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Effect of endometriosis on the protein expression pattern of follicular fluid from patients submitted to controlled ovarian hyperstimulation for *in vitro* fertilization

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BACKGROUND: The aim of this study was to evaluate protein expression profile and quantify the proteins present in follicular fluid (FF) samples from women with endometriosis and pregnant women without endometriosis.

METHODS: A prospective case–control study was carried out including women with Stage III or IV endometriosis (Group I) and pregnant women without endometriosis (Group II), both at the maximum age of 35 years. Women were submitted to controlled ovarian stimulation for *in vitro* fertilization, and FF was collected after ultrasound-guided ovarian aspiration. FF from both ovaries was pooled, and patient samples were pooled according to Group I or II. Pooled protein samples were separated and analyzed by MudPIT (multidimensional protein identification technology followed by Expression^E and label-free quantification with ProteinLynxGlobalServer 2.4v, Identity^E and Expression^E software).

RESULTS: A total of 416 proteins or randomic sequence were identified, 62 proteins differentially expressed between Groups I and II. One (1.6%) was expressed at a higher level and 36 (58.1%) were uniquely expressed in Group I, whereas 8 (12.9%) were expressed at a higher level and 17 (27.4%) were uniquely expressed in Group II. Of all these, 15 (24.2%) are related to binding, 1 (1.6%) to immune response, 8 (12.9%) to cell division, 3 (4.8%) to cellular metabolism, 16 (25.8%) to general function and 19 (30.6%) do not yet present an identified function.

CONCLUSIONS: Protein expression profiles of patients with and without endometriosis identified at least 64 proteins differentially expressed, which may be related to the physiopathology of endometriosis. These proteins may additionally be useful in determining potential biomarkers for diagnostics, as well as for therapeutic intervention in women with infertility due to endometriosis.

Key words: biomarkers / endometriosis / IVF / follicular fluid / proteomics

Introduction

During follicular maturation, follicular fluid (FF) provides the microenvironment for the oocyte and contains many substances involved in oocyte maturation, possibly affecting fertilization and embryo development (Schweigert *et al.*, 2006). This microenvironment may be altered by conditions such as endometriosis, resulting in ovulatory dysfunction, poor oocyte quality, reduced fertilization rate, low-grade embryos and reduced implantation rates (Pellicer *et al.*, 2000a; Garrido *et al.*, 2003).

Endometriosis is a chronic gynecological disease characterized by the presence of functional endometrial tissue outside the uterine cavity (Koninckx *et al*., 1991; Liu *et al*., 2008). This disease affects 10–32% of women at reproductive age and can result in pelvic pain and infertility (Ballard *et al*., 2006). The most common form of endometriosis, which accounts for ~55% of all cases, is the ovarian endometrioma (Jenkins *et al*., 1986). Many studies have reported that pregnancy rates are lower in women with endometriosis than in controls, but the mechanism accounting for this difference is poorly understood (D'Hooghe *et al*., 2003; Gupta *et al*., 2008; Tatone *et al*., 2008). Numerous putative mechanisms have been described for decreased fertility, such as altered folliculogenesis (Doody *et al*., 1988) leading to ovulatory dysfunction and poor oocyte quality, as well as luteal phase defects (Grant, 1966), reduced fertilization rates (Wardle *et al*., 1985) and abnormal embryogenesis (Garrido *et al*., 2002).

Recently, the development of high-throughput proteomic technologies (Geromanos *et al*., 2009) has led to the promise of early diagnosis of endometriosis by comparing the protein composition in affected and normal tissues. Aside from accelerating diagnosis, proteomics may lead to a better understanding of the physiopathology of disease development by identifying proteins involved in its different stages (Taylor, 2004) and, ultimately, present targets for endometriosis-specific therapeutic intervention.

Among high-throughput proteomic approaches, multidimensional protein identification technology (MudPIT) is a recently developed, powerful research tool to separate and identify proteins in body fluids (Gonzalez-Begne *et al*., 2009). MudPIT has rapidly become a popular approach for shotgun proteomics since it combines high-resolution separation with tandem mass spectrometry (MS/MS; Washburn *et al*., 2001). In the present study, we therefore used a MudPIT approach via nanoUPLC tandem nanoESI-MS^E to identify proteins with high resolution and accuracy. To our knowledge, this is the first of the proteomics of human endometriosis or in FF using the MudPIT nanoESI-MS^E technology.

This study was therefore aimed to identify and quantify the protein profiles of FF of woman with endometriosis and controls (women without endometriosis who achieved pregnancy), aiming at identifying possible protein targets for diagnostics and therapeutic intervention against endometriosis-derived infertility.

Materials and Methods

Study group

A prospective case–control study was carried out, FF samples obtained from 10 women submitted to *in vitro* fertilization (IVF). This study received the Institutional Review Board approval from the São Paulo Federal University Research Ethics Committee. Patients were subdivided into two groups. The study group consisted of five patients with endometriosis grade III or IV with the presence of peritoneal lesions and ovarian endometriomas, diagnosed by videolaparoscopy (age 32.12 ± 3.41 years, mean ± SD) referred to the IVF program at the São Paulo Federal University. The control group consisted of five women without endometriosis referred to the IVF program and who achieved pregnancy in that treatment cycle (age 31.88 ± 3.28 years, mean ± SD). Controls underwent IVF due to either a tubal factor for female infertility and/or a mild male infertility factor (at least 3 million sperm ml^{-1} and over 5% strict morphology; Kruger *et al*., 1987). Further inclusion criteria for both groups were only couples with a female age of up to 35 years and a serum follicle-stimulating hormone (FSH) level of between 3 and 9 μg/ml on Day 3 of the menstrual cycle previous to the treatment cycle. Finally, only couples who had not been submitted to previous IVF cycles were included in the study.

We only included women who had received a similar ovarian stimulation protocol for IVF. Controlled ovarian stimulation was achieved through the use of exogenous recombinant gonadotrophins (225 IU/day of Gonal-F, Merk-Serono, Darmstadt, Germany) starting on cycle day 2. When the leading follicle reached 14 mm in diameter, endogenous LH release was suppressed by use of a GnRH antagonist analog (Cetrorelix—Cetrotide; Merk-Serono) until the day of hCG administration. When the leading follicle reached 17 mm in diameter, a total dose of 250 μg of hCG was administered. Ultrasound-guided transvaginal oocyte retrieval was performed 36 h after hCG administration. For all patients, FF was obtained from the group of follicles present in each ovary. Ovarian endometriomas were not aspirated for the study.

Protein identification and quantification

Protein concentration was initially evaluated using a Bradford (1976) assay. FF samples from different patients were pooled according to the group and normalized according to the protein concentration. Two pools were formed: (i) women with endometriosis grades III and IV (study group) and (ii) women without endometriosis who achieved pregnancy (control group).

The FF pools and an internal standard (alcohol dehydrogenase, spiked to 50 fmol, SwissProt accession number P00330) were submitted to tryptic digestion. Briefly, the protein (fluid extract) samples were denatured with 0.1% RapiGest^{TM} SF Protein Digestion surfactant (Waters, Milford, USA), reduced (10 mM dithiothreitol), alkylated (10 mM iodoacetamide) and enzymatically digested with trypsin at 1:50 (w/w) enzyme:protein ratio.

NanoUPLC tandem nanoESI-MS^E (MudPIT) conditions

Qualitative and quantitative nanoUPLC tandem nanoESI-MS^E experiments were conducted using either a 1.5-h reversed phase gradient from 5% to 40% (v/v) acetonitrile (0.1% v/v formic acid) at 600 nl/min on a nanoACQUITY UPLC core system. A nanoACQUITY UPLC C18 BEH 1.7 μm, 100 μm × 10 cm column was used in conjunction with an SCX 5 μm, 180 μm × 23 mm column. Typical on-column sample loads were 250 ng of total protein digests. For all measurements, the mass spectrometer was operated in the 'W' mode with a typical resolving power of at least 20 000. All analyses were performed using electrospray ionization in the positive ion mode ESI(+) and a NanoLockSpray source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a GFP solution (100 fmol/μl) delivered through the reference sprayer of the NanoLockSpray source. The doubly-charged ion ($[M + 2H]^{2+}$) was used for initial single-point calibration (L_{teff}), and MS/MS fragment ions of GFP were used to obtain the final instrument calibration. Data-independent scanning (MS^E) experiments were performed with a Synapt HDMS mass spectrometer (Waters, Manchester, UK), which was automatically planned to switch between standard MS (3 eV) and elevated collision energies MS^E (12–40 eV) applied to the trap 'T-wave' CID (collision-induced dissociation) cell with argon gas; the transfer collision cell was adjusted for 1 eV, using a scan time of 1.0 s, both in low-energy and in high-energy CID orthogonal acceleration time-of-flight (oa-TOF) MS^E from *m/z* 50 to 3000. The RF offset (MS profile) was adjusted such that the LC/MS data were effectively acquired from *m/z* 300 to 3000, which ensured that any masses observed in the LC/MS^E

data less than m/z 300 were known to arise from dissociations in the collision cell.

Database searching

Protein identifications and quantitative data packaging were generated by the use of dedicated algorithms (Silva *et al.*, 2005) and searching against a species-specific database (Krämer-Albers *et al.*, 2007). The utilized databases were randomized 'on-the fly' during the database queries and appended to the original database to access the false-positive rate of identification. For proper spectra processing and database searching conditions, a ProteinLynxGlobalServer v.2.4 (PLGS) with an Expression^E informatics v.2.4 license installed was used. A UniProtKB/Swiss-Prot Release 57.1 and a UniProtKB/TrEMBL Release 40.1 database were used and the search conditions were based on taxonomy [*Homo sapiens* (human)], maximum missed cleavages by trypsin allowed up to 1, variable modifications by carbamidomethyl (C), acetyl N-terminal and oxidation (M). Proteins obtained were organized by the PLGS into a list corresponding to a unique protein for both conditions (study or control group), and a logarithmic ratio between the different groups was plotted onto a scatter plot to observe differences between groups. Only proteins in attendance scores and confidence higher than 50% and 99%, respectively, were considered in order to accept these database searches, and when the same protein was identified for different MS/MS fragment ions, those presenting the highest score were considered for comparisons and data presentation (Chambery *et al.*, 2009; Cockman *et al.*, 2009; Levin *et al.*, 2009; Li *et al.*, 2009).

Results

Table I presents clinical results for both groups. Only serum LH was lower in the study group, when compared with the control group. All peptide spectra displaying high-resolution precursor ions were previously de-convoluted into singly charged ions obtained through the nanoLC-MS^E acquisition mode and were selected for cluster analysis. Around 1500 MS/MS experiments were performed, and data were divided into protein data sets (Fig. 1).

Only LH levels were lower in patients from the study group. In FF, a total of 416 proteins and/or randomic sequences were identified for the study and the control groups, of which 39 were randomic sequences and 377 were predicted (cDNA) or observed proteins. Figure 2 demonstrates a scatter plot of the logarithmic values of the average intensity for each protein in the study group (x-axis) and the control group (y-axis). Proteins presenting similar expression values should be mirrored between each side of the regression line, and uncorrelated (thus, differentially expressed) proteins are not mirrored. Because 207 of these proteins were repeated products (proteins identified at different peaks), a total of 170 different proteins were identified in the study (Supplementary Table SI).

Of the 416 initial products observed, 315 were equally observed among both groups (39 randomic sequences and 276 predicted or observed proteins) and 101 products were differentially or uniquely expressed in the study or the control groups (only predicted or observed proteins were identified in this case; Fig. 3). When accounting for repeated products reporting to a same protein, a total of 62 proteins were differentially or uniquely expressed in the study. Of these proteins, 1 was more expressed and 36 were uniquely expressed in the study group, whereas 8 were more expressed and 17 were uniquely expressed in the control group. Regarding function, 15 proteins were related to binding or apoptosis, 1 to immune response, 8 to cell division, 3 to cellular metabolism, 16 to general function and 19 do not yet present an identified function (Tables II and III, Fig. 4).

Table I Clinical characteristics of patients from the study (endometriosis stage III or IV) and control (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) groups.

	Control group	Study group	*P*-value
Age (years)			
Mean; SD	32.2; 2.2	31.8; 3.42	0.830
95% CI	29.5–34.9	27.6–36.1	
FSH ($\mu g\ ml^{-1}$)			
Mean; SD	5.5; 1.4	5; 2.1	0.683
95% CI	3.7–7.2	2.4–7.6	
LH ($\mu g\ ml^{-1}$)			
Mean; SD	5; 1.4	2.6; 1.1	**0.015**
95% CI	3.3–6.8	1.2–3.9	
Follicles (*n*)			
Mean; SD	30.8; 6.5	29.6; 8.7	0.812
95% CI	22.7–38.9	18.8–40.5	
Oocytes (*n*)			
Mean; SD	18.6; 6.5	12; 5.5	0.121
95% CI	10.5–26.7	5.2–18.8	
MII oocytes (%)			
Mean; SD	84.6; 10.5	83.5; 22.2	0.920
95% CI	71.5–97.6	55.9–100.0	
Fertilization rate (%)			
Mean; SD	62.5; 22	79.9; 15.6	0.187
95% CI	35.2–89.8	60.5–99.3	

Bold indicates statistical significance.

To verify interactions between identified proteins and current interactome databases, proteins from our study were submitted by further interactome analysis using Cytoscape (www.cytoscape.org). Figures 5–8 show interaction maps where proteins overexpressed or exclusively expressed in patients with endometriosis are shaded in red, whereas those overexpressed or exclusively expressed in controls are shaded in green. ***Interacting proteins not differentially expressed in our study are shaded in grey.

Endometriosis patients' interactomes demonstrated proteins participating in inflammation and in apoptosis mainly, whereas controls presented an interactome containing transcription factors, oncogenes and proteins participating in cell signaling.

Discussion

FF is comprised of a variety of chemicals, such as metal ions, enzymes, steroids, proteoglycans, hormones and prostaglandins (Yen, 1986). Currently, over 200 different proteins have been identified in FF (Von Wald *et al.*, 2009). FF provides a substrate for oocyte growth,

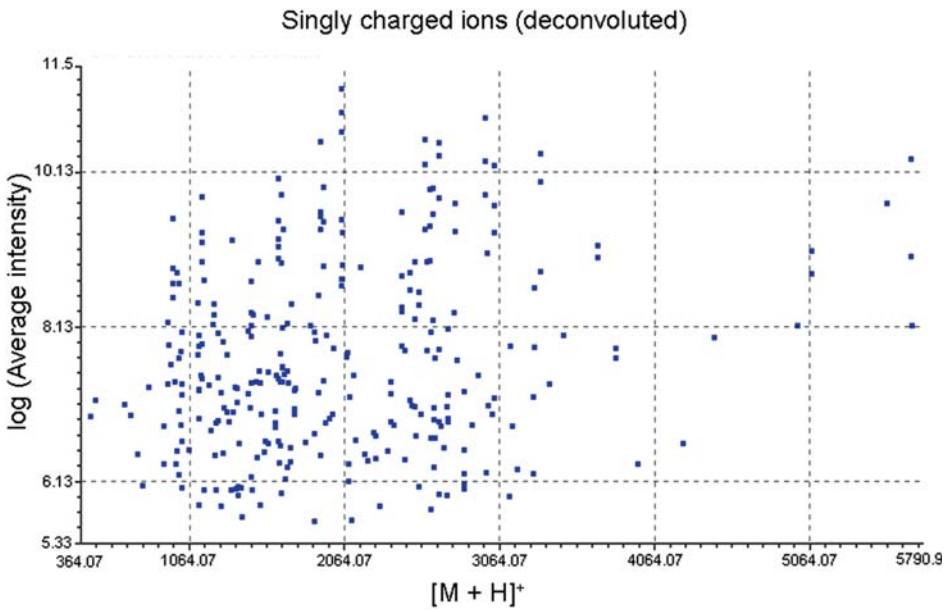

Figure 1 AMRT (accurate-mass retention time) component plot of singly charged peptides found in both groups (deconvoluted) selected for clustering processing.

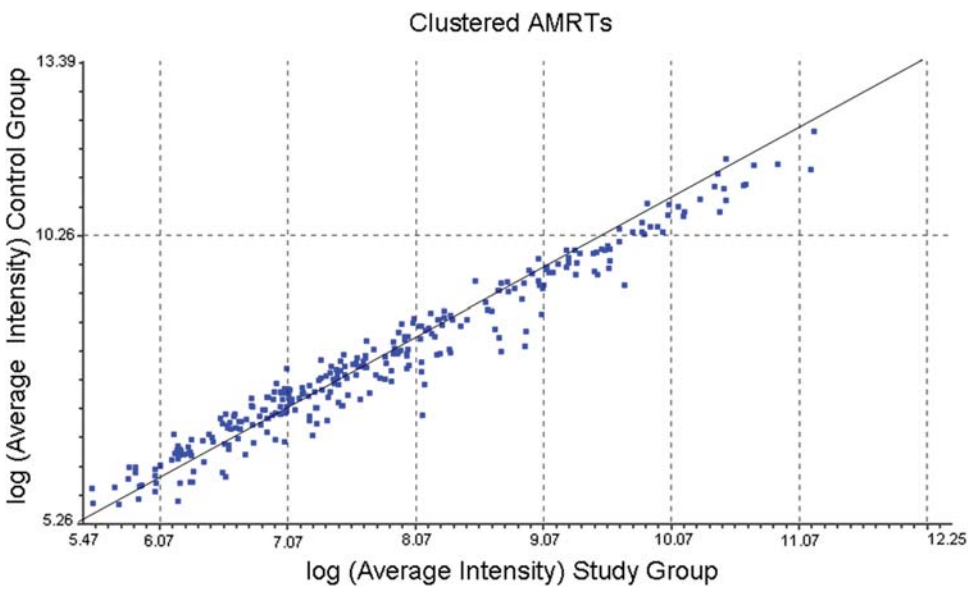

Figure 2 Comparison of clustered AMRTs between human FF (group I) versus human FF (group II) with 1.0 pmol of exogenous protein, demonstrating 417 proteins which were present in groups I and II. For each matched AMRT component, the average log intensity from each condition is plotted along each of the two axes. These data have been filtered using a number of the available statistical measures obtained from the clustering tool of the Expression Informatics software.

and because endometriosis may alter the follicular microenvironment, oocyte quality and embryo development are compromised in this disease (Pellicer *et al*., 2000b). Most studies report that pregnancy rates are lower in women with endometriosis. Nonetheless, specific mechanisms that may account for this decrease are poorly understood (Garrido *et al*., 2003).

A few studies have been performed aimed at determining proteomics of FF. A recent study evaluating FF from the leading follicle

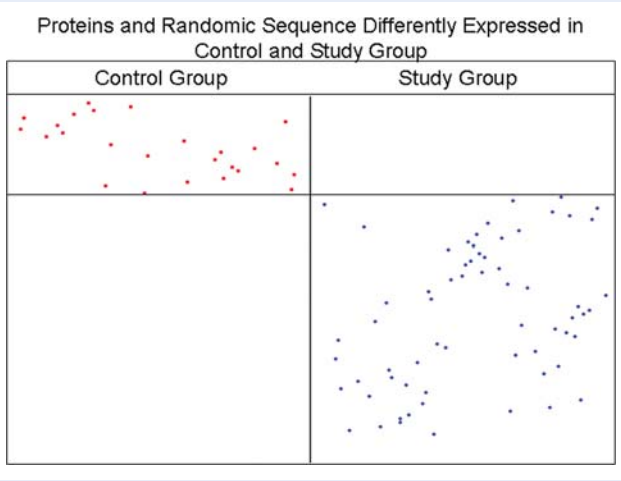

Figure 3 FF expression plot indicating 102 proteins and randomic sequences which were expressed differently in the two groups.

during hormonal stimulation for IVF in women up to 32 years old demonstrated increased expression of haptoglobin, predominantly fetal expressed T1 domain, mitochondrial genome integrity gene, apolipoprotein H, dihydrolipoyl dehydrogenase, lysozyme C, fibrinogen α-chain and Ig heavy-chain V-III region BRO and decreased expression of antithrombin, vitamin D-binding protein and complement 3 in women with successful outcome (live birth). These authors carried out two-dimensional electrophoresis (2D SDS–PAGE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) of FF samples (Estes *et al*., 2009).

Another study evaluating FF from patients with recurrent spontaneous miscarriage including six women (three study and three controls) utilizing 2D SDS–PAGE followed by MALDI-TOF and LC-MS/MS identified that women with recurrent spontaneous miscarriage presented expression of complement component C3c chain E, fibrinogen γ, antithrombin, angiotensin and hemopexin precursor, not observed in controls. This result indicated that these proteins may participate in determining alterations to oocytes and embryos (Kim *et al*., 2006).

Other studies also reported thioredoxin peroxidase-1, transthyretin, retinol-binding protein (Anahory *et al*., 2002), hormone sensitive lipase, apolipoprotein IV (Lee *et al*., 2005)), haptoglobin α-1 and α-2 chains and haptoglobin 1 (Schweigert *et al*., 2006). Moreover, a recent study utilizing different proteomic approaches determined proteins specifically accumulated into or depleted from the FF, when compared with blood plasma, which demonstrated that different approaches may lead to increased capacity to observe protein profiles (Jarkovska *et al*., 2010). To observe proteins present in lower concentrations, the authors depleted FF and blood plasma from some of the more abundant proteins.

In our study, we utilized the MudPIT achieving higher accuracy than other related protocols. When combined with bioinformatics, a larger number of proteins are potentially identified by MudPIT when compared with other proteomic approaches (Steel *et al*., 2005). We were able to statistically identify 416 proteins and randomic sequences in human FF samples, of which 62 were differentially expressed between women with endometriosis group III or IV (study group) and women without endometriosis who achieved pregnancy (control group).

Proteins identified in this study may represent potential biomarker targets for tailored diagnostic and therapeutics according to the oocyte requirements made specific in women with endometriosis. Of the differentially expressed proteins, 24.19% are related to binding and apoptosis, 1.61% to immune response, 12.9% to cell division, 4.48% to cellular metabolism, 25.81% to general function and 30.65% do not yet present an identified function (Fig. 4).

Binding and apoptosis

Twenty proteins identified may be related to binding to DNA, RNA, pro- and anti-apoptotic factors and other proteins. In our study, PBX3 post-meiotic segregation factor, exclusively expressed in controls, may act by increasing expression levels of BMI1 (overexpressed in controls) and may therefore be related to pre-transcriptional control through binding to DNA, ultimately inducing gene expression or silencing. In turn, post-meiotic segregation increased 2-like protein 3, exclusively expressed in the study group, interacts selectively and non-covalently with ATP, a universally important co-enzyme and enzyme regulator (Somervaille and Cleary, 2006; Faber *et al*., 2009).

We also observed 2 proteins overexpressed and 12 only expressed in the study group and 3 only present and 2 overexpressed in controls. Our findings indicate that endometriosis alters substantially the follicular microenvironment, by inducing overexpression of some proteins that have binding as a main function, whereas also partially suppressing expression of proteins participating in regulation of apoptosis. Figure 5 shows an interaction map for FAN (exclusively expressed in endometriosis), a protein participating in the apoptosis cascade. A better overall comprehension of apoptosis pathways in endometriosis may assist in understanding pathogenesis during initial development of the disease.

Immunologic function

Numerous proteins identified in the present study have some relationship with immunologic functions. In samples from the study group, IGL@, IGLC1 and serotransferrin participate in a large immune response cycle (Fig. 6). On the other hand, controls presented expression of IL-2 (Fig. 7).

IL-2 (score: 713.11) is produced by activated T cells and has an immunoregulatory effect on a variety of immune cells (Paul and Seder, 1994). This protein is probably inhibited in endometriosis, an effect that, added to overexpression of the serotransferrin-IGL@ cycle, induces an unbalanced inflammatory response in the follicular microenvironment.

Cell division

Cell division is orchestrated by interaction of numerous proteins. We observed five proteins that have some relation with cell division. Two of these proteins showed a high score: cell division cycle-associated protein 2 (CDCA2; score: 201.52) and TGF-β-activated kinase 1 (TAK-1; score: 311.75).

CDCA2 up-regulation may involve activators of cell cycle progression, DNA replication and repair (Trinkle-Mulcahy *et al*., 2006).

Table II Proteins exclusively or overexpressed in women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle when compared with the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle).

Gene name	Description	Score	Ratio endometriosis:control	Aminoacids
Binding				
PMS2L3	Post-meiotic segregation increased 2-like protein 3	60.04	Only in endometriosis	264
B4E0T2	cDNA FLJ56404, highly similar to peroxisomal targeting signal 1 receptor	69.38	Only in endometriosis	660
B4DZ45	cDNA FLJ52372, highly similar to peroxisomal targeting signal 1 receptor	70.02	Only in endometriosis	654
PEX5	Peroxisomal targeting signal 1 receptor	71.66	Only in endometriosis	639
APBA3	Amyloid-β (A4) protein-binding, family A, member 3 variant	82.7	Only in endometriosis	337
B4DWW0	cDNA FLJ50721, highly similar to peroxisomal targeting signal 1 receptor	112.07	Only in endometriosis	519
Q5HYG5	Putative uncharacterized protein DKFZp686N0152	147.94	Only in endometriosis	110
RIPP2	Protein ripply2	312.04	Only in endometriosis	128
A6NBZ8	Putative uncharacterized protein ALB	500.83	1.04	627
A8K891	cDNA FLJ78290, highly similar to peroxisomal C-terminal targeting signal import receptor	71.66	Only in endometriosis	639
Without specific function				
Q6DHW4	Putative uncharacterized protein	165.23	Only in endometriosis	237
Q6P5S3	Putative uncharacterized protein	165.93	Only in endometriosis	236
Q6GMW6	Putative uncharacterized protein	166.64	Only in endometriosis	235
IGL@	IGL@ protein	166.64	Only in endometriosis	235
Q5CZ94	Putative uncharacterized protein DKFZp781M0386	167.35	Only in endometriosis	234
IGL@	IGL@ protein	167.35	Only in endometriosis	234
IGL@	IGL@ protein	168.07	Only in endometriosis	233
Q6ZP19	cDNA FLJ26714 fis, clone PNC01043	252.49	Only in endometriosis	200
C1 segment	C1 segment protein	372.95	Only in endometriosis	105
Q96E61	Putative uncharacterized protein	165.93	Only in endometriosis	236
Q6GMV7	Putative uncharacterized protein	165.93	Only in endometriosis	236
Q6P2J1	Putative uncharacterized protein	166.64	Only in endometriosis	235
P01842	Ig lambda chain C regions	168.07	Only in endometriosis	105
Q8N355	IGL@ protein	168.07	Only in endometriosis	234
Metabolic function				
PPP1R3B	Protein phosphatase 1 regulatory subunit 3B	66.61	Only in endometriosis	285
General function				
Q56G89	Serum albumin	237.26	1.04	609
B4DPP6	cDNA FLJ54371, highly similar to serum albumin	251.11	1.04	618
B2RBS8	cDNA, FLJ95666, highly similar to *Homo sapiens* albumin (ALB), mRNA	296.77	1.01	609
A8K9P0	cDNA FLJ78413, highly similar to *Homo sapiens* albumin, mRNA	297.26	1.04	608
Q8IUK7	Albumin protein	397.62	1.04	396
Q53H26	Transferrin variant	83.75	Only in endometriosis	698
B4DI57	cDNA FLJ53691, highly similar to serotransferrin	95.56	Only in endometriosis	571
Q1HBA5	Transferrin	116.71	Only in endometriosis	698
Q06AH7	Transferrin	120.91	Only in endometriosis	698
ALBU	Serum albumin	296.77	1.04	609
B4DFB0	cDNA FLJ58357, highly similar to protein FAN	66.13	Only in endometriosis	948
A8K9G4	cDNA FLJ77745, highly similar to sphingomyelinase (N-SMase)	69.29	Only in endometriosis	917
B2RA06	cDNA, FLJ94643, highly similar to sphingomyelinase (N-SMase)	69.29	Only in endometriosis	917
P02787	Serotransferrin	120.91	Only in endometriosis	698

Continued

Table II *Continued*

Gene name	Description	Score	Ratio endometriosis:control	Aminoacids
Cell division				
LRC50	Leucine-rich repeat-containing protein 50	50.51	Only in endometriosis	725
FAN	neutral sphingomyelinase (N-SMase) activation-associated factor	69.29	Only in endometriosis	917
B3KRS8	cDNA FLJ34845 fis, clone NT2NE2011221, cell division cycle-associated protein 2 (CDCA2)	201.52	Only in endometriosis	422
A8K8Z0	cDNA FLJ78 763 cell division cycle-associated protein 2 (CDCA2), mRNA	84.37	Only in endometriosis	1008

Table III Proteins exclusively or overexpressed in the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) when compared with women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle.

Gene Name	Description	Score	Ratio endometriosis:control	Aminoacids
Binding				
PBX3	Pre-B-cell leukemia homeobox 3 (PBX3)	56.42	Only in controls	367
PBX3	Pre-B-cell leukemia homeobox 3 (PBX3)	117.64	Only in controls	176
BMI1	BMI1 polycomb ring finger oncogene	173.48	0.68	184
CN143	Chromosome 14 open reading frame 143, isoform CRA_a	207.51	Only in controls	163
CN143	EF-hand domain-containing protein C14orf143	262.01	Only in controls	163
Without specific function				
Q8TCL9	Putative uncharacterized protein DKFZp564A247	191	Only in controls	142
A6NIZ7	Putative uncharacterized protein TMEM141	201.51	Only in controls	157
B4DW36	cDNA FLJ58862, highly similar to THUMP domain-containing protein 2	246.88	Only in controls	290
Q53TT8	Putative uncharacterized protein C2orf8	268.14	Only in controls	267
B5MD15	Putative uncharacterized protein THUMPD2	295.85	Only in controls	242
Metabolic function				
PLGLA	Plasminogen-related protein A	390.66	Only in controls	68
PLGLA	Plasminogen-related protein A	183.99	Only in controls	96
General function				
B4E1B2	cDNA FLJ53691, highly similar to serotransferrin	91.64	0.88	678
A8K7I7	cDNA FLJ36261 fis, clone THYMU2002695	236.29	Only in controls	303
Cell division				
THUMPD2	THUMP domain-containing protein 2	77.05	Only in controls	473
B0EVZ7	TAK-1-like protein	311.75	Only in controls	87
B0EVZ8	cDNA FLJ31779 fis, clone NT2RI2008204, highly similar to TAK-1-like protein	191	Only in controls	142
B4DFW0	cDNA FLJ60482, highly similar to TAK-1-like protein	199.43	Only in controls	136
Immunologic function				
O95974	IL-2 protein	713.11	Only in controls	41

Ryu *et al*. (2007) observed this gene expression in patients with melanoma. We observed CDCA2 expression only in women with endometriosis (B3KRS8), which seems to indicate that this disease leads to changes in cell cycle progression factors (Gupta *et al*., 2008). Gupta *et al*. (2006) have also suggested that CDCA2 expression may lead to follicular oxidative stress, probably from erythrocytes and apoptotic endometrioma cells (Gupta *et al*., 2006).

TAK-1 is involved in regulation of the TGF-beta-activated kinase function by alternative splicing of its mRNA and by TAK-1-like (TAKL) proteins. We identified TAK-1 as a mitogen-activated protein kinase-like protein activated by TGF-β and BMP signaling (Yamaguchi *et al*., 1995).

TAK-1 may be activated by interleukin -1 (IL-1) and TNF-α (Takii *et al*., 1999; Holtmann *et al*., 2001). Ectopic expression of TAK-1 in

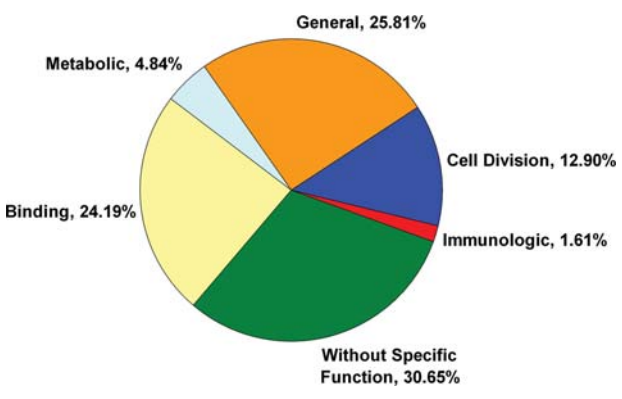

Figure 4 Percentage of proteins according to a function indicating all proteins that were expressed differently in the two groups.

Xenopus embryos causes cell death or ventralization of embryos when apoptosis is blocked by Bcl-2 expression (Shibuya *et al*., 1998). In the present study, we observed TAK-1 only in controls, indicating a likely physiological process.

Metabolic function

The present study observed two proteins that may be related to metabolic functions, plasminogen-related protein A (PLGLA; score: 390.65) and protein phosphatase 1 regulatory subunit 3B (PPR3B; score: 66.61; Fig. 5).

PLGLA may be involved in the regulation of fibrinolysis and thrombosis, and its sequence displays high homology with plasminogen, the precursor of plasmin, which is a fibrinolytic and pericellular proteolytic enzyme. This protein is considered a secreted extracellular protease that degrades extracellular or cell surface components (Judex and Mueller, 2005). Because only controls presented the expression of PLGLA, it may be suggested that endometriosis alters coagulation mechanisms within the follicle (Krikun *et al*., 2008).

PPR3B, which was only observed in endometriotic samples, acts as a glycogen-targeting subunit for phosphatase (PP1) that is a member of the Ser/Thr phosphatases and widely distributed in many organisms. PP1 enzyme regulates many important physiological processes, including gene transcription, translation, metabolism, cell growth and division (Wang *et al*., 2008).

PPR3B facilitates interaction of the PP1 with enzymes of the glycogen metabolism and regulates its activity. It also suppresses the rate at which PP1 dephosphorylates (inactivates) glycogen phosphorylase and enhances the rate at which it activates glycogen synthase and therefore limits glycogen breakdown (Montori-Grau *et al*., 2007). Thus, endometriosis may determine alteration in metabolic pathways as well, limiting follicular glucose uptake by follicular and/or germ cells.

General function

In the study group, we observed 14 proteins with general function, 7 overexpressed and 7 exclusively expressed in this group. In the control group, we observed one protein overexpressed and another one exclusively expressed in pregnant woman without endometriosis. Most of these proteins have cellular transport as the primary function.

Proteins participating in ferric iron transmembrane transport are exclusively found or overexpressed in patients with endometriosis. Endometriosis may be involved with iron influx to the cell interior,

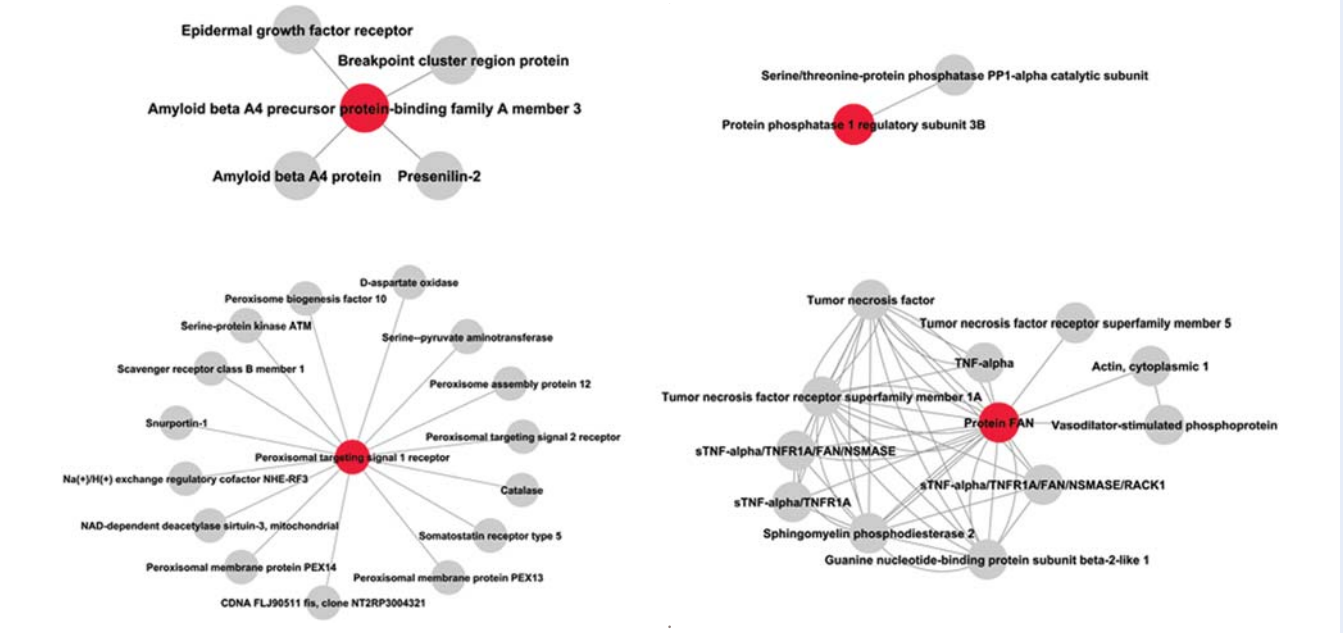

Figure 5 Interactome maps demonstrating principal interactions for amyloid-β A4 precursor protein-binding family A member 3, peroxisomal targeting signal 1 receptor, PPR3B and protein FAN, proteins with cell division, metabolic, binding or apoptotic functions exclusively expressed in women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle when compared with the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle).

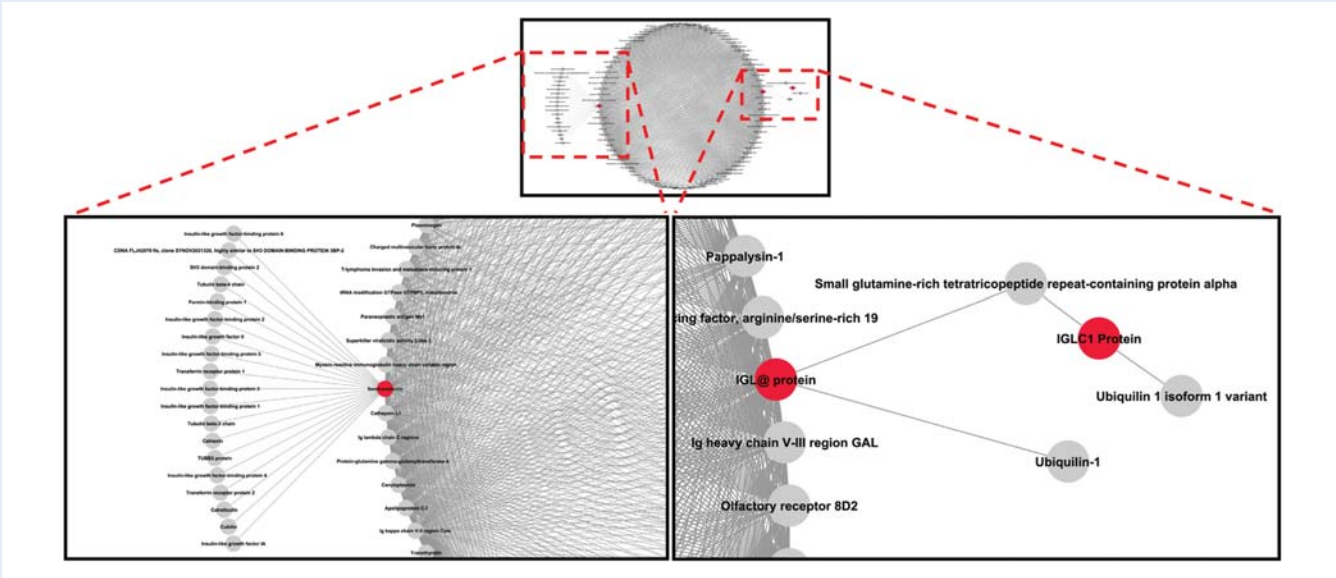

Figure 6 Interactome maps demonstrating principal interactions for Serotransferrin, IGL@ and IGLC1 Protein, proteins with inflammatory or general function exclusively expressed in women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle when compared with the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle).

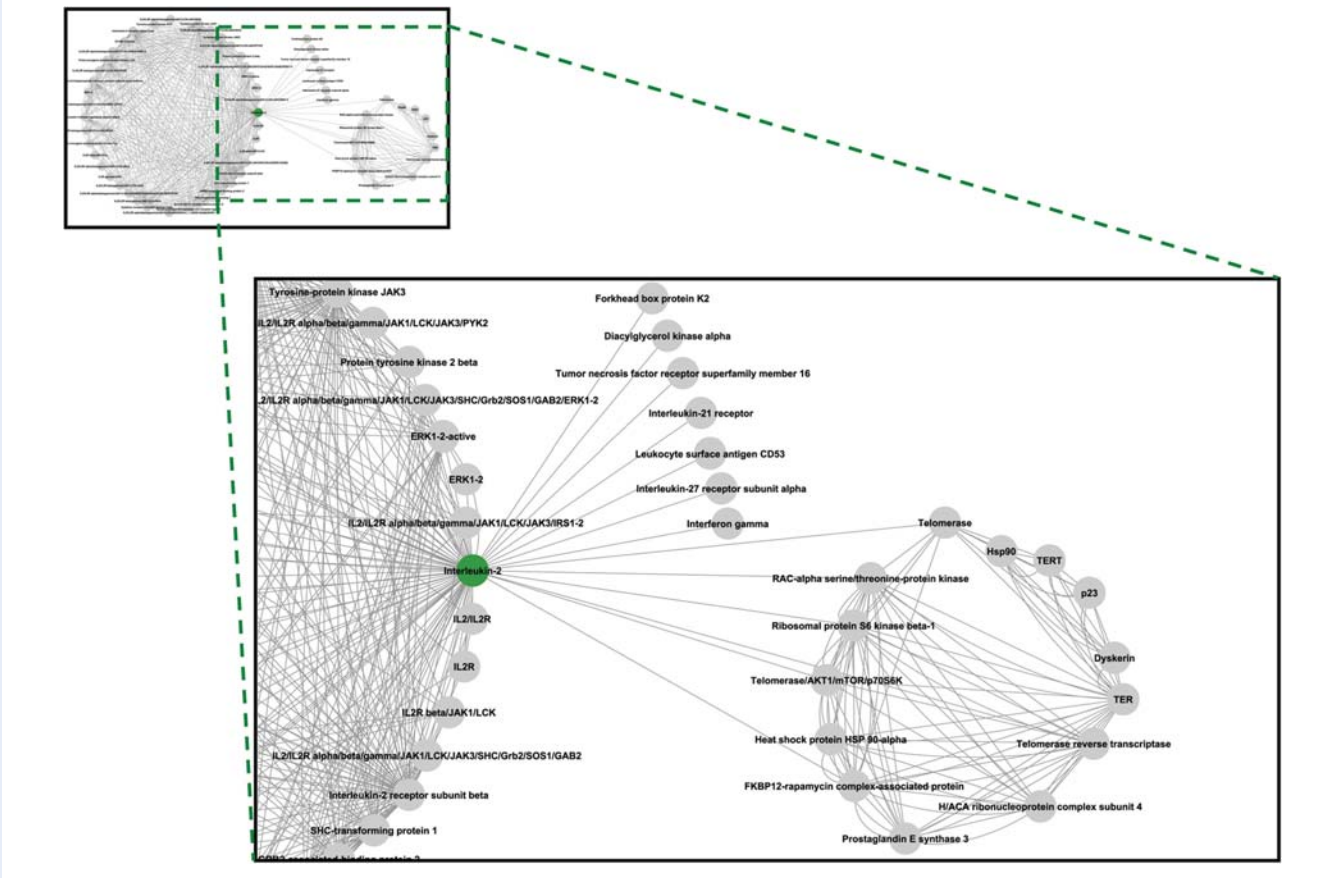

Figure 7 Interactome maps demonstrating principal interactions for IL-2, an inflammatory protein exclusively expressed in the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) when compared with women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle.

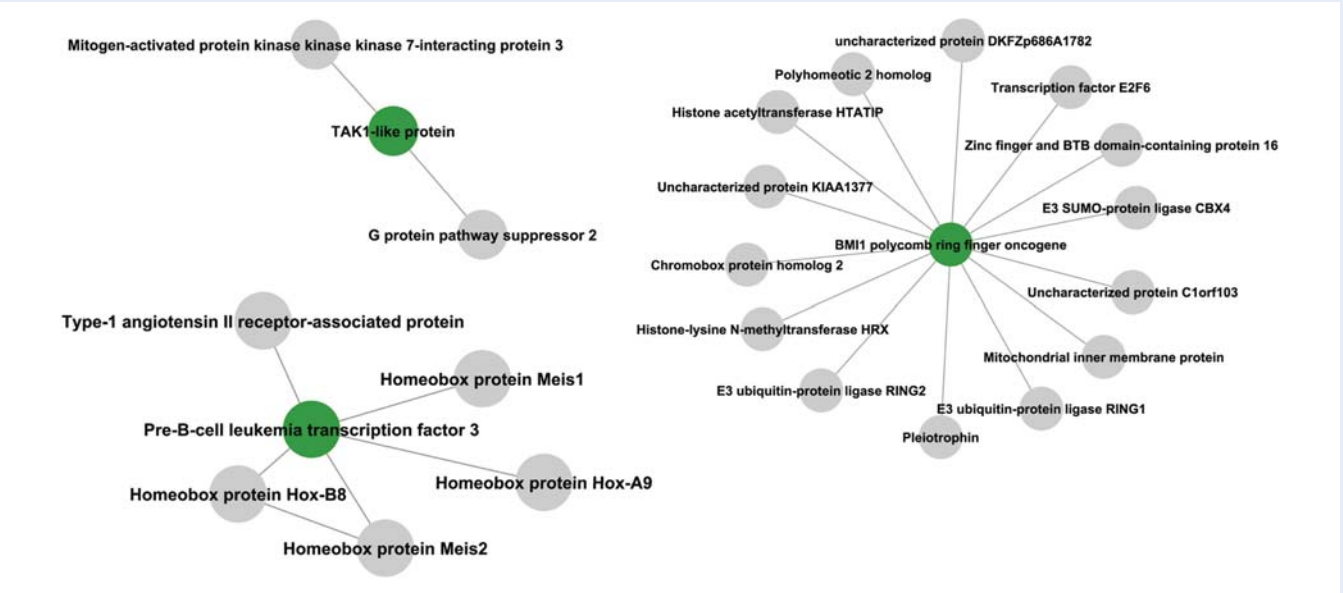

Figure 8 Interactome maps demonstrating principal interactions for TAKL protein, pre-B-cell leukemia transcription factor 3 (PBX3) and BMI1 polycomb ring finger oncogene, proteins with cell division or binding functions exclusively or overexpressed in the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) when compared with women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle.

resulting in the oxidative modification of lipids and proteins, which leads to cell and DNA damage, and subsequently fibrosis development (Kobayashi *et al*., 2009).

A number of different proteins and pathways were observed in this study which may contribute to elucidating endometriosis since this disease determines alterations to oocyte competence and an overall decrease in fertility. Although only five patients were included in each group, we utilized inclusion and exclusion criteria to select samples appropriate to represent each group. Although the sample size is still very small, MudPIT associated with MS^E is a robust and proven technique which helps to identify even small protein differences that may effect alterations under different biological conditions. Because MudPIT-MS^E does not require separation of proteins in 2D gels, a lot of experimental variation is removed. Owing to the dynamic range and selectivity, techniques for depletion of albumin and other abundant proteins were not employed, which also greatly decreases variability. Currently, MudPIT utilizes pooling of samples in groups prior to trypsin digestion. We wish to build larger pools in the future and to also be able to do individual paired analyses, but our results in this study are encouraging in showing that MudPIT-MS^E assists in determining a great number of proteins associated with endometriosis.

Conclusion

Protein profiles of women with endometriosis and pregnant women without endometriosis have been obtained by MudPIT-MS^E analysis. Patients with endometriosis expressed at least 62 proteins that may be related to endometriosis physiopathology. The identification of these potential biomarker proteins may assist in understanding the mechanisms of endometriosis and in determining potential target biomarkers for diagnosis and prevention of endometriosis. This information may also be useful for the search of effective therapies in women with infertility due to endometriosis.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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