FOXO1 and c-jun transcription factors mRNA are modulated in endometriosis

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Endometriosis is a polygenic gynaecological condition affecting 5–15% of women of childbearing age. Major symptoms of the disease are pelvic pain and infertility. No clear link has been established between symptoms and the stage of the disease. Although some aspects have begun to be clarified, clinical understanding of endometriosis remains partial at the molecular level. In this perspective, we targeted isolation of differentially expressed genes in the eutopic endometrial tissue. Our assumption was that the endometrial cells of patients presented an unusual gene expression profile, allowing their implantation and survival in an ectopic site, leading to endometriotic lesions. Here, we report that mRNA steady-state levels of two key transcription factors are modulated in endometriosis. FOXO1 (also known as FKHR) levels were 1.6-fold lower in endometriosis compared to the control group at the onset of the secretory phase (day 15–21), while c-jun mRNA was present at higher amounts in endometriosis (1.5-fold) at the proliferative phase of the menstrual cycle. These results were derived from a large sample composed of 157 control subjects and 209 patients with endometriosis. Gene profiling was conducted by real-time quantitative PCR, and data were quality controlled before statistical analysis. Whether protein levels are affected as well remains to be investigated.

Key words: c-jun/differential display/endometriosis/FOXO1

Introduction

Endometriosis is one of the most common gynaecological disorders, affecting up to 10–15% of women of reproductive age. It is mainly associated with severe pelvic pain and/or infertility, but also with dysmenorrhea, dyspareunia, and several other symptoms, such as intractable bleeding, back pain, constipation and/or diarrhoea. Endometriosis is characterized by the implantation and growth of endometrial cells that normally constitute the lining of the uterus, in extra-uterine sites, most frequently in the peritoneal cavity. The severity of the disease can be graded. According to the American Society of Reproductive Medicine (ASRM) (Schenken, 1998), the disease is classified into four stages, namely, minimal (stage I), mild (stage II), moderate (stage III) and severe (stage IV). Although the aetiology and pathogenesis of endometriosis remain unclear, the theory of retrograde menstruation is the most widely accepted to explain the presence of endometrial cells in ectopic sites.

However, retrograde menstruation occurs in most women. Thus, a certain genetic potential or predisposition, present in the endometrial eutopic cells might be responsible for the presence of the disease. Several studies support the idea that endometriosis is a polygenic disease (reviewed by Simpson et al., 2003).

Initially, this genetic potential may relate to mutations in the genome, but in addition, it may also lead to subsequent altered gene expression. On this basis, many differentially expressed genes have been isolated to date, and some of them have already been extremely helpful to understand better some aspects of endometriosis, in addition to providing disease-related markers. It was initially hypothesized that to become implanted, endometrial cells might exhibit a peculiar extracellular matrix profile. Studies reporting abnormal expression levels for matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (Sharpe-Timms et al., 1998; Bruner et al., 1999) as well as integrins (Lessey et al., 1994) definitely support this hypothesis.

As a fundamental phenomenon originating downstream cascades of abnormal gene expression, unopposed estrogen action has been reported in endometriosis, essentially through clinical observations such as progesterone’s beneficial effect on symptom manifestations. This observation recently found a molecular explanation through modulation of the genes involved in the metabolism of estradiol (E2), cyp19 encoding P450 aromatase (Noble et al., 1996) and 17β-hydroxysteroid dehydrogenase type 2 enzyme (Zeitoun et al., 1998). Furthermore, a variety of potential markers were recently discovered through the use of microarray chips (Kao et al., 2003).

We aimed at expanding the discovery of genes that are modulated in the endometrium of females suffering from endometriosis. Our approach was mainly based on the use of differential display (DD) and cDNA array and the analysis of reported parameters, such as relationship between genes and pathological conditions implicating proliferation, steroid responsiveness and citation index. In the second step, candidate genes were validated on a large number (n = 366) of samples by real-time quantitative RT–PCR. Both the endometriosis patients and the control subjects were enrolled in the study according to strict inclusion criteria. As the technical approach, the real-time PCR was chosen because of its sensitivity and throughput capacities, while inherent variations of this method were extensively measured prior to this validation step and taken into account in the quality-control algorithm applied to the outcome. We report for the first time endometriosis-related modulations in
mRNA steady-state levels of the proto-oncogene c-jun and FOXO1 (Forkhead box, class O) transcription factors. The product of c-jun belongs to the API forming family of proteins acting as immediate early response nuclear factors, while FOXO1 is a member of forkhead pro-apoptotic transcription factors.

Materials and methods

Patient recruitment

Women who provided endometrial biopsies for this study were undergoing laparoscopy for various medical indications such as tubal ligation, tubal anastomosis, hysterectomy and diagnostic surgeries. The inclusion criteria of the study were the following: premenopausal age; not currently menstruating; menstrual cycles between 21 days and 35 days; no acute salpingitis; no prior HIV diagnosis; no prior hepatitis B or C infection; not currently pregnant or in the last 3 months; not currently breastfeeding; no current use of a compound selected from the group consisting of GnRH agonists, progestins, Danazol and oral contraceptives or in the last 3 months; no current use of intruterine device or in the last 3 months. Endometrial biopsies were routinely carried out with a Pipelle, as described by Wagaarachchi and Sirisena (2000).

Patients were subdivided into proliferative (P) and secretory (S) phase groups based on the phase of the menstrual cycle indicated by last menstruation and confirmed by histological examination from a section of formalin-fixed endometrial tissue (Fox, 1995). Subjects ranged from 29 years to 38 years of age. Metrogene’s Ethics Review Board as well as the Internal Review Board of each participating clinical institution approved this study. Endometrial biopsies were obtained from women who had signed a consent form.

Isolation and preparation of RNA from biopsies

A portion of each endometrial biopsy was processed for histological confirmation, and the remainder was immediately transferred in RNAlater™ (Ambion, Inc., TX, USA), maintained on ice during transport from the hospital to our laboratory, and then stored at −80°C until RNA extraction. Total cellular RNA was isolated directly from endometrial biopsies using the RNeasy® Maxi Kit (QIAGEN, Inc., Ontario, Canada), according to the manufacturer’s protocol. Briefly, 300–900 μg of biopsy tissue was rinsed in phosphate-buffered saline, pH 7.4, and then homogenized using an electric homogenizer. The concentration of the purified RNA was determined by spectrophotometry (OD260). For further analysis, equal RNA loading and integrity was confirmed by showing consistent intensities of 28S and 18S rRNA bands on RNase-free agarose gel electrophoresis.

Differential display (DD)

DD was performed according to the protocol of (Liang and Pardee, 1998) using total RNA samples, extracted from 24 P-phase and 24 S-phase endometrial biopsies. Briefly, first strand cDNA synthesis was performed using 200ng RNA in the presence of 0.01 M diethiothreitol (DTT), 20 μM 5NTP, 0.2 μM of anchor primers H-T11M (where M is G or A or C; Genset Corp, CA, USA) and 200 U of M-MLV reverse transcriptase (RT) in the appropriate reaction buffer (Invitrogen Life Technologies, Ontario, Canada). RT reactions were performed in a total volume of 20 μl at 65°C for 5 min, followed by 37°C for 1 h and 5 min at 75°C. S-phase specific DD PCR products were isolated by subjecting 2 μl of the RT reaction to PCR amplification in the appropriate buffer containing 2 μM 5NTP, 1 U rTaq DNA polymerase and α32P-dATP (2500 Ci/mmol) [(all from Amersham Pharmacia Biotech)] and 0.2 μM of anchor and arbitrary primers (Genset Corp). A total of 18 different combinations of primer pairs (3 anchor primers and 6 arbitrary primers) were used for the PCR reactions. Amplifications were done in a Stratagene Robocycler and involved 40 cycles of 94°C (1 min), 40°C (2 min) and 72°C (1 min) with a final extension of 7 min at 72°C.

Equivalent amounts of DNA samples were electrophoresed on a 6% denaturing polyacrylamide gel, and the dried gels were exposed to film. Differentially modulated bands were excised from the gel and DNA was reamplified according to the same PCR reactions described above and cloned using the TA Cloning® Kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. Clones containing the desired fragments were identified by colony-PCR; at least three clones per cDNA candidate were picked and validated by reverse Northern blotting to decrease as much as possible the number of false positive clones. Briefly, this consisted of spotting PCR-amplified DNA fragments purified from the DD gels on two identical nylon membranes (Boehringer Ingelheim Ltd, Ontario, Canada) and to hybridize with two 32P-labelled DD PCR reactions, one from each experimental group, as probes. Confirmed differential signals were kept in the study for the next step. Following Escherichia coli transformation with the cloned DNA fragments into a plasmid vector, three clones per candidate were selected for nucleotide sequence determination, performed using the ABI Prism Rhamidine cycle sequencing kit™ and run on an automated ABI Prism 310™ Sequencer (Applied Biosystems Canada). Nucleotide sequence alignments were performed with Genbank™ through the BLAST software available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nih.gov). The fragment was included into the study if the three sequences were identical with each other.

Quantitative real-time RT–PCR

Total cellular RNA, previously isolated from endometrial biopsies as described above, was used as a template for the production of cDNA. Briefly, 2 μg of RNA was digested in a total of 20 μl with 1 U DNase I (Amplification Grade, Invitrogen Life Technologies), in 20mM Tris–HCl (pH 8.4), 2mM MgCl2, 50 mM KCl at room temperature for 5 min, then at 65°C for 10 min. The reaction was terminated with 2.5 mM EDTA. DNase-treated RNA was then reverse transcribed into cDNA in a total of 50 μl using 400 U M-MLV RT in the presence of 100ng random primers, 10mM DTT, 0.5mM (final) of each of dGTP, dATP, dTTP, and dCTP (all from Invitrogen Life Technologies). Following incubation at 37°C for 1 h, heating to 70°C in the presence of 1mM EDTA for 15 min terminated the reaction.

A relative standard curve (SC) for real-time PCR, representing six 10-fold dilutions of a pool of cDNA was used as a common set of samples that linked the 16 experimental PCR plates together and permitted the overall analyses of 366 samples (see Statistical data analysis below). Preparation of this SC involved pooling 10 μg of each of 10 P-phase and 10 S-phase biopsy RNA samples. The pools were redistributed into aliquots containing 8 μg/tube, and the DNase and RT reactions were carried out as described above, with the proper augmentation of materials corresponding to 8 μg RNA instead of 2 μg. Upon completion of the RT reactions, the cDNA aliquots were pooled, yielding 200 μl of 20X cDNA. This stock was then serially diluted into 6X’, 1X’, 1/6X’, 1/36X’, 1/216X’ and 1/1296X’. These standard curve dilutions were then distributed into 45 μl aliquots and kept at −80°C until use.

FOXO1 and c-jun mRNAs were measured by the SYBR Green® chemistry, while for TATA binding protein (TBP) Taqman® chemistry was used (Applied Biosystems Canada, Ontario, Canada). PCR primers and a fluorogenic probe were designed using primer Express, version 1.5 (Applied Biosystems Canada, Ontario, Canada) to amplify unique sequences in each gene of interest and are presented in Table I. The TBP fluorogenic probe was labelled with the reporter dye 6-carboxyfluorescein (6-FAM) at the 5’ end.

Table I. Oligonucleotide sequences used in the real-time PCR experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>Genebank</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Fluorogenic probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO1</td>
<td>AFO32885</td>
<td>GCCATGTAAGCTCCATCAGGA</td>
<td>ATCGGACAAAGAAGCTGGAAC</td>
<td>N/A</td>
</tr>
<tr>
<td>c-jun</td>
<td>J04111</td>
<td>CAAAGTGGATTGCATCACAAGT</td>
<td>TAAACATTTATAGTGTCAGACACAG</td>
<td>N/A</td>
</tr>
<tr>
<td>TBP</td>
<td>XM_004534</td>
<td>GCCCGAAGCGCCAATA</td>
<td>CTGGGCTCTTCTATCCATG</td>
<td>ATCCCGCCCGTTCCTGCG</td>
</tr>
</tbody>
</table>

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and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end.

Although no major difference was observed between results obtained via Sybyr Green and Taqman chemistries, we decided here to use the Taqman-derived data for TBP, because they were experimentally more homogenous between plates, and therefore allowed the inclusion of a large amount of data in the final analysis after the QC process.

All three primer sets were intra-exonic. Therefore, the risk of a potential PCR signal due to contaminant genomic DNA was highly reduced by the DNase I treatment of all RNA samples prior to the RT step.

The c-jun gene is transcribed into five mRNA species with 5’ end length variations (Hattori et al., 1988). Primers used here were both located at the 3’ end of the c-jun mRNA, covering all five variants.

A large-scale population study of real-time PCR reactions involved eight 96-well plates per phase of the menstrual cycle, where each plate contained duplicates of each dilution of the SC, duplicates of a no template control, and duplicates of the 366 endometrial samples.

**QC algorithm and statistical data analysis**

Cycle threshold (Ct) values were directly used as a measure of gene expression, without conversion into relative copy numbers. Final results (ΔCt) were transformed from exponential into linear space through 2(-ΔΔCt) calculation method (ABI user bulletin #2, 1997). A Ct value is inversely proportional to the initial concentration of a given mRNA, since the higher the level of expression, the lower the number of PCR cycles needed to reach the threshold value of detection of the fluorescent signal.

All Ct values above 35 were excluded from the study because of the accuracy limits of the PCR machine. Briefly, data from each plate were analysed for experimental errors on duplicates and for detection of ‘bad runs’ (Tukey’s mean-difference plot). In the second step, all plates were assembled; SC as well as sample data were compiled in two series of data and analysed in parallel for inter-plate variations and for potential statistical bias in the population distribution.

Quality-controlled data were then submitted to statistical comparison tests according to their distribution. In this report, all distributions were normal, allowing the use of Student’s t-tests for the statistical analysis. Statistics Package for Social Sciences and Microsoft Excel softwares were used for the statistical analysis. For all analyses, P-values of ≤0.05 were considered as the limit of significance.

**Results**

The experimental design of this work was based on the belief that a large number of patients are required to validate possible gene modulation in the eutopic endometrium of patients suffering from endometriosis compared to disease-free individuals. This reasoning was based on two facts. Firstly, the disease is multifactorial with various symptoms and stages, and thus its study requires patient stratification. Secondly, intrinsic inter-individual variation in gene expression needs to be taken into account as a potential source of misinterpretation of gene profiling studies. An integrated measure of such hurdles on a large sample size was predicted to stabilize median values of the final outcome, resulting in more statistically and biologically relevant conclusions.

Another important issue to consider in the data management was the menstrual cycle phase-dependent variations of expression in endometrium. Sex steroid hormones, namely E2 and progesterone, have a tremendous influence on transcription of a large number of genes in this exceptionally plastic tissue. Therefore, experimental data were analysed first between the two phases in the control group. If a significant difference was observed, then data were compared between the control and endometriosis (Endo) populations separately for each phase of the cycle, and even more tightly for the S-phase temporal subcategories on the basis of the histological dating of tissue samples. Due to the physiological nature of the proliferative phase, no dating information is available in this phase.

### FOXO1 as a differentially expressed gene in endometriosis

As a discovery approach to identify differentially expressed genes in endometriosis, we applied the DD technique on total RNA extracted from endometrial biopsy tissues. The endometriosis group consisted of 20 patients representing all four stages of the disease in similar proportions as the entire tissue library. The gene expression profile from patients was compared to that of the control group composed of 10 disease-free subjects. Both P-phase and S-phase of the menstrual cycle were equally represented in these two groups. Visual examination of autoradiograms suggested a lower signal intensity in the S-phase, for four of six bands in 500 in the endometriosis group when compared to five of six bands from the control group (Figure 1). This signal and two others were isolated among approximately 500 cDNA amplicons present on the gel. The differential signal was further confirmed by the reverse Northern approach (data not shown).

Nucleotide sequence analysis of several signals showed 100% identity with the 3’ end of the mRNA encoding the FOXO1 transcription factor (Genbank AF032885).

### Normalization of mRNA levels and data quality control

Quantitative gene profiling data of target genes need to be normalized by the mRNA levels of another gene that does not vary in the condition of interest. Therefore, we aimed at the identification of a gene that showed no significant difference of expression levels between the control and endometriosis groups.

We measured by real-time PCR the levels of TBP mRNA in all the samples of the cohort (n = 366; Table II), and showed no significant difference between the control and endometriosis groups in both phases of the menstrual cycle (Figure 2). Thus, we ascertained that mRNA levels of the genes analysed throughout this report could be normalized with TBP mRNA levels.

Prior to the normalization procedure, data from the analysed gene and from TBP were quality controlled (see Materials and methods section). Therefore, final sample sizes (n) correspond to data successfully filtered for both the marker and the normalizer genes.

### Validation of FOXO1 as an endometriosis marker

In order to validate the differential levels of FOXO1 detected by DD, we measured this specific mRNA by real-time PCR in

**Table II. Composition of initial experimental populations (before QC)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Endo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I/II</td>
<td>III/IV</td>
</tr>
<tr>
<td>P-phase</td>
<td>68</td>
<td>87</td>
</tr>
<tr>
<td>S-phase</td>
<td>89</td>
<td>77</td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>164</td>
</tr>
</tbody>
</table>
the experimental cohort representative of all stages of the disease and in both phases of the menstrual cycle. Data were quality-controlled and normalized on TBP levels. Statistical analyses were conducted as described in the Materials and methods section.

As mentioned earlier, the first step of the data analysis consisted of the comparison of the P-phase and S-phase levels for the control group. A significant increase (1.7-fold, \( P < 0.01 \)) of the FOXO1 mRNA was found in the S-phase compared to the P-phase of the menstrual cycle, as initially suggested by DD (Figure 3A). This result is in agreement with a microarray-based report performed on progesterone-induced decidual fibroblasts (Brar et al., 2001), as well as a semi-quantitative study by immunostaining of the FOXO1 protein (Christian et al., 2002).

Data between the control and endometriosis groups were compared separately in each phase of the menstrual cycle. The comparison of the endometriosis group to the control group, both in the secretory phase, showed a statistically significant decrease of the FOXO1 mRNA in the endometriosis group of 1.3-fold (\( P < 0.05 \), \( n = 73 \) control and \( n = 76 \) endo; Figure 3A). This result confirmed the DD observation described above, although the fold difference is quite subtle. Moreover, when the S-phase data were subcategorized according to the histological dating of the biopsy, namely S1 (days 15–21) and S2 days (days 22–28), a significant decrease of FOXO1 was confirmed with a higher fold decrease (1.6×) in the S1 endo group (\( P < 0.03 \), \( n = 39 \) control and \( n = 33 \) endo; Figure 3B), while the S2 subcategory did not show any significant difference between the two groups. This suggested that the initial 1.3-fold S-phase difference was essentially due to the larger difference (1.6-fold) present in the S1 subgroup.

No statistical difference was noted for FOXO1 mRNA levels between the endometriosis and the control group when compared in the proliferative phase (Figure 3A).

**c-jun modulation in endometriosis**

The c-jun gene was targeted as a prime candidate to be implicated in endometriosis using a proprietary decision algorithm taking into account several factors such as documented links between the given gene products and pathological conditions implicating proliferation, sex steroid hormone responsiveness and citation index. Such an assumption was first tested by performing a real-time PCR analysis on a small subset of samples, suggesting the presence of a positive candidate marker (data not shown). We measured c-jun mRNA levels throughout the menstrual cycle, in samples collected from both control and endometriosis groups of patients.

As the first step of data analysis, when mRNA levels in the control group as reference were compared between the two phases (\( n = 53 \) in P and \( n = 80 \) in S) of the menstrual cycle, a highly significant (\( P < 0.001 \)) increase of 2.6-fold in the S-phase was measured (Figure 4). These results are in agreement with prior data reporting a 3–5-fold increase of c-jun promoter induction by progestins in target cell-lines such as T-47D (Alkhalaaf and Murphy, 1992).

We, therefore, analysed disease-related data separately for P-phase and S-phase populations. When only populations collected at the proliferative phase were compared (\( n = 63 \) CTL and \( n = 97 \) endo), c-jun mRNA levels showed a 1.5-fold increase in the endometriosis group (\( P < 0.01 \); Figure 4). No statistically significant difference was observed in the S-phase between the two groups (\( n = 80 \) control and \( n = 86 \) endometriosis; Figure 4).
In both figures, the fold of statistically significant mRNA decrease in the endometriosis (endo) group is indicated above the corresponding bar. The number of remaining samples (N) after the QC in each group is indicated inside each bar. The Cr value is inversely proportional to the initial concentration of the mRNA. Error bars correspond to 95% CI. P = proliferative; S = secretory.

**Discussion**

In this study we report the modulation of mRNA steady-state levels of two major transcription factors, FOXO1 and c-jun, in the eutopic endometrium of endometriosis patients compared to disease-free (control) individuals. FOXO1 mRNA levels were found to be lower (1.6-fold, \( P < 0.03 \)) in the early secretory phase (between day 15 and day 21) of the menstrual cycle in endometriosis patients. On the other hand, c-jun transcript was present at higher levels (1.5-fold, \( P < 0.01 \)) in the proliferative phase in endometriosis of diseased patients. To our knowledge, this is the first time that both transcription factors have been shown to be differentially expressed in endometriosis on large patient cohorts.

Independent of the disease state, we also report a menstrual cycle phase-specific modulation of both genes, probably under sex steroid hormone transcriptional effect. FOXO1 mRNA was found to be increased by 1.7-fold at the S-phase of the control group (\( P < 0.01 \)). c-jun transcript was shown here to be increased by 2.6-fold in the S-phase of the same control group (\( P < 0.001 \)).

In the disease-free human endometrium, FOXO1 mRNA was reported to be induced by progestins in decidual fibroblast cells (Brar et al., 2001), and to be present at higher levels in decidualizing endometrial stromal cells compared to the proliferative phase (Christian et al., 2002). Our observations are in agreement with the FOXO1 responsiveness towards progesterone. Two reports, both derived from a chip-based approach, do not mention FOXO1 among the differentially expressed genes between the two phases (Kao et al., 2002; Borthwick et al., 2003). This could be due to the lower sensitivity of the chip technique and/or the decision algorithms used in data treatment, or yet the simple fact that a small number of distinct subjects were analysed.

FOXO1 is a transcription factor involved in metabolism, cell cycle, growth, oxidative stress and survival (reviewed by Tran et al., 2003). Its initial isolation was done in a rhabdomyosarcoma in fusion with PAX3 as the result of a genetic translocation (Fredericks et al., 1995). The present knowledge in mammals was built upon the nematode Caenorhabditis elegans model, in which the FOXO1 counterpart, daf-16, was identified (Lin et al., 1997) as being part of a signalling pathway including daf-2 (insulin receptor homologue), and age-1 (phosphatidylinositol 3 kinase homologue; Morris et al., 1996). The high degree of conservation in evolution of this signal transduction pathway supports FOXO1’s central position in cell physiology.

Regulation of expression of the FOXO1 gene has been poorly investigated to date. Nevertheless, a recent study in rat liver showed that fasting and dexamethasone induced FOXO1 mRNA levels by 1.5-fold and 1.8-fold, respectively, while drug-induced diabetes reduced its expression (Imae et al., 2003). It is of interest to note that levels of modulation are comparable with the present result in endometriosis and also to stress that FOXO1 has been linked to different diseases such as cancer (rhabdomyosarcoma, prostate cancer), diabetes and obesity.

Our results do not show whether the observed modulation operates at the transcriptional level. Nevertheless, one can hypothesize that the decrease in FOXO1 mRNA levels observed in endometriosis could be the result of a delay in the onset of progesterone-driven transcription early after ovulation, while later (day 21) FOXO1 mRNA reached comparable levels with the control group of the menstrual cycle. Although there is no clear mechanism to explain the altered responsiveness of target genes towards progesterone in endometriosis, this phenomenon has already been widely reported in the eutopic tissue of endometriosis patients (e.g. Bruner et al., 1999). Abnormal key enzyme levels such as aromatase (reviewed by Bulun et al., 2004), and severe decrease of \( 3\beta \)-HSD mRNA levels, the enzyme responsible for the biosynthesis of progesterone (Kao et al., 2003), in the endometrium of women with endometriosis have been proposed as possible explanations.

Potential consequences of FOXO1 mRNA decrease in endometriosis during the reported temporal window are to be further investigated. Nevertheless, some literature-based evidence supporting the involvement of the FOXO1 gene in endometriosis can be revisited in the context of the present study. Firstly, insulin-like growth factor binding protein-1 (IGFBP-1), which has recently been reported to be present at lower levels in endometriosis (Cunha-Filho et al., 2003), is a target for FOXO1 (Guo et al., 1999). IGFBPs are inhibitory factors modulating IGF’s activity by preventing its bioavailability. Down-regulation of IGFBP5 could well lead to higher levels of proliferation and angiogenesis via IGF’s action. In addition, in non-human primate endometrium, FOXO1 was reported to bind the IGFBP-1 promoter in cooperation with HoxA10 as a heterodimer and induce its expression (Kim et al., 2003, reviewed by Fazleabas et al., 2004). Interestingly, HoxA10 was previously shown to be abnormally expressed in endometriosis. Its expression was reported to increase at the mid-secretory phase in a control group, while this variation was not observed in the endometriosis population (Taylor et al., 1999). This abnormal expression pattern is quite similar to what was observed for FOXO1 and reported in the present work. Secondly, FOXO1 protein was shown to cooperatively bind to the promoter with CEBPbeta and to induce transcription of the prolactin gene, a marker for endometrial decidualization (Christian et al., 2002). Higher modulations for FOXO1 between the proliferative and secretory phases compared to the present observations were recently reported with the microarray approach (Brar et al., 2001). Other than inter-technical possible differences, it has been documented that inter-individual changes in expression levels, based on genetic differences (Oleksiak et al., 2002; Morley et al., 2004) can be high enough to explain, at least in part, the outcome differences between the two reports. It would be of interest to measure these levels on a larger sample size, expected to normalize genetic variations. These issues are common to the growing field of molecular diagnosis, but the major limitation in the field of diagnostic tool discovery for endometriosis is the uncertainty associated with the present diagnosis and classification of the disease. Laparoscopy leading to a visual
scoring of endometriosis according to AFS guidelines are widely practised as the gold standard reference, but is nonetheless quite inaccurate. This controversial paradigm, therefore, constitutes a fragile basis to all marker discovery approaches and the presented work herein does not escape this reality. Again, the use of a large number of samples is the safest approach to limit the impact of this inevitable inaccuracy.

As of c-jun, its expression has been studied in the human endometrium and appears to follow mainly the levels of the estrogen receptor (Salmi et al., 1996). c-jun’s promoter was shown to be induced by estrogens in rat endometrium (Cicatiello et al., 1992; Hyder et al., 1995) and differentially controlled by progesterone (Alkhalaf and Murphy, 1992; Salmi et al., 1998; Dai et al., 2003). c-jun proto-oncogene encodes one of the AP1 transcription complex major components, and as an immediate early response gene, has a wide-range action in different tissues and physiological states, such as proliferation, transformation and death (reviewed in Shaullian and Karin, 2002). C-jun protein is activated through Jun N-terminal Kinase phosphorylation. When over-expressed, c-jun was described to confer on the MCF-7 cell-line invasiveness and higher motility, as well as induction of some extracellular matrix markers such as MMPs (Smith et al., 1999).

Abnormal c-jun activity was reported in different disease situations, such as cellular transformation and tumorigenesis (reviewed by Vogt, 2002), and neurodegenerative disorders (reviewed by Bozychko-Coyne et al., 2002). Danazol, one of the most widely used therapies for endometriosis, showed a preventive effect in an endometrial carcinogenic mouse model through estrogen-specific c-jun down-regulation (Niwa et al., 2000). TNFα, also reported to act in endometriosis (Braun et al., 2002; D’Hooghe et al., 2003), activates specifically AP-1 and NFκB to induce survival, inflammation and cell growth (reviewed by Leong and Karsan, 2000).

Overall, we have demonstrated that in normal individuals, eutopic endometrial FOXO1 and c-jun mRNA levels are increased in the S-phase compared to the P-phase of the menstrual cycle. We also reported that these normal cycle-specific modulations are disturbed in patients suffering from endometriosis. Hence, the normal FOXO1 increase accompanying the early S-phase (days 15–21) is reduced in endometriosis patients, while the normal increase in c-jun mRNA levels observed in the S-phase is less pronounced in endometriosis patients. As mentioned, modulations at the protein level and functional studies are now required to unequivocally link FOXO1 and c-jun with endometriosis. Furthermore, identification of target genes downstream of these two transcriptional factors would allow better understanding of their respective roles in the aetiology of endometriosis.

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FOXO1 and c-jun in endometriosis