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ARTICLE

Endometrial expression of selected genes in patients achieving pregnancy spontaneously or after ICSI and patients failing at least two ICSI cycles

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Adolfo Allegra obtained his medical degree in 1978 and his specialization in obstetrics and gynaecology in 1982 at the University of Palermo and his specialization in endocrinology in 1985 at the University of Rome. From 1984 to 1992, he was director of the reproductive medicine unit of the department of obstetrics and gynaecology at 'Civico' Hospital, Palermo, Italy. Since 1985, he has directed the reproductive medicine unit of Andros Day Surgery, one of the most important reproductive clinics in Italy. From 2003 to 2007, he was a member of the National Committee of SIdR (Italian Society of Reproduction). Since 2004, he has been aggregate professor of gynaecological surgery at the University of Palermo. His main clinical and research interests are reproductive endocrinology, reproductive endoscopy and endometrial receptivity.

Abstract The objective of this study was to identify the endometrial gene expression profile in receptive phase, which could represent a useful prognostic tool for selecting IVF patients. Endometrial expression of 47 selected genes biopsied during the window of implantation in natural cycles was compared between patients who achieved a successful pregnancy spontaneously or after subsequent intracytoplasmic sperm injection (ICSI) cycles and patients who did not achieve a pregnancy after at least two failed ICSI cycles. The comparative analysis showed significantly different levels of expression in 19 genes, five implicated in apoptosis (*CASP8*, *FADD*, *CASP10*, *APAF1*, *ANXA4*), three in immunity (*LIF*, *SPP1*, *C4BPA*), five in transcriptional activity (*MSX1*, *HOXA10*, *MSX2*, *HOXA11*, *GATA2*), two in lipid metabolism (*LEPR*, *APOD*) and four in oxidative metabolism (*AOX1*, *ALDH1A3*, *GPX3*, *NNMT*). The evidence for these genes being differently expressed could represent the starting point of identifying the ideal receptive endometrial gene expression profile, which could be used in the future as a prognostic tool for IVF patients.

KEYWORDS: endometrium, gene expression profile, implantation window, pregnancy

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Introduction

Implantation rates in stimulated cycles are lower than those of natural cycles. In a review of the efficacy of natural IVF cycles (Pelinck et al., 2002), implantation rates ranged from 0% and 50% in natural cycles, compared with 7–9% in stimulated cycles. The problem of reduced implantation rates in stimulated IVF cycles is compensated by the transfer of multiple embryos, but this choice has resulted in an increase in multiple pregnancies (Bergh et al., 1999; Luke et al., 2010), which are associated with a high rate of morbidity and mortality (Helmerhorst et al., 2004; Pinborg, 2005).

In order to avoid multiple pregnancies, elective single-embryo transfer seems to be a very promising tool (Gelbaya et al., 2010). In a very recent publication, it has been shown that birth rates were significantly lower after fresh transfer of one versus two embryos (28% versus 43%, $P < 0.001$) but this difference was ameliorated when, after unsuccessful fresh elective single-embryo transfer, a frozen embryo was subsequently transferred (Practice Committee, 2012). Thus, it is fundamental to select the better-prognosis patients to undergo single-embryo transfer.

It would be valuable to have a prognostic endometrial tool specific to the patient that could predict the chance of pregnancy well. It is known that the embryo is only able to attach itself to the uterine wall during the so-called window of implantation, a short period of time in which the endometrium clearly shows important modifications compared with the pre-receptive period (Harper, 1992; Wilcox et al., 1999; Lessey, 2011). Many growth factors, cytokines, transcription factors and apoptotic factors are involved in the typical modifications of this period (Hoozemans et al., 2004) and these molecules seem to establish a coherent dialogue with other factors secreted by the blastocyst (Haouzi et al., 2011).

To date, many studies of endometrial gene expression have been published which demonstrate a modification in the gene expression profiles of pre-receptive and receptive endometria (Carson et al., 2002; Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Ponnampalam et al., 2004; Mirkin et al., 2005; Talbi et al., 2006; Haouzi et al., 2009a; Tseng et al., 2010; Diaz-Gimeno et al., 2011), although these functional studies have not provided a coherent, complete knowledge of the human endometrial receptivity (Horcajadas et al., 2007). The above-mentioned studies identified changes during the window of implantation in the expression of several genes, but there is a great variability in the number of up- and down-regulated genes: for example, in Talbi et al. (2006), 49.2% of the total genes were up-regulated during the window of implantation, but in Haouzi et al. (2009a), the percentage of up-regulated genes increased to 93.4%. The reasons for this high variability depend on many factors: type of DNA microarray, the fold changes utilized for defining the significance, different statistical methodologies, incomplete validation by real-time PCR and the populations analysed (Haouzi et al., 2012). Another factor that may contribute to this variability is the fact that different kinds of cells are analysed in a single sample. In this sense, laser capture microdissection could be an important tool in producing more reliable results (Franchi et al., 2008); however, it is not simply achievable.

Nevertheless, some genes with a high fold change are common throughout the different microarray data, which suggests the teleological significance of these genes in determining endometrial receptivity. A previous work (Allegra et al., 2009) selected genes which, in other studies, had shown the strongest up- (23) or down- (11) regulation between pre-receptive and receptive endometrium and 13 genes involved in apoptosis pathways. Six of these genes (*VEGFA*, *PLA2G2A*, *ALPL*, *LIF*, *NNMT* and *STC1*) were homogeneously expressed during the window of implantation of patients that became pregnant in subsequent intracytoplasmic sperm injection (ICSI) cycles. These results should be tested in larger cohorts (Haouzi et al., 2012).

The aim of the present study was to compare the expression profiles of the genes selected by Allegra et al. (2009) in receptive endometria of patients who had been pregnant before biopsy spontaneously or after biopsy and ICSI and patients who failed to become pregnant after two ICSI cycles. The identification of differentially expressed genes in these two groups of patients could represent a useful prognostic tool for identifying good- and poor-prognosis IVF patients.

Materials and methods

Patient recruitment

Two groups of patients were selected. Group A consisted of 24 women who had achieved a successful pregnancy, comprising of 19 women who became pregnant after no more than two ICSI cycles performed at Andros Day Surgery based on a severe male infertility factor according to WHO criteria and five fertile oocyte donors recruited at Tambre Clinic who had had a previous natural pregnancy. Group B consisted of 10 women who failed to achieve a pregnancy after at least two ICSI cycles performed at Andros Day Surgery and who transferred at least one top-quality embryo in each cycle.

All patients underwent endometrial biopsy, under sterile conditions, using a Pipelle Catheter (Laboratoire CCD, Paris), in the respective clinics, during the window of implantation of a natural cycle, 7–9 days after the urinary LH surge. Patients attending their first ICSI at Andros Day Surgery were biopsied in an unstimulated cycle one or two cycles before the first ICSI cycle, whereas the oocyte donors at Tambre Clinic were biopsied up to 3 months before the stimulation cycle for oocyte donation.

The patients were selected on the basis of the following criteria of eligibility: age <35 years old; regular menstrual cycles (26–32 days); body mass index 18–30 kg/m^2; normal basal serum FSH (≤12 mIU/ml); and normal uterine cavity assessed by hysteroscopy (only for the ICSI patients).

In the ICSI patients, ovarian stimulation was carried out using a standard long protocol. Recombinant FSH (Gonal F; Serono, Italy) was initiated after at least 12 days of pituitary down-regulation. If biopsy was performed during the last menstrual cycle before ICSI, gonadotrophin-releasing hormone (GnRH) agonist was always started after the biopsy itself.

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The protocols and procedures during the ICSI cycles and pregnancy are fully explained in Volpes et al., 2004. All embryo transfers, at day 2 or day 3 post insemination, were performed by the same well-experienced physician (AV). The embryos were classified as top quality when on day 2 or day 3 they had 4 or 8, respectively, even blastomeres with <10% fragmentation (grade 1). Good-quality embryos were those with a number of stage-specific blastomeres, with no evidence of multinucleation and a 10–25% fragmentation (grade 2). Poor-quality embryos were those with a number of uneven blastomeres not stage specific, with evidence of multinucleation and >25% fragmentation (grade 3).

All patients gave written informed consent. The study was approved by the local ethics committee (Ethics Committee of Andros Day Surgery, Reproductive Medicine Unit, Palermo, approved 9 April 2009, reference number 002/MR/09).

Concerning the study population, in comparison to the 19 patients who became pregnant after ICSI, the patients who did not achieve pregnancy showed no difference in age, basal FSH, menstrual cycle length (data not shown), total FSH units administered, days of treatment with FSH, oestradiol concentrations and the number of follicles ≥16 mm and endometrial thickness on the day of human chorionic gonadotrophin administration, number of oocytes retrieved, number of mature oocytes, number of fertilized oocytes and number and quality of transferred embryos (**Table 1**).

Tissue collection and histological evaluation

This study performed a comparative analysis between endometrial expression of selected genes in 24 patients who had been pregnant before the biopsy spontaneously or after the biopsy and ICSI and 10 patients who did not achieve pregnancy after biopsy and ICSI. Endometrial tissues were divided into two parts, one for histological evaluation and the other, stored at −80°C in lysis buffer (Promega, USA) containing guanidine thiocyanate, for total mRNA extraction. All endometrial biopsies were evaluated in accordance with the histological criteria of Noyes et al. (1950, 1975). Biopsies with a discordance between the histological data and the day of the cycle were excluded from the subsequent analysis of the gene expression.

For the biopsies collected at Tambre Clinic, samples were appropriately labelled with the donor's name, the date of birth and an identification code. They were stored in the same lysis buffer as used at Andros Day Surgery. Subsequently, the samples were frozen in liquid nitrogen (−196°C) and sent to Andros Day Surgery, according to the 2004/23/CE, 2006/17/CE and 2006/86/CE European Directives describing the correct preservation, storage, transport and distribution of human tissues.

Table 1 Characteristics of ICSI patients and cycles.

Characteristic	*Pregnant after ICSI* (n = 19)	*Not pregnant after ICSI* (n = 10)
Age (years)	28.9 ± 3.4	28 ± 3.4
Infertility duration (years)	1.96 ± 1.5	3.1 ± 2.7
Basal FSH (mIU/ml)	6.73 ± 1.7	6.4 ± 1.8
Cycles performed[a]	1.4 ± 0.5	2.4 ± 0.5
Total FSH dose (IU)	2201.3 ± 1048.6	1975.8 ± 543.8
Duration of stimulation (days)	12.79 ± 2.2	11.89 ± 0.7
Follicles ≥16 mm on HCG day	7.8 ± 2.2	8.5 ± 1.1
17-β-Oestradiol on HCG day (pg/ml)	1188.7 ± 540.8	1260.15 ± 384.8
Endometrial thickness on HCG day (mm)	11.3 ± 1.6	11.2 ± 1.9
Oocytes retrieved	7.68 ± 1.8	7.75 ± 1.4
Mature oocytes	6.05 ± 1.5	6.12 ± 1.1
Oocytes inseminated[b]	3 ± 0	3.16 ± 0.5
Oocytes fertilized	2.37 ± 0.7	2.3 ± 0.5
Embryos transferred	2.26 ± 0.7	2.17 ± 0.2
Embryo score		
Grade 1	21/43 (48.8)	22/52 (42.3)
Grade 2	9/43 (20.9)	5/52 (9.6)
Grade 3	13/43 (30.2)	25/52 (48.1)

Values are mean ± SD or *n*/total transferred embryos (%). Unpaired t-test was applied to all variables apart from embryo score, for which Fisher's Exact test was used to test for significant differences.
HCG = human chorionic gonadotrophin.
[a] $P = 0.001$, no significant differences in any other variable.
[b] All the ICSI cycles of the patients who became pregnant were performed when the limit of three oocytes to be inseminated was in effect, as stated by the Italian Law 'Norms on the matter of medically assisted reproduction, 19th February 2004, Number 40'. In 2009, this limit was eliminated due to the sentence of the Italian Constitutional Court (n. 151/09) which allowed, in specific cases, the insemination of more than three oocytes, with the consequent possibility of cryopreserving the surplus embryos. Some of the ICSI cycles of non-pregnant patients were carried out after this sentence.

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RNA extraction and gene expression analysis of 47 selected genes

Total RNA was isolated using the Maxwell total RNA purification kit (Promega), in accordance with the manufacturer's protocol. Genomic DNA decontamination was carried out using Clearing Agent (Promeya, Italy). The isolated total mRNA was then eluted into RNase-free water.

TaqMan Low Density Array Formats 48 (Applied Biosystems, USA) was used in order to test gene expression of 47 selected genes (**Table 2**). An aliquot of each total RNA sample was used in a reverse-transcription reaction for the conversion into cDNA single-strand copy using a High Capacity cDNA Archive kit (Applied Biosystems).

Real-time PCR was carried out in an Applied Biosystems 7900HT Fast Real-Time PCR System using a standard program. The housekeeping gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was amplified in the same samples in order to have a qualitative evaluation of an optimal PCR reaction, as well as to quantify the relative expression of target genes per samples. *GAPDH* expression was always present and, in all samples, was above the threshold of significance, and was at similar levels in different samples.

The genes analysed in this study were the same as selected for the first study (Allegra et al., 2009) and also previous literature (Carson et al., 2002; Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Ponnampalam et al., 2004; Mirkin et al., 2005) for their presumable involvement in endometrial receptivity. Thirty-four genes were chosen particularly because they were up- (23) or down- (11) regulated during the window of implantation, while 13 further genes were selected from the apoptosis pathway, due to the fact that implantation seems to be characterized by a local immune response with the activation of the FAS/FAS ligand system (Aagaard-Tillery et al., 2006). The selection criteria were based on the fact that each of these selected genes was found in common among all the different microarray studies (Carson et al., 2002; Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003; Ponnampalam et al., 2004; Mirkin et al., 2005) and all with a high significance. One of the strongest sources of bias in microarray studies is the high variability among the different laboratories so that it is really difficult to reach clear conclusions. The selected genes showed a fold change >3 between pre-receptive and receptive endometrium (24 ± 30.22 for up-regulated genes and 9.8 ± 3.06 for down-regulated genes). Quantitative gene expression was calculated by the ABI Prism 7700 Sequence Detection System software (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using the significance analysis of microarrays (SAM; Tusher et al., 2001). The SAM software can perform up to 500 random permutations of group labels expressed in ΔCt, $\log_{10}$ of quantity of target genes, normalized in comparison to *GAPDH* as the control gene, to calculate the false-discovery rate (q-value%) on the basis of t-statistics or scores. This approach permitted this study to verify which genes were differently expressed. Statistical significance was calculated on the basis of the *d*-score value ($d_i = r_i/s_i + s_0$) where $r_i = \Sigma x_{ij}/n$ and $s_i = \{\sum(x_{ij} - \mu_i)^2/(n(n-1))\}^{1/2}$. Once all random permutations were performed, the SAM software reported a set of empirical *d*-scores (observed and theoretical *d*-scores (expected). As the empirical d_i values became different from theoretical d_i values, the points on the graph outdistance themselves from the straight oblique line passing through the origin.

Statistical significance was established on the basis of the Delta range which defines the distance between an upper and a lower line in respect to the straight oblique line passing through the origin. SAM was used in the response type 'two class' for unpaired data, using non-parametric test (Wilcoxon test), to evaluate the difference in gene expression between group A and group B. Moreover, for each gene, the significance of the distributions was validated using the non-parametric two-tailed Kolmogorov–Smirnov test, which quantifies the differences of the empirical cumulative distribution functions between the two groups.

Results

This study compared the endometrial gene expression profiles of 24 patients achieving pregnancy with 10 patients who failing to achieve pregnancy. Only 36 out of 47 genes were expressed in all patients: 11 genes (*LAMB3*, *VEGFA*, *HBEGF*, *LEP*, *KCNG1*, *CALB2*, *TRH*, *BCL2*, *CASP9*, *DFFB*, *FASLG*) were not expressed in one or more patients and were excluded from the analysis.

Of the 36 included genes, 19 were significantly different: *MSX1*, *HOXA10*, *CASP8*, *SPP1*, *LEPR*, *ANXA4*, *GATA2*, *MSX2*, *C4BPA*, *APOD*, *AOX1*, *ALDH1A3*, *FADD*, *HOXA11*, *CASP10*, *APAF1*, *GPX3*, *LIF* and *NNMT*. **Figure 1** summarizes the fold changes and false-discovery rates. The significant differences observed for all 19 genes were confirmed by the Kolmogorov–Smirnov test. The genes could be classified into the following biological groups: apoptosis (*CASP8*, *FADD*, *CASP10*, *APAF1*, *ANXA4*), immunity (*LIF*, *SPP1*, *C4BPA*), transcriptional activity (*MSX1*, *HOXA10*, *MSX2*, *HOXA11*, *GATA2*), lipid metabolism (*LEPR*, *APOD*) and oxidative metabolism (*AOX1*, *ALD1A3*, *GPX3*, *NNMT*). The data are expressed as ΔCt (a lower value indicating higher expression).

The strength of difference was not equal for all 19 genes. Indeed, the major gene expression difference, evaluated in terms of the combination of two parameters (the fold change and the better false-discovery rate), was observed for *MSX1* (fold change = 1.89) and *HOXA10* (fold change 1.39) having a null percentage error described by the false-discovery rate (q-value% = 0). In detail, *MSX1* expression was significantly higher in group A (mean ± SD, ΔCT = 7.4 ± 2.04) versus group B (ΔCT = 9.02 ± 1.1; $P = 0.0055$), and the same was found for *HOXA10* expression (ΔCT = 3.4 ± 1.7 in group A versus 4.5 ± 0.5 in group B; $P = 0.0129$). Moreover, for both *MSX1* and *HOXA10*, the expression distributions in the two groups were totally different as the probability of false-positive classification resulted equal to zero.

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Table 2 List of genes selected on the basis of possible importance to endometrial receptivity.

Gene symbol	*Gene name*	*ID gene bank*	*Assay ID*[a]
Up-regulated genes			
PLA2G2A	Phospholipase A2 group IIA	M22430	Hs00179898_m1
PAEP	Progestagen-associated endometrial protein	J04129	Hs00171462_m1
GPX3	Glutathione peroxidase 3	D00632	Hs00173566_m1
ALDH1A3	Aldehyde dehydrogenase 1, member A3	U07919	Hs00167476_m1
CD55	CD55	M31516	Hs00167090_m1
ANXA2	Annexin II	D28364	Hs00733393_m1
ANXA4	Annexin IV	M82809	Hs00154040_m1
SPP1	Secreted phosphoprotein 1	J04765	Hs00167093_m1
APOD	Apolipoprotein D	J02611	Hs00155794_m1
SCGB1D2	Secretoglobin	AJ224172	Hs00255208_m1
APOE	Apolipoprotein E	M12529	Hs00171168_m1
NNMT	Nicotinamide N-methyltransferase	4837	Hs00196287_m1
C4BPA	Complement component 4 binding protein	M31452	Hs00426339_m1
AOX1	Aldehyde oxidase 1	AF017060	Hs00154079_m1
LAMB3	Laminin, beta 3	U17760	Hs00165078_m1
STC1	Stanniocalcin 1	U25997	Hs00174970_m1
LIF	Leukaemia inhibitory factor	M63420	Hs00171455_m1
HOXA10	Homeobox A10	BC013971	Hs00538183_m1
HOXA11	Homeobox A11	AF071164	Hs00194149_m1
VEGFA	Vascular endothelial growth factor A	BC058855	Hs00173626_m1
HBEGF	Heparin binding EGF-like growth factor	M60278	Hs00181813_m1
LEPR	Leptin receptor	AA813024	Hs00174497_m1
LEP	Leptin (obesity homologue, mouse)	U43653	Hs00174877_m1
Down-regulated genes			
MSX2	Msh homeobox homologue 2	D89377	Hs00741177_m1
GATA2	GATA binding protein 2	M68891	Hs00231119_m1
MSX1	Msh homeobox homologue 1	M97676	Hs00427183_m1
CCR1	Chemokine receptor 1	D10925	Hs00174298_m1
HPGD	Hydroxyprostaglandin dehydrogenase 15-NAD	L76465	Hs00168359_m1
ALPL	Alkaline phosphatase, liver/bone/kidney	AB011406	Hs00758162_m1
KCNG1	Potassium voltage-gated channel, subfamily G, member 1	AL050404	Hs00383304_m1
SLC15A2	Solute carrier family 15 member 2	S78203	Hs00221539_m1
CALB2	Calbindin 2	X56667	Hs00242372_m1
TRH	Thyrotropin-releasing hormone	M63582	Hs00175078_m1
CTNNA2	Catenin (cadherin-associated protein), alpha 2	M94151	Hs00189285_m1
Apoptosis genes			
APAF1	Apoptotic peptidase activating factor 1	AF013263	Hs00559421_m1
BAX	BCL2-associated X protein	L22474	Hs00180269_m1
BAK1	BCL2-antagonist/killer1	BC004431	Hs00832876_g1
BCL2	B-cell CLL/lymphoma 2	AC021803	Hs00153350_m1
CASP3	Caspase 3	BU753483	Hs00234385_m1
CASP8	Caspase 8	U58143	Hs00236278_m1
CASP9	Caspase 9	AB015653	Hs00154260_m1
CASP10	Caspase 10	U60519	Hs00154272_m1
DFFA	DNA fragmentation factor, 45 kDa, alpha polypeptide	AU121791	Hs00189336_m1
DFFB	DNA fragmentation factor, 40 kDa, beta polypeptide (caspase-activated DNAse)	AF409062	Hs00237077_m1
FADD	Fas (TNFRSF6)-associated via death domain	AL575732	Hs00538709_m1
FAS	Fas (TNF receptor superfamily member 6)	D31968	Hs00163653_m1
FASLG	FAS ligand (TNF superfamily member 6)	X89102	Hs00181225_m1

[a]Assay ID refers to TaqMan Gene Expression Assays (Applied Biosystems).

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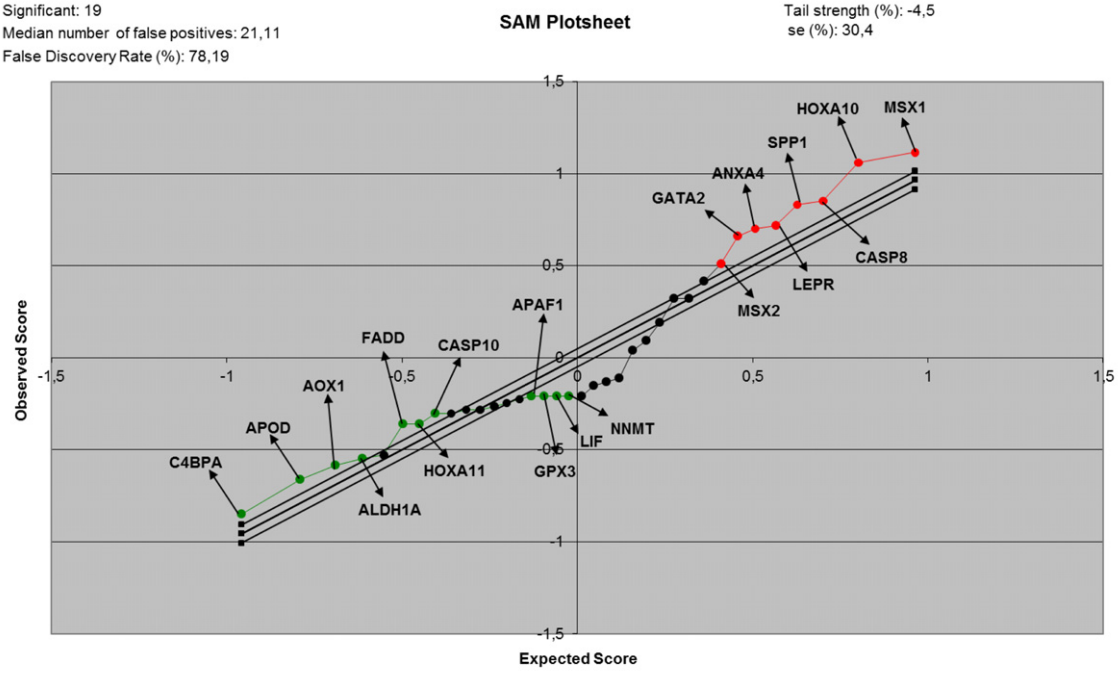
Discussion

This paper investigated the basal endometrial expression of selected genes in 24 women who shared a common characteristic (pregnancy) and in 10 patients who failed to achieve a pregnancy after at least two ICSI cycles, even with at least one top-quality embryo in each cycle. Nineteen genes were differently expressed: five implicated in apoptosis (*CASP8*, *FADD*, *CASP10*, *APAF1*, *ANXA4*), three in immunity (*LIF*, *SPP1*, *C4BPA*), five in transcriptional activity (*MSX1*, *HOXA10*, *MSX2*, *HOXA11*, *GATA2*), two in lipid metabolism (*LEPR*, *APOD*) and four in oxidative metabolism (*AOX1*, *ALDH1A3*, *GPX3*, *NNMT*). This evidence could provide the starting point of identifying an ideal gene expression profile of a functional endometrium which, after a broader confirmation, could be used as a prognostic tool for couples undergoing IVF.

The ICSI patients who achieved a pregnancy are considered to be equivalent to the five oocyte donors due to the fact that each one had a functional endometrium based

(a)



(b)

Gene ID	Score (d)	Wilcoxon Test: Numerator (r)	Wilcoxon Test: Denominator (s+s0)	Wilcoxon Test: Fold Change	Wilcoxon Test: q-value (%)	Kolmogorov-Smirnov test: p-value
MSX1	1,12	59	52,92	1,89	0	< 0,0001
HOXA10	1,1	56	52,92	1,39	0	< 0,0001
CASP8	0,85	45	52,92	1,42	47,62	< 0,0001
SPP1	0,83	44	52,92	1,99	47,62	< 0,0001
LEPR	0,72	38	52,92	1,26	47,62	< 0,0001
ANXA4	0,7	37	52,92	1,21	47,62	< 0,0001
GATA2	0,66	35	52,92	1,28	47,62	< 0,0001
MSX2	0,51	27	52,92	1,27	76,92	< 0,0001
C4BPA	-0,85	-45	52,92	0,073	66,67	< 0,0001
APOD	-0,66	-35	52,92	0,79	66,67	< 0,0001
AOX1	-0,59	-31	52,92	1,36	66,67	< 0,0001
ALDH1A3	-0,55	-29	52,92	0,73	66,67	< 0,0001
FADD	-0,36	-19	52,92	0,63	66,67	< 0,0001
HOXA11	-0,36	-19	52,92	0,56	66,67	< 0,0001
CASP10	-0,3	-16	52,92	0,75	66,67	< 0,0001
APAF1	-0,21	-11	52,92	0,76	66,67	< 0,0001
GPX3	-0,21	-11	52,92	0,79	66,67	< 0,0001
LIF	-0,21	-11	52,92	1,32	66,67	< 0,0001
NNMT	-0,21	-11	52,92	0,95	66,67	< 0,0001

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on pregnancy, even if this event was obtained before (oocyte donors) or after (ICSI patients) the biopsy itself. Each ICSI patient enrolled in this study was selected for a severe male factor, thus excluding any evident female factor of infertility. Nevertheless, while the patients of group A had shown a good functional endometrium by achieving pregnancy either naturally or by ICSI, it is much more difficult to be sure for the patients of group B that the endometrium was not functionally adequate and therefore responsible for the failed implantation. As shown, in comparison to the ICSI patients who achieved a pregnancy, the patients who did not become pregnant showed no difference for variables that can affect the reproductive outcome (**Table 1**). The exclusion of the effect of all these factors eliminates any possible bias that could have affected the comparison.

Not all biopsies were carried out on the same cycle day but during a period of 3 days (7–9 days after LH surge) because of couples' prior commitments. This is common to many studies (Carson et al., 2002; Kao et al., 2002; Riesewijk et al., 2003; Borthwick et al., 2003) and, given that the endometrium is a dynamic tissue, may have influenced the endometrial gene expression results, but this problem is by no means solvable. Nevertheless, all samples were collected during the implantation window.

The present work represents one of the first attempts to relate the gene expression profile to the clinical outcomes of IVF cycles. In the last 4 years, other data relating biomolecular analysis to clinical outcomes and features have been published (Bersinger et al., 2008; Haouzi et al., 2009a,b; Van Vaerenbergh et al., 2009, 2011a,b; Altmäe et al., 2010; Blockeel et al., 2011). Nevertheless, it is extremely difficult to reach a clear conclusion because the designs of these studies differ greatly and are not homogeneous. In contrast, the objective of this study was simpler and based on the analysis of differences in endometrial gene expression between patients with a potential functional endometrium and patients with an impaired endometrium. It is very problematic to give sure answers on this topic but some of the genes found to be differently expressed clustered in common functional pathways, and some of these (immunity, transcriptional activity, lipid metabolism) have been widely highlighted in literature.

Concerning the apoptosis pathway, apoptosis is predominant in the normal endometrium during the secretory phase and peaks in the menstrual phase (Toki et al., 1998; Jones et al., 1998). Specifically for the implantation process, a co-ordinated embryonic regulation of apoptosis of human endometrial epithelial cells has been demonstrated (Galàn et al., 2000). In the adhesion phase, the blastocyst may induce a paracrine apoptotic reaction, mediated by the FAS/FAS ligand system.

Regarding the differently expressed genes associated with immunity, a recent paper highlighted the importance of some (such as *SPP1* and *LIF*) in the human receptive endometrium (Altmäe et al., 2012). Other studies have suggested a major role for LIF (leukaemia inhibitory factor, a member of the interleukin-6 family) in embryo implantation and endometrial receptivity, especially in mice but also in humans (Yue et al., 2000; Cheng et al., 2001; Lass et al., 2001; Aghajanova, 2004; Nakamura et al., 2006). The role of LIF in successful implantation of murine models has been known for many years; Stewart et al. (1992) demonstrated that blastocysts transferred into murine females lacking a functional LIF gene fail to implant, although the same blastocysts implant if transferred to wild-type mice. The role of LIF in humans is more debatable. As stated in a recent review, in fertile patients, high LIF concentration signals a receptive endometrium, but, in infertile patients, the data on its significance are controversial, so that its detection is not sufficient to establish the implantation potential in women with unexplained infertility (Aghajanova, 2010).

LIF transduces a signal for the transcription of a set of genes, by inducing the translocation into the nucleus of the transcription factor STAT3 (signal transducer and activator of transcription 3), through the specific phosphorylation of the Tyr705 residue. In the murine model, STAT3 has been described as localized in the nucleus in its active form in a temporally regulated manner at day 4 (implantation day), whereas, on days 3 and 5 post coitus, STAT3 is predominantly cytoplasmic (Cheng et al., 2001). On the other hand, in humans, Dimitriadis et al. (2007) observed a significant decrease of the phosphorylated form of STAT3 in uterine glandular epithelia of women with unexplained infertility compared with fertile women. Some of the genes found in this study to be differently expressed could be

Figure 1 (a) Gene expression analysis elaborated by SAM two-class test comparing patients achieving pregnancy with patients not achieving pregnancy. The Y-axis plots the observed score values and the X-axis plots the expected score for each gene. The diagonal straight line at an angle of 45°, passing through the origin, shows the equality of the empirical d_i values with the theoretical d_i values. The distance between the dashed lines is the Delta threshold, which is used for the detection of genes which are homogeneously (points inside the Delta threshold) as well differently (points outside the Delta threshold) expressed between the two groups. Nineteen genes were found to be differently expressed: *MSX1*, *HOXA10*, *CASP8*, *SPP1*, *LEPR*, *ANXA4*, *GATA2*, *MSX2*, *C4BPA*, *APOD*, *AOX1*, *ALDH1A3*, *FADD*, *HOXA11*, *CASP10*, *APAF1*, *GPX3*, *LIF* and *NNMT*. (b) Statistical analysis of the 19 differently expressed genes. Score (*d*) is the result of the ratio between the numerator (*r*) and denominator (*s* + *s*0). The numerator represents the difference between the sum of the positions occupied by the ordered units of the two groups and the expected theoretical values for each position when the two distributions come from the same population ($r = U - \mu_u$). The denominator represents the mean square deviation of the positions occupied by the ordered units of the two groups (*s*) plus a factor for exchangeability, which is 5% in the Wilcoxon test. The q-value measures the proportion of false-positives incurred (the false-discovery rate) when that particular test is called significant and allows the calculation of the robustness of the significance of the difference between the two distributions (a higher q-value indicates a lower grade of the statistical robustness of the significant differences). The results of a non-parametric two-tailed Kolmogorov–Smirnov test are reported.

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under the control of LIF. Indeed, a sequence element responding to STAT3 has been described for NNMT (nicotinamide N-methyltransferase) and LEPR (leptin receptor) (Tomida et al., 2008; Israel and Chua, 2010). These overall observations support data in the literature where a cytokine-homeobox signalling network was necessary for implantation success (Taylor, 2000).

Moreover, regarding the role of apoptosis in the context of the present observations, Tanaka et al. (2011) recently found that some STAT3-activating cytokines, such as LIF, oncostatin M and leptin, enhanced the Fas-mediated apoptosis of human endometrial epithelial cell.

Concerning transcriptional activity, the group of *HOX* genes has been identified as having a crucial role in implantation process (Taylor, 2000, 2002). The genes *MSX1* (Msh homeobox homologue 1), *HOXA10* (homeobox A10), *MSX2* (Msh homeobox homologue 2) and *HOXA11* (homeobox A11), which showed the highest expression in patients with a functional endometrium, are part of the highly conserved *HOX* gene family and share a homeodomain of a 61-amino-acid, helix–turn–helix DNA-binding domain and act as regulator of morphogenesis and differentiation during embryo development (Taylor, 2000). Their role in driving the correct embryogenesis of the uterus and embryo implantation via regulation of down-stream genes is widely attested (Taylor, 2000; Zanatta et al., 2010). Cyclic endometrial expression of *HOXA10*, with a peak of expression during the window of implantation, is observed in response to oestrogen and progesterone in the adult in humans as well as in mice (Taylor, 2000; Daikoku et al., 2004).

Nevertheless, it must be remembered that the majority of studies regarding these genes derive from the murine model. In humans, there is great variability and the effect of these genes is much less defined. *HOXA10* or *HOXA11* mutant mice show implantation and decidualization failure, although they produce normal embryos able to develop in wild-type mice (Satokata et al., 1995). Very recently, a main role for *HOX* genes in endometriosis was also demonstrated, as patients do not demonstrate the expected mid-luteal rise of *HOXA10* expression, which might partially explain the infertility observed in many of these patients (Zanatta et al., 2010). In mice, an independent action for *MSX1* and *HOXA10* has been observed both in normal and mutant *HOXA10* mice; *MSX1* expression increased on implantation day) and dramatically decreased during the following 12 h.

Concerning lipid metabolism, *APOD* (apolipoprotein D) was previously identified as one of the genes with high up-regulation during window of implantation (Kao et al., 2002). The higher expression of *APOD* in the endometria of patients who achieved a pregnancy could be related to the cholesterol transport, probably for steroid hormone biosynthesis or binding.

LEPR is the receptor of leptin, the product of the *ob* gene. The pathway LEP/LEPR seems to play a role in embryo–maternal cross-talk during the window of implantation. In normal human endometria, LEPR concentrations are high during the mid-secretory phase, corresponding to the period of implantation, and LEPR expression is lower in the same phase in women with polycystic ovary syndrome or endometriosis (Wang et al., 2003; Kao et al., 2003). Furthermore, patients with endometrial maturation defects show a deficiency in a functional leptin receptor (Alfer et al., 2000). In the present paper, the higher expression of *LEPR* in patients with a functional endometrium is in accordance with the previous data. Nevertheless, Dos Santos et al. (2012) demonstrated that endometrial *LEPR* expression was higher in patients with implantation failure by IVF whereas leptin expression was significantly lower. The discordance of the conclusions among the different papers indicates the need for further research in this field.

Until now, there has been little evidence to support the role of oxidative metabolism in embryo implantation. Focusing on the imbalance between reactive oxygen species production and antioxidant systems, oxidative stress seems to have a negative impact on reproductive processes (Al-Gubory et al., 2010).

There remains a crucial question to be addressed: can natural cycles be representative of endometrial conditions in stimulated cycles? To date, there are many papers comparing natural and stimulated cycles (Mirkin et al., 2004; Horcajadas et al., 2005, 2008; Simon et al., 2005; Martinez-Conejero et al., 2007; Liu et al., 2008, 2010; Haouzi et al., 2009b, 2010) and it seems that ovarian stimulation can induce some important modifications in gene expression. A correspondence between the pharmacological protocol and the modifications in gene expression has been shown: GnRH antagonists seem to be associated with a gene expression profile more similar to a natural cycle compared with GnRH agonists (Simon et al., 2005; Haouzi et al., 2010).

Because of different methodologies in published studies, it is not simple to draw a final conclusion. Also, it is ethically questionable to perform an endometrial biopsy in a stimulated cycle after embryo transfer during the window of implantation. Some authors have tried to study the endometrial gene expression profile during the same stimulated cycle in which a fresh embryo transfer has been performed (Haouzi et al., 2009b; Van Vaerenbergh et al., 2009; Blockeel et al., 2011). In two of these (Van Vaerenbergh et al., 2009; Blockeel et al., 2011), the biopsies were carried out on the day of oocyte retrieval, before the window of implantation and the endometrial priming of progesterone; in the other (Haouzi et al., 2009b), although the biopsies were performed on the day of embryo transfer, the authors themselves state that the clinical consequences of this procedure are not known. Thus, this problem is by no means solvable.

In conclusion, the present results highlight some groups of genes related to each other by common pathways (e.g. *HOXA10* and *LIF*-dependent pathways) that could represent, if confirmed by broader analyses, the basis of receptive endometrial gene expression profiling, which could be used as a prognostic tool for couples undergoing IVF procedures. The analysis of these pathways could be helpful in the selection of couples who have an optimal chance of pregnancy and could receive elective single-embryo transfer to minimize the risk of multiple pregnancy. On the other hand, some patients with an abnormal endometrial gene expression profile could avoid undergoing IVF cycles with a negative outcome.

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