMP7, catenin α 2, hyaluronan binding protein 2, collagen type IX α 1 (Fig. 3A). PR-B appears to interact with the following genes: Chromosome 20 open reading frame 103, calpain 6, thrombospondin 4, leucine rich repeat containing 17, periostin, collagen type IV α 6 chain,

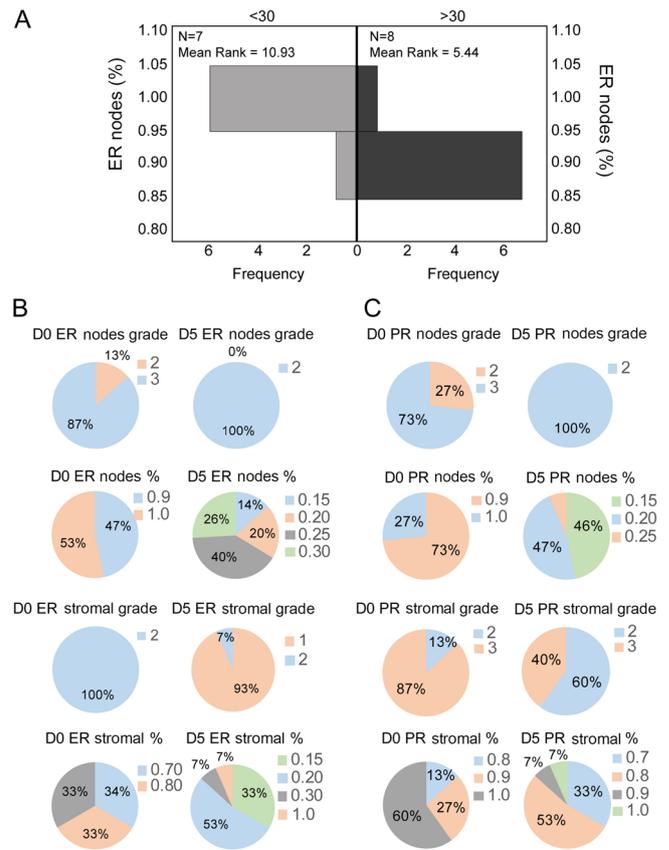


Figure 2. Differences in the expression grade and location of ER α and PR-B. (A) Frequency of ER expression stratified by age. ER α and PR-B expression on days 0 and 5. Pie charts of histopathology results for positive nuclei percentage and receptor grade per location for both (B) ER α and (C) PR-B. ER α , estrogen receptor; PR-B, progesterone receptor-B; D, day.

TRH, TRHR, nidogen 2, MMP7, MMP26, oviductal glycoprotein 1 (OVGP1), Jun dimerization protein 2, endothelin converting enzyme like 1, insulin like growth factor binding protein 1 (IGFBP1), Kruppel-like factor 9 (Fig. 3B).

In the case of previously identified RIF overlapping genes (21), potential interactions with ER include: Prostaglandin E receptor 3, Wilms tumor 1 (WT1), progesterone-associated endometrial protein (PAEP), odd-skipped related transcription factor 2, progesterone receptor, nidogen 1, nuclear factor I/A, splicing factor 1 (SF1; Fig. 4A). PR-B interacts with the following genes: Clusterin, solute carrier family 9 member A3, OVGP1, PAEP, superoxide dismutase 3, extracellular, ribonucleotide reductase regulatory TP53 inducible subunit M2B, ephrin B2, hydroxysteroid 17- β dehydrogenase 2 (HSD17B2), kallikrein related peptidase 3, and IGFBP1 (Fig. 4B).

Discussion

Successful implantation following IVF is a complex procedure that is very much dependent on the fertilized egg's progression into a blastocyst, synchronized with the differentiation of the endometrium (1,2). This 'implantation window' initiates a molecular dialogue of sorts that has not yet been fully clarified (1-4).

The aim of the present study was to elucidate a small part of this dialogue. These results, in combination with the

Table I. Descriptive statistics of immunohistochemical analysis.

Parameter	N	Minimum	Maximum	Mean	SDEV
Age (years)	15	25	32	28.93	2.89
A, Day 0					
ER nodal grade	15	2.00	3.00	2.87	0.35
ER stromal grade	15	2.00	2.00	2.00	0.00
ER nodal %	15	0.90	1.00	0.95	0.05
ER stromal %	15	0.70	0.90	0.80	0.08
PR nodal grade	15	2.00	3.00	2.73	0.05
PR stromal grade	15	2.00	3.00	2.87	0.35
PR nodal %	15	0.90	1.00	0.93	0.05
PR stromal %	15	0.80	1.00	0.95	0.07
B, Day 5					
ER nodal grade	15	1.00	1.00	1.00	0.00
ER stromal grade	15	1.00	2.00	1.07	0.26
ER nodal %	15	0.15	0.30	0.24	0.05
ER stromal %	15	0.15	1.00	0.24	0.21
PR nodal grade	15	1.00	1.00	1.00	0.00
PR stromal grade	15	2.00	3.00	2.40	0.51
PR nodal %	15	0.15	0.25	0.18	0.03
PR stromal %	15	0.70	1.00	0.89	0.06

ER, estrogen receptor; PR, progesterone receptor; SDEV, mean \pm SD.

number of ERs and PRs, play an important role in the success of IVF, since their expression causes a series of paracrine and autocrine signals, which through adhesion molecule processes ultimately lead to the successful adhesion and penetration of the endometrium by blastocysts (22). As far as permeability is concerned, the effect of ovarian steroids on the uterus is achieved through their receptors (23). Immunohistochemistry results for ER receptors showed a significant reduction in D5, as compared to D0, while an equally significant increase in the PR receptors was observed on the same days. Nodal ER and PR receptors with a strong presence on day 0 showed a very limited presence on day 5, while only PR receptors were strongly represented in the stroma. Estrogens in the follicular phase prepare the endometrium for the action of progesterone in the subsequent secretory phase of the cycle (24). Having stratified our samples into two age-groups (<30 and >30 years), it was found that the ER receptor nodal staining percentage on day 0 was age-related, with patients aged <30 years showing 100% staining and those aged >30 years showing 90% staining. This was consistent with potential age-related complications associated with maternity and successful pregnancy. Progesterone is another determining factor in the creation of the implantation window and the maintenance of pregnancy. The stromal cells differentiate into progesterone-responsive peristaltic cells during the perforation process, which is characterized by morphological changes (25). In the present study, changes in the expression of PR-B were observed between days 0 and 5. This was consistent with a study showing that stimulation of the endometrium with ganirelix acetate (a GnRH antagonist) and gonadotropins led to the increase of PR-B gene expression

at the time of embryo transfer (26). Of note, in the same study it was only the expression of PR-B that changed, while PR-A, the other splice isoform of the PR, was undetectable. It should be noted that, in addition to the two well studied variants of PR-A and -B, other splicing isoforms have also been detected including PR-C and PR-M (27). Moreover, it is now well accepted that both steroids can activate membrane-bound receptors acting in a non-genomic manner (28,29). Future studies should also concentrate on elucidating the expression of these receptors during the implantation window. Finally, evidence of a potential crosstalk between these receptors and genes implicated in RIF and UIF during the implantation window was provided herein, using data previously generated (21). This is of interest, given that these genes are involved in key physiological processes, such as the regulation of the protein activation cascade, complement activation, humoral immune response, acute inflammatory response or protein processing in the case of the gene cluster, which is common in RIF. One of the most interesting interactions of both ER and PR is with TRH and TRHR in UIF. Previous studies have corroborated these *in silico* findings in other *in vitro* or *in vivo* systems. For example, 17 β -estradiol (E2) was found to modulate the prolactin secretion induced by TRH in a female anterior pituitary primary cell culture. This response was mediated by a membrane-ER, but this finding provided an insight into a potential crosstalk (30). Similarly, E2 appears to inhibit TRH expression in the hypothalamic paraventricular nucleus of female rats (31). Similarly, TRHR immunoreactivity in the myometrium of cynomolgus macaques was increased when they were treated with conjugated equine estrogens alone or in

Table II. Full names of genes studied *in silico* including genes that have been identified from the previous referenced study (21).

Official gene symbol	Name
POSTN	Periostin, osteoblast specific factor
RBP4	Retinol binding protein 4, plasma
MMP26	Matrix metalloproteinase 26
WT1	Wilms tumor 1
LRRRC17	Leucine rich repeat containing 17
OSR2	Odd-skipped related transcription factor 2
HABP2	Hyaluronan binding protein 2
HSD17B2	Hydroxysteroid (17-β) dehydrogenase 2
PTGER3	Prostaglandin E receptor 3
SOD3	Superoxide dismutase 3, extracellular
PAEP	Progesterone-associated endometrial protein
THBS4	Thrombospondin 4
EFNB2	Ephrin-B2
TRHR	Thyrotropin-releasing hormone receptor
ECEL1	Endothelin converting enzyme-like 1
GLI1	GLI family zinc finger 1
MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)
COL9A1	Collagen type IX, α 1
SERPINA1	Serpin peptidase inhibitor, clade A, member 1
CTNNA2	Catenin (cadherin-associated protein), α 2
KLK3	Kallikrein-related peptidase 3
JDP2	Jun dimerization protein 2
IGFBP1	Insulin-like growth factor binding protein 1
KLF9	Kruppel-like factor 9
C20orf103	Chromosome 20 open reading frame 103
NID2	Nidogen 2 (osteonidogen)
CAPN6	Calpain 6
OVGP1	Oviductal glycoprotein 1, 120kDa
NID1	Nidogen 1
SLC9A3	Solute carrier family 9 (sodium/hydrogen exchanger), member 3
PGR	Progesterone receptor
SF1	Splicing factor 1
CLU	Clusterin
COL4A6	Collage type IV α 6 chain
RRM2B	Ribonucleotide reductase regulatory TP53 inducible subunit M2B
TRH	Thyrotropin-releasing hormone
NFIA	Nuclear factor I/A

combination with medroxyprogesterone acetate (32). Another common pathway that estrogen and progesterone appear to modulate is that of MMP7. Again, previous studies have corroborated this interaction. There has been an association between cellular and molecular responses in the rat mammary gland and E2, including MMP7 and MMP9 (33). When female

sheep pups were treated with P4, it led to a reduced expression of MMP7. Moreover, P4 inhibited uterine gland development in the uterus of a neonatal mouse; a process that involved the downregulation of MMP7 (34). Endometriotic cells have been found to contain the full complement of steroidogenic genes such as SF1 and WT-1, which can influence the transcription of steroidogenic genes necessary for E2 synthesis in endometriosis (35). A similar interplay between ER-SF1-WT1 was produced as interactions under RIF conditions. On the other hand, P4 inhibited the stimulatory effect of E2 on the expression of oviductin in the cervix of rhesus macaques (36). In addition, HSD17B2 expression in endometrial epithelial cells was found to be regulated by downstream molecules of progesterone (37).

Finally, we have shown using qRT-PCR that patients that were E-Cadherin-positive between days 0 and 5 were also all PR Grade 3. The small sample of this study clearly limits its scientific value, particularly with regards to very low gene expression, and lack of any statistical strength. We appreciate that this is a limitation of the current study. However, it should be noted that endometrial sampling at Day 5 is considered an invasive procedure and a cause of some discomfort to the individual, so it is very hard to recruit large numbers. Despite close follow-ups of the oocyte recipients, matching the morphological-structural changes of the donor endometria and the IVF result was not feasible, due to the anonymous nature of the oocyte donation procedure. It should be noted that the present study is limited to young women (due to better quality of oocytes and lower incidence of trisomy 21) and future studies should aim to test whether the present findings would hold true in other age ranges, such as women >35 years old.

Another limitation of the present study is that immunohistochemistry is less quantitative than western blotting. Performing this analysis using a housekeeping protein as a loading control, such as GAPDH or β-actin, would have been useful. Alternatively, ELISA or a gene expression assessment of both receptors using RT-qPCR could have been conducted. However, due to ethical restrictions, sufficient tissue for protein extraction could not be obtained in order to pursue this further.

As mentioned, synchronization between blastocyst development and the acquisition of endometrial receptivity is a prerequisite for the success of IVF, a process that appears to be dependent on a number of different events at the hormonal and cellular levels. In this study, novel evidence of differential expression and potential involvement of two key steroid hormone receptors in this process was provided; namely, in women undergoing treatment during an oocyte donation program. The present results, combined with those of *in silico* analyses, suggested that the changes in ER and PR expression and cellular distribution are crucial events that can impact implantation. This should be investigated further in studies with a larger population, which should further validate the original findings using alternative, more quantitative approaches, and further explore changes between D0-5 in a 'non-biased' way through RNAseq or proteomic analysis instead. Expanding our knowledge on this field beyond the two steroid hormone receptors described herein, would augment our understanding of the signalling mechanisms implicated in infertility, and potentially provide novel therapeutic targets. It would have

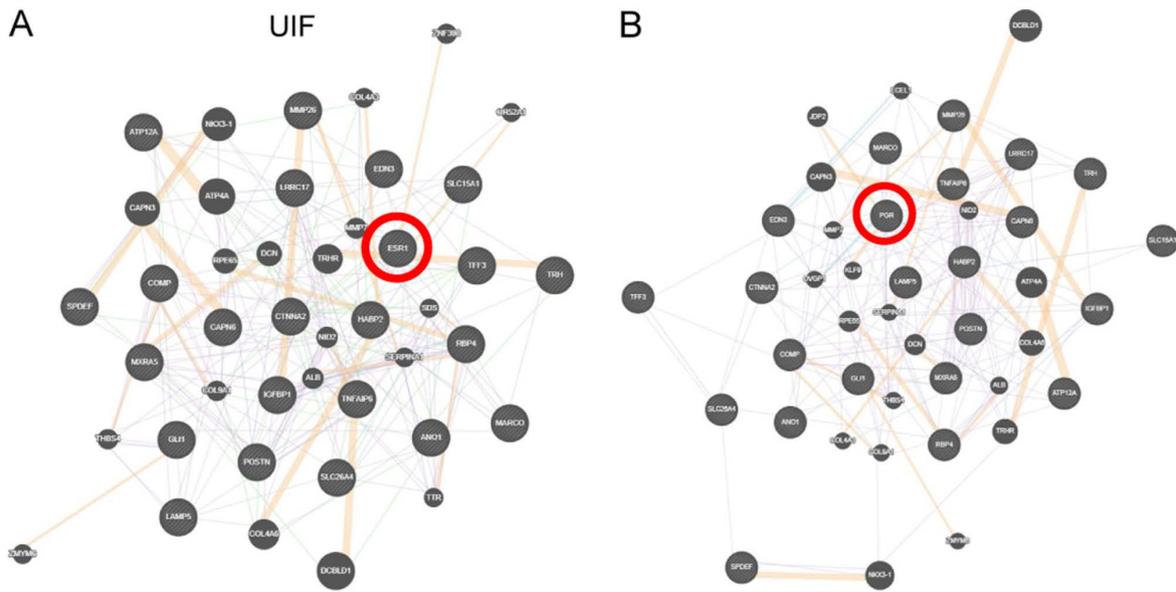


Figure 3. Predictive interactions of ESR1 and PGR during UIF. Network annotation of previously identified genes involved in UIF with (A) estrogen receptor alpha (ESR1 in red circle) and (B) progesterone receptor (PGR in red circle). ESR1, estrogen receptor α ; PGR, progesterone receptor; UIF, unexplained infertility.

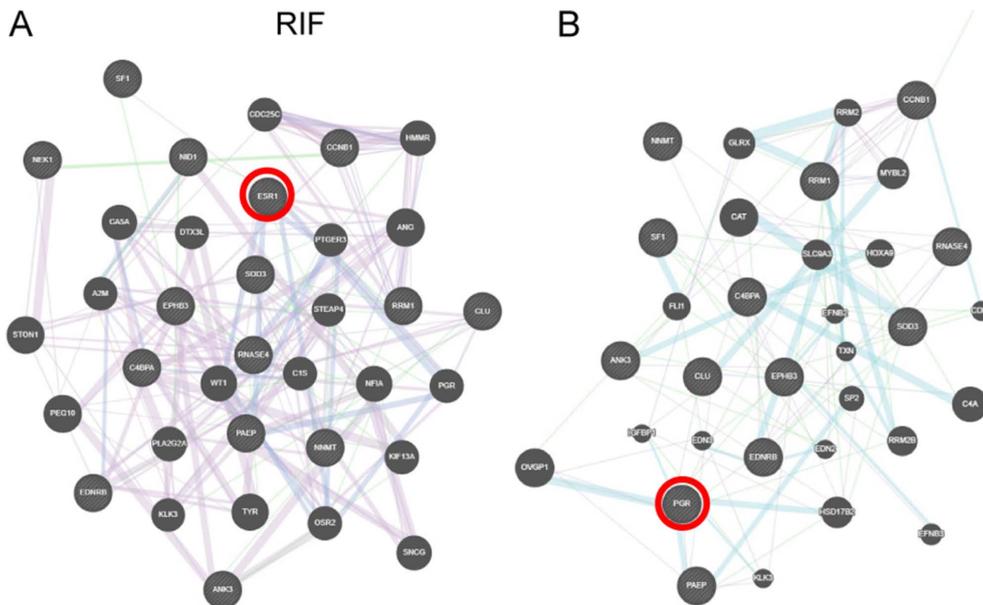


Figure 4. Network annotation of previously identified genes involved in RIF with (A) ESR1 (red circle) and (B) PGR (red circle). RIF, recurrent implantation failure; ESR1, estrogen receptor α ; PGR, progesterone receptor.

been useful to investigate the downstream signalling as well. However, for this to happen we would need to generate primary cell cultures from the biopsies. This was impossible due to the small size of the study. There are no commercially available cell lines to mimic this pharmacological milieu. We mention this under limitations in our discussion. It is for the very reason that we embarked on bioinformatic analysis to we provide novel evidence for a potential crosstalk of these receptors during the implantation window with genes that are implicated in RIF and UIF.

In conclusion, successful implantation implies synchronization between a blastocyst and the endometrium, which undergoes

structural and functional remodelling. It was shown herein that both ER- α and PR-B were expressed abundantly on days 0 and 5, showing significant variation in the nodal and stromal preparations. Age appeared to be a critical factor, since ER- α nodal staining showed higher values in the age group of oocyte donors <30 years old. Therefore, focusing on the functional characteristics of the endometrium will provide a better insight into successful embryo implantation, thus improving IVF results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EKa, EP, BT and GP conceived the current study. EK1, PK and EKa performed the experiments. EK1, PK, Eka and GP performed *in silico*, bioinformatics and statistical analyses. EKa, EP, BT and GP performed the investigation provided funding and materials. EK1 and PK integrated the data and created figures. EK1, PK, Eka and GP wrote the original draft, writing review and editing, EK1, EKa, EP, BT and GP reviewed and edited the manuscript. EK1 and GP supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Committee for Medical Ethics and Deontology, School of Medicine, Aristotle University of Thessaloniki (approval no. A 13032 15/7/10). Written informed consent was obtained from patients prior to enrolment.

Patient consent for publication

Consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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