Global gene analysis of late secretory phase, eutopic endometrium does not provide the basis for a minimally invasive test of endometriosis

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BACKGROUND: Endometriosis occurs in 10% of women and is currently diagnosed by invasive laparoscopic testing. We tested the hypothesis that endometrial gene expression in late secretory phase endometrium differs between patients with and without endometriosis. METHODS: Ten patients with laparoscopically proven endometriosis (minimal/mild n = 5 and moderate/severe n = 5) and six controls, underwent endometrial biopsy in the late secretory phase (Day 23 onwards). Microarray interrogation of eutopic endometrial gene expression was performed. RESULTS: Microarray data were obtained for all control samples and eight samples from the endometriosis patients (n = 4 minimal/mild, n = 4 moderate/severe disease). Eight genes were identified as up-regulated and one gene was down-regulated in all endometriotic samples (more than 1.75-fold, P < 0.01). Real-time PCR analysis of protocadherin-17 (PCDH17), protein tyrosine phosphatase, receptor type, R (PTPRR) and interleukin-6 signal transducer (IL6ST) expression validated the microarray findings. CONCLUSIONS: Expression of very few transcripts differs, in late secretory eutopic endometrium, between controls and patients with endometriosis. The median fold changes of these genes are small. No transcripts were identified that could discriminate between minimal/mild and moderate/severe endometriosis. Therefore, interrogation of the late secretory endometrial transcriptome is not likely to form the basis of a minimally invasive diagnostic test for endometriosis.

Keywords: endometrium; endometriosis; protocadherin-17; protein tyrosine phosphatase, receptor type, R; microarray

Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity and occurs in 6–10% of women in the general population and 35–50% of women with pain and/or infertility (Eskenazi and Warner, 1997). These women may present with dysmenorrhoea, dyspareunia, chronic pelvic pain with or without subfertility (Rice, 2002). Currently, laparoscopy is the gold standard method of diagnosing endometriosis (Kennedy et al., 2005); however, there is an urgent need to develop minimally invasive diagnostic tests.

A number of studies have utilized large-scale genomics techniques to compare eutopic and ectopic endometrial lesions (Eyster et al., 2002; Lebovic et al., 2002; Arimoto et al., 2003; Matsuzaki et al., 2004, 2006; Hu et al., 2006; Wu et al., 2006; Mettler et al., 2007); however, only two studies have compared eutopic endometrial gene expression in biopsies from women with endometriosis and controls (Kao et al., 2003; Matsuzaki et al., 2005). These two studies identified a large number of genes in the early and mid-secretory phases of the menstrual cycle, whose endometrial expression differed between women with and without endometriosis. Neither of these studies exclusively analysed late secretory phase endometrium and so gave little insight into the potential use of genomics interrogation of the late secretory endometrial transcriptome, in diagnosing endometriosis. We chose to analyse late secretory phase samples as a well characterized sample set had been collected and this would address an unanswered question. We used oligonucleotide microarrays to test the hypothesis that endometrial gene expression in the late secretory phase differs between patients with laparoscopically proven endometriosis and those without disease. We also sought to identify transcripts that could discriminate between women with minimal/mild and moderate/severe disease.
Methods and Materials

Tissue collection
Ten patients with laparoscopically proven endometriosis (five women with minimal/mild and five women with moderate/severe disease as classified by the modified AFS scoring system) and six controls, who were laparoscopically proven not to have endometriosis, were recruited from the Leuven University Fertility Center. Written informed consent was obtained from all patients and the study was approved by the local research ethics committee. All subjects and controls reported regular menstrual cycles (cycle length 25–32 days) and no women had received hormonal preparations in the 3 months preceding biopsy collection. Endometrial biopsies were obtained in the late secretory phase using a pipelle sampler (Pipelle Laboratories CCD, Paris, France). All biopsies were divided into two pieces, one part being flash frozen in liquid nitrogen for RNA isolation and the second fixed in formalin for routine histology. Biopsies were dated according to the stated, last menstrual period and dating was confirmed by histological assessment, to be Days 23–26 of the cycle, according to published criteria (Noyes et al., 1950). Total RNA was extracted from each endometrial tissue using Trizol Reagent (Invitrogen Life Technologies, Carlsbad CA, USA) and RNA quality was assessed by loading 200 ng of total RNA onto an RNA Labchip and analysis on an A2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany).

For evaluation of gene expression changes throughout the menstrual cycle, additional endometrial samples were collected from 36 normal fertile women attending Addenbrooke’s Hospital, Cambridge for sterilization. These women were not using hormonal contraception or IUCD, had regular menstrual cycles and were laparoscopically proven not to have endometriosis. These endometrial biopsies were taken throughout the menstrual cycle and histologically dated according to the Noyes criteria (Noyes et al., 1950). Written informed consent was obtained from all patients and this study was approved by the local research ethics committee.

Microarray gene profiling
Microarray analysis was performed using a custom made array (Microarray Core Facility, Department of Pathology, University of Cambridge) containing oligonucleotides specific for 22,000 different human transcripts (for further details of the array see http://www.path.cam.ac.uk/resources/microarray/microarrays/humanrefset23k.html). A reference cDNA was made from pooled endometrial total RNA samples from two of the control and four of the endometriotic, eutopic endometrial RNA samples. The pooled reference cDNA was labelled with Cy3-deoxyuridine triphosphate. Endometrial samples taken from control patients and from those with endometriosis were labelled with Cy5-deoxyuridine triphosphate (Amersham-Pharmacia, Little Chalfont, UK), using a BioPrime DNA Labelling kit (Invitrogen, Paisley, UK). Each Cy5-labelled control or endometriotic sample was hybridized from the two endometriotic samples (n = mild/mild, n = moderate/severe disease). cDNA from the two endometriotic samples that gave discordant array data had not been used to generate the common pooled reference. Eight genes were identified as down-regulated using the same criteria (Table I). No transcripts were identified as significantly different between the minimal/mild and moderate/severe disease groups.

In order to validate changes in abundance of these RNA transcripts, real-time RT–PCR was performed for protocadherin-17 (PCDH17), protein tyrosine phosphatase, receptor type, R (PTPRR) and interleukin-6 signal transducer (IL6ST). Real-time PCR analysis using the same RNA samples as used for the array analysis, confirmed a statistically significant increase in the expression of these genes in the eutopic endometrial biopsies from women with endometriosis.

Array analysis
Hybridization signals were quantified using BlueFuse 2.1 software (BlueGnome Ltd, Cambridge, UK). The raw spot intensity data were normalized per spot and per chip using GeneSpring 7.2 software with intensity dependent (Lowess) normalization (percentage of the data used for smoothing 10% and cut-off value of 0.01). Spots with poor morphology and low hybridization signals were removed using default confidence values with the BlueFuse software. Genes that showed statistically significant changes were identified using the parametric Welch t-test, using a Benjamini and Hochberg false discovery rate correction for multiple testing.

Real-time RT–PCR
Array verification was preformed by real-time RT–PCR analysis of selected genes using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Warrington, UK). For ribosomal 18S RNA, pre-validated primers and probes were purchased (Applied Biosystems: hs99999901_s1). Primers for real-time RT–PCR using SYBR Green detection for IL6ST (Forward: tgtatcagacgctaaccag, Reverse: gcatttgctctctgctaagttcc) and PTPRR (Forward: gcaagttcatcatcttactcaca, Reverse: catgcgggctataacattc) were designed using Primer Express v5.0. Primers and probes for real-time ‘Tagman’ PCR assay of PCDH17 abundance (Forward: aagccggtcaaatgtgatcgtc, Reverse: tgaaggctttcaggaatc, Probe: tgttgaggctcagctatacagct) were designed with the same software. Real-time PCR analysis was performed as previously described (Sherwin et al., 2007). Expression levels in endometrial biopsies from the control and endometriotic patient groups patient were compared using the non-parametric Mann–Whitney test; statistical significance was accepted when P < 0.05.

Results
Identification of genes dysregulated in late secretory, eutopic endometrium of women with endometriosis
Endometrial cDNAs from controls (n = 6) and patients with endometriosis (n = 10) were hybridized to oligonucleotide arrays and the detected intensity signals were normalized and statistically analysed using GeneSpring 7.2 software. Array data from two patients with endometriosis were excluded from subsequent analysis as the expression profiles from these arrays were not representative of the other samples within the disease group (see Supplementary Data). These outliers were not the result of either uncorrected image artefacts or abnormal hybridization of the common reference probe. Therefore, microarray data were obtained for six control and eight endometriotic samples (n = 4 minimal/mild, n = 4 moderate/severe disease). cDNA from the two endometriotic samples that gave discordant array data had not been used to generate the common pooled reference. Eight genes were identified as up-regulated in all endometriotic samples, by more than 1.75-fold (P < 0.01) and one gene was identified as down-regulated using the same criteria (Table I). No transcripts were identified as significantly different between the minimal/mild and moderate/severe disease groups.
compared with that from controls (Mann–Whitney test; \( P \leq 0.05 \); see Fig. 1). PCDH-17, PTPRR and IL6ST expression increased significantly, 2.4-fold (\( P = 0.0007 \), 4.2-fold (\( P = 0.008 \)) and 1.4-fold (\( P = 0.04 \)), respectively. Real-time RT–PCR was also performed for cysteine-rich secretory protein 3 (CRISP3), a gene transcript whose expression was shown by microarray analysis, not to differ between control and endometriotic eutopic endometrium. This confirmed that expression of CRISP3 was not different between the control and endometriotic samples (data not shown).

**Endometrial expression of PCDH17 and PTPRR during the menstrual cycle**

Quantitative analysis of transcript abundance for PCDH-17 and PTPRR has not previously been reported in human endometrium. Real-time RT–PCR was used to examine endometrial expression of PCDH-17 and PTPRR throughout the menstrual cycle, in menstrual (\( n = 7 \)), proliferative (\( n = 11 \)) and secretory (\( n = 18 \)) phase biopsies. Median gene expression for PCDH-17 increased 6-fold (\( P = 0.0006 \)) and PTPRR increased 47-fold (\( P = 0.0002 \)) from the proliferative to the secretory phase (Fig. 2a and b).

**Discussion**

This study has demonstrated that the expression of only a relatively small number of genes differs in late secretory phase endometrium, between patients with endometriosis and controls. We have also shown that the median fold changes of these genes are small. Previous studies have shown that gene expression in eutopic endometrium from women with endometriosis differs significantly from those who are disease free (Kao et al., 2003; Matsuzaki et al., 2005). Kao et al. (2003) studied global gene expression in mid-secretory phase eutopic endometrium (LH+8 to +10) from fertile controls (\( n = 7 \)) and women with mild/moderate endometriosis (\( n = 8 \)). Using Affymetrix Hu95A oligonucleotide arrays representing 12 500 genes, 91 genes were up-regulated and 115 down-regulated, more than 2-fold/\( P < 0.05 \). Northern blot analysis of endometrium from two patients with endometriosis and two controls validated the up-regulation of six genes. However, there was little correlation between the northern blot and microarray estimates of gene expression fold change. Matsuzaki et al. (2005) compared global gene expression in eutopic endometrium, from controls and patients with deep endometriosis, at various time points throughout the menstrual cycle. Laser capture microdissection of glandular epithelium and stroma was performed and transcript abundance was assayed using Clonetech 1.2k cDNA arrays. No genes were up- or down-regulated in all phases of the cycle, in either tissue compartments. The largest number of differentially expressed genes was found in the early secretory phase (Day 15–18) of the menstrual cycle, where 39 genes were dysregulated in the epithelium and 51 genes were dysregulated in the stroma. In the late secretory phase endometrium, 20 genes were up-regulated and 5 were down-regulated in the epithelial compartment and 2 genes were up-regulated and 1 was down-regulated in the stromal compartment. None of the genes from this study that had been identified as differentially expressed in either the stromal or epithelial compartments was shown to be differentially expressed in our study, even though the transcripts were present on our array. This may be due to heterogeneity of eutopic endometrium from patients with endometriosis or the relative contribution that the epithelial and stromal transcriptomes make to whole tissue gene expression, as assayed in our study.

A recent study by Burney et al. (2007) studied proliferative, early secretory and mid-secretory eutopic endometrium (up to Day 23), from women with endometriosis and controls. These authors found that endometrial gene expression differed most, between these groups, in the early secretory phase (Days 15–18), where 85 transcripts were altered more than 4-fold. They found far fewer differences in the mid-secretory phase where no transcripts were found to be up- or down-regulated 4-fold. They did not examine later than Day 23 in the menstrual cycle. The fact that we found relatively few differentially expressed genes in eutopic mid- to late secretory phase endometrium when comparing patients with endometriosis and controls is in line with these recently published findings. The molecular phenotype of mid- and late secretory, eutopic endometrium from women with endometriosis and from controls appears to be very similar.

We recently published data from experiments that adopted a candidate based approach to identify genes that are dysregulated in luteal (\( n = 20 \)) and menstrual phase (\( n = 15 \)), eutopic endometrium of patients with endometriosis (Kyama et al., 2006a). The expression of interleukin-8 (IL-8), tumour necrosis factor alpha (TNF-\( \alpha \)) and matrix metalloproteinase 3 (MMP-3) were increased in menstrual phase, eutopic endometrium of women with endometriosis. No significant differences in

<table>
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<th>Gene name (gene symbol / gene ID)</th>
<th>GenBank accession</th>
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<th>( P )-value</th>
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<td>2.17</td>
<td>0.00237</td>
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Microarrays of late secretory endometriosis
gene expression were found in luteal phase biopsies, for any of the eight candidates that were tested. In a separate study, using surface enhanced laser desorption/ionization time of flight mass spectroscopy (SELDI-TOF MS), we have identified a number of proteins/peptides that are present in luteal phase, endometrial biopsies from patients with endometriosis, but absent in endometrium from matched controls (Kyama et al., 2006b). The identity of these proteins and peptides is currently being established.

None of the genes that have so far been identified as differentially expressed, in eutopic endometrium from women with endometriosis compared with controls, has been identified in more than one study. Obviously, the choice of microarray platform and post-hybridization analysis will affect the composition of any microarray gene list (Sherwin et al., 2006) and given the rapid changes in endometrial gene expression that occur throughout the menstrual cycle (Kao et al., 2002; Borthwick et al., 2003), the phase of the menstrual cycle, in which the endometrial biopsy is taken, will significantly affect the number of gene differences that are detected. Also minimal endometriosis may be an intermittent condition (Harrison and Barry-Kinsella, 2000) and so the effects of ectopic lesions on eutopic endometrium may vary with time, in the same patient. Therefore, in some women with endometriosis, eutopic endometrial gene expression may not differ from women who are disease free, as is evidenced by the spectrum of clinical, endometrial dysfunction that is observed (Barnhart et al., 2002).

From the small number of genes that were identified in our microarray analysis, there are some candidates that may offer insight into the aetiology of endometriosis. PTPRR is a member of the protein tyrosine phosphatase family of signalling molecules that regulate a variety of cellular processes including cellular growth, differentiation and mitosis (Neel and Tonks, 1997). PTPRR contains a 16-amino-acid, kinase-interaction motif that binds specifically to, and inhibits, extracellular-signal-regulated kinase 1/2, a member of the mitogen-activated protein kinase family (Munoz et al., 2003). PTPRR is expressed predominantly in brain, but is also found in cartilage (Augustine et al., 2000), and alternative splicing produces transmembrane and cytosolic variants. The rat orthologue of this gene is regulated by the nerve growth factor, which suggests a role for PTPRR in neuronal growth and differentiation (Sharma and Lombroso, 1995). The identification of specific inhibitors of PTPRR function (Barr and Knapp, 2006) raises the possibility of novel treatments for PTPRR mediated diseases. PTPRR is known to be expressed in the cervix; however, this current study is the first report showing endometrial expression of the receptor. It can be speculated that the up-regulation of PTPRR in late secretory phase endometrium of women with endometriosis compared with controls, may prevent normal differentiation of endometrial cellular function and may be a predisposing factor in the aetiology of endometriosis.

We have identified a small number of genes whose endometrial expression in late secretory phase of the cycle differs between women with endometriosis and controls. One of the limitations of our study is the small number of biological
replicates that were arrayed. We did not perform sample size estimations, as no pilot data were available for late secretory phase endometrial gene expression, in controls and patients with endometriosis. Larger numbers in each sample group would increase the power of the study and reduce the false discovery rate (Jorstad et al., 2007). However, previous studies using similar sample sizes (Horcajadas et al., 2004; Matsuzaki et al., 2005; Burney et al., 2007) were sufficient to identify transcripts that discriminate between women with endometriosis and controls.

We have verified that median fold changes of the differentially expressed genes are small and as such it is very unlikely that analysis of late secretory phase endometrial gene expression is able to form the basis of a minimally invasive diagnostic test for endometriosis. Second, we found no significant evidence at this time of the cycle that eutopic endometrial gene expression differs between women with minimal/mild and moderate/severe endometriosis. In light of our data and the recent publication by Burney et al., which shows major differences in endometrial gene expression, between controls and women with endometriosis, in the early secretory phase of the cycle, we suggest that current efforts to develop minimally invasive diagnostic tests for the presence of endometriosis and also tests to distinguish minimal/mild and moderate/severe disease, by sampling the endometrium, should be focused on the early secretory phase of the menstrual cycle, where the presence of endometriosis, appears to maximally perturb eutopic endometrial gene expression.

Acknowledgements
We should like to thank our colleagues in the microarray core facility, Department of Pathology, Cambridge University for microarrays and technical support.

Conflict of interest: TD is or has been an advisor/consultant/research collaborator to the following pharmaceutical companies: Merck-Serono, Ferring, Centocor, Pfizer, Ipsen, Organon, Schering and Genentech. He currently holds the Serono Chair for Reproductive Medicine at Leuven University, Belgium. The other authors have nothing to disclose.

Funding
This study was supported by the RCOG Millennium Research Fund; Microarray Core Facility is supported by the BBSRC of the UK (BBSRC grant no 8/EGH16106); Meres senior research fellowship from St John’s College, Cambridge to A.M.S.

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Submitted on May 13, 2007; resubmitted on February 11, 2008; accepted on February 22, 2008