Cyr61, a deregulated gene in endometriosis

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Introduction

Endometriosis is characterized by the presence of endometrial tissue fragments outside the uterine cavity. It is one of the most common gynaecological diseases and affects >10% of all pre-menopausal women (Mahmood and Templeton, 1991). Though considered a benign gynaecological disorder, endometriosis causes pelvic pain, dysmenorrhoea and is often accompanied by infertility (Schweppe, 1990; Garrido et al., 2002). The aetiology and exact mechanisms for the development of endometriosis are unclear, but implantation of viable endometrium refluxed into the peritoneal cavity during menstruation may explain its origin (Thomas, 1993). It is thought that retrograde menstruation occurs to some extent in almost all women, without necessarily leading to the development of endometriotic lesions. This and the frequent reappearance of endometriotic lesions within 1 year after apparently successful hormonal and/or surgical treatment (Wheeler et al., 1993; Regidor et al., 1996) suggest that cell-physiological differences between endometria of women with and without endometriosis play a role in this disease. Differences in hormonal regulation of the endometrium resulting in an abnormal gene expression pattern may be responsible for adhesion and invasion properties of the shed endometrial fragments. Based on the observation that endometriosis is often associated with infertility, it has been hypothesized that the disease is caused by an inappropriate differentiation of the endometrium. Indeed, nearly 60% of women examined for fertility problems are diagnosed with endometriosis (Ledger, 1999).

Symptoms do not appear before menarche and they typically regress spontaneously after menopause (Thomas, 1995), suggesting an important role for estrogen in the establishment and maintenance of endometriosis. In an attempt to elucidate the mechanisms responsible for endometriosis, we sought to identify genes that were differently regulated between eutopic endometrium of ‘normal’ and ‘endometriotic’ women using the microarray technology. Among the genes identified as differently regulated, we focused on the highly up-regulated cysteine-rich 61 (Cyr61) gene. Cyr61 belongs to a recently discovered family of conserved and modular proteins with diverse functions (Babic et al., 1998, 1999). Cyr61, connective tissue growth factor (CTGF) and gephroblastoma overexpressed (NOV) were the first family members identified, hence the name CCN family (Cyr61, CTGF, NOV) (Bork, 1993). The family now consists of six distinct members which are all secreted, extracellular matrix-associated proteins involved in the regulation of multiple cellular processes such as adhesion, migration, proliferation, differentiation and survival. These proteins also regulate processes such as angiogenesis and chondrogenesis, and have been implicated in wound healing, tumorigenesis as well as fibrotic and vascular diseases (Lau and Lam, 1999).

The human Cyr61 cDNA encodes a cysteine-rich secreted heparin-binding protein of 381 amino acids with a predicted molecular mass of 42 kDa (Brigstock, 1999). Expression of the Cyr61 gene is rapidly induced in an immediate early fashion by a spectrum of stimuli such as growth factors or cytokines (Lau and Nathans, 1985, 1987; Nathans et al., 1988). It may also have oncogenic bioactivity (Lau and Lam, 1999). The proangiogenic function of Cyr61 may be mediated through integrins. It has been shown that Cyr61 induces cell adhesion and tubule formation through interaction with integrin α6β1 in early
passage human umbilical vein endothelial cells where integrins have not been activated. In endothelial cells where integrins have been activated, Cyr61 promotes cell adhesion, migration, survival, growth factor-induced mitogenesis and endothelial tubule formation through integrin α₅β₃ (Leu et al., 2002).

There is evidence that Cyr61 expression is regulated by estrogen. Cyr61 transcript levels are strongly up-regulated by 17β-estradiol in ovariectomized rat uteri (Rivera-Gonzalez et al., 1998). Moreover, Cyr61 has also been described as an estrogen target gene in the myometrium as well as in breast cancer (Sampath et al., 2001a,b).

As endometriosis is an estrogen-dependent disease, the current therapy is based on the administration of inhibitors of endogenous estrogen production and hormonal castration (Giudice et al., 1998). It has recently been reported that in addition to ovarian estrogen synthesis, estrogen is produced locally within endometriotic cells (Zeitoun and Bulun, 1999). This local estrogen biosynthesis is due to aromatase activity and cannot be blocked by the currently used GnRH agonists. In addition to aromatase activity, imbalance in 17β-hydroxysteroid dehydrogenase (HSD) expression may further increase the local concentrations of estrogen in endometriotic lesions (Zeitoun et al., 1998).

Here we present evidence that Cyr61 is a deregulated gene in endometriosis. Its expression is enhanced in endometria of women suffering from endometriosis and in endometriotic lesions. Estrogen control of Cyr61 was observed in eutopic endometria but the unchanged levels found in lesions of women treated with a GnRH agonist suggest the existence of other regulatory factors besides ovarian hormones.

Materials and methods

Patients

Endometrium and endometriotic tissues were obtained from patients in the proliferative or secretory phase of the menstrual cycle who were undergoing endometrial biopsy or hysterectomy at the Department of Gynecology, University Hospital Duisburg–Essen, Germany. A total of 84 patients was investigated, 41 women without and 43 women with endometriosis. The women were aged between 19 and 48 years and did not receive any hormonal treatment before surgery, unless otherwise reported. All the patients were living in the mid-western region of Germany. Most of the patients were Caucasians, mainly of German origin. Less than 30% of the patients were orientals.

Blood of all patients was collected 24–48 h before surgery and sera were tested for 17β-estradiol and progesterone concentrations with a commercially available radioimmunoassay (Sorin Biomedica, USA). The hormonal profile was used to determine the status of the menstrual cycle. Samples from women with a progesterone concentration >1.0 ng/ml were allocated to the secretory phase, samples with a concentration below that level were allocated to the proliferative phase.

In addition to the eutopic endometrial samples (41 endometria without and 43 with endometriosis) a total of 19 endometriotic lesions was examined from a different cohort of patients. For investigation of endometriotic lesions of women whose ovarian estrogen synthesis has been suppressed (n = 3), patients were treated six times with one injection per month of 3.57 mg Leuprolelin (Eanatone-Gyn; Takeda), a GnRH agonist. One of these patients additionally received Tibolone (Livialla; Organon, Nourypharma), a synthetic steroid with partially estrogenic function, to alleviate side-effects. Tibolone has no effects on the uterus (Albertazzi et al., 1998). In these patients, estrogen levels were between 11 and 23 pg/ml compared to a mean serum estrogen level of 130 pg/ml in the proliferative phase of untreated patients.

Fresh endometrial tissue as well as endometriotic lesions were collected in Moscona solution (Gibco, Germany) and either frozen in liquid nitrogen for RNA isolation or embedded in 10% formaldehyde for immunohistochemical analysis.

These studies were approved by the local ethics committee at the Medical Faculty of the University Duisburg–Essen, Germany.

RNA extraction and cDNA synthesis

Total RNA was isolated from endometrial and endometriotic tissue as well as from endometrial tissue grown in nude mice using a Qiagen RNeasy Kit (Hilden, Germany) according to the manufacturer’s protocol.

Total RNA (2 μg) was digested with DNase I (Invitrogen, Germany) for 15 min at room temperature and converted to cDNA by RT with M-MLV Reverse Transcriptase (Invitrogen) in a total volume of 50 μl. RT was performed at 37°C for 60 min in a thermocycler (Bioterma, Germany). Four microliters of the reaction mixture were used for PCR experiments and 1 μl was used for real-time RT–PCR experiments.

Microarray analysis

One to two micrograms of poly A+ RNA or 8–10 μg of total RNA were converted into double-stranded cDNA using a modified oligo-dT primer including a 5' T7 RNA polymerase promoter sequence and the Superscript Choice system for cDNA synthesis (LifeTechnologies, USA). In vitro transcription was performed with T7 RNA polymerase (T7 Megascript kit; Ambion, USA) and 0.5–1 μg of double-stranded cDNA template in the presence of a mixture of ATP, CTP, GTP, biotin-11-CTP, and biotin-16-UTP (Enzo Diagnostics, USA). Of the 30–100 μg obtained by in vitro transcription, 20 μg of cRNA were fragmented randomly by incubating in 40 mmol/l Tris-acetate pH 8.1, 100 mmol/l K+ acetate, and 30 mmol/l Mg2+ acetate at 94°C for 35 min. Human Genome U95A arrays (Affymetrix Inc., USA) were hybridized, washed and stained according to standard protocols (Mahadevappa and Warrington, 1999), involving a three-step staining and amplification procedure with streptavidin–phycoerythrin (SAPE), a biotinylated anti-streptavidin antibody, and a final SAPE step. Each gene on an Affymetrix chip is represented by 20 perfect and 20 mismatches, that is 40 data points. The results were only taken into account if the signals originating from perfect matches were consistently higher than those originating from the mismatches. The fluorescence intensities for the individual oligonucleotide probes on the arrays were determined with a confocal scanner from Affymetrix. Following inter-array normalization of overall fluorescence intensities, the expression ratios for individual genes between two arrays were determined by a statistical analysis of the distribution of expression ratios as derived from the individual oligonucleotide detector pairs, and genes were then ranked according to coherent regulation over multiple pairwise comparisons, using a proprietary software tool (Kassel et al., 2002).

Every endometrial tissue sample was analysed on an individual gene array. Differently expressed genes in endometria of women with endometriosis were determined by comparison of each normal endometrial sample with every sample of endometrium from the group of women with endometriosis.

The range of the progesterone concentrations in the secretory phase was between 1.67 and 9.52 ng/ml, with a median of 8.52 ng/ml. The range in the proliferative phase was between 0.2 and 0.7 ng/ml, with a median of 0.3 ng/ml. The difference in the progesterone concentrations between the proliferative and secretory phase was statistically significant (P < 0.001). In total, we analysed 20 different endometrial samples, 11 were taken from the proliferative phase (five samples from women without endometriosis, six samples from women with endometriosis) and nine from the secretory phase (five samples from women without and four from women with endometriosis) respectively.

Standard RT–PCR

RT–PCR experiments were performed in a thermocycler (Bioterma, Germany). Total RNA from endometrial and endometriotic tissue as well as from endometrial tissue grown in nude mice was reverse-transcribed into cDNA. The target and the endogenous control (β-actin) amplifications were run in one tube (for primers see Table I). PCR was performed in a 50 μl volume using BioTherm Taq polymerase (Genecraft, Germany) for different amplification cycles depending on the target gene. The generated PCR amplification products were analysed by agarose gel electrophoresis and detected with ethidium bromide staining. PCR products were normalized to β-actin and relatively quantified (Gelscan Professional V4.0; BioSciTec, Germany) by densitometric analysis using Gel imager (Intas, Germany).
Real-time RT–PCR
total RNA samples of endometrial tissue and endometriotic lesions were reverse-transcribed to cDNA and further analysed by real-time RT–PCR using an SYBR Green I dye. Using this technique, detection of accumulated PCR products could be monitored by an increase in fluorescence, which occurs when the dye SYBR Green I binds to double-stranded DNA. Non-specific binding of SYBR Green I was ruled out by analysing the PCR amplification products by agarose gel electrophoresis. Reactions were performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Germany). The target and the endogenous control (β-actin) amplifications (for primers see Table II) were run in separate tubes. It was ensured that the dynamic ranges of both the target and the reference were similar to ensure that both amplifications performed equally efficiently. The threshold cycle (CT value) is the cycle at which the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. The higher the initial amount of cDNA, the lower the CT value. For relative quantification of gene expression, the comparative CT method \((ΔΔCT)\) was used. To calculate the normalized quantification, the amplification products were normalized to β-actin \((ΔCT)\). The normalized values of the amplification were used as a calibrator summarized as a mean value for each cyclic phase. The fold change given for EnEMT relative to En or EMT relative to EnEMT was determined by evaluating the expression \(ΔΔCT\) with \(ΔΔCT \pm \text{s}\) and \(ΔΔCT \pm \text{s}\), where \(s\) is the standard deviation of the \(ΔΔCT\) value and \(ΔΔCT\) is the CT value minus the CT calibrator value. The fold change was calculated as \(2^{−ΔΔCT}\).

Statistical analysis
Values derived from real-time RT–PCR experiments were analysed for statistical significance by Wilcoxon rank sum test for combined samples. \(P < 0.05\) was considered significant. Values derived from standard RT–PCR experiments were analysed for statistical significance by Student’s t-test. \(P < 0.05\) was considered significant.

Immunohistochemistry
Immunostaining of Cyr61 was performed on paraffin sections from endometrial tissue and endometriotic lesions. Briefly, sections were washed three times (5 min each) with TBS solution (0.5 mol/l Tris, 150 mmol/l NaCl, pH 7.2) at room temperature and incubated with the primary antibody [anti-mouse CYR61 antibody (Munin Corp., USA), diluted 1:100 with TBS] at 4°C overnight. Further steps were carried out at room temperature. Following three 5 min washes with TBS, a monoclonal mouse anti-EST antibody IgG (Dako, Germany, diluted 1:50 in a solution consisting of 100 μl human AB plasma in 700 μl PBS) was added (100 μl per section) and incubated for 30 min. Following another TBS treatment for 5 min, the rabbit anti-mouse IgG antiserum similarly diluted 1:25 was added and incubated for 10 min. Sections were washed in TBS for 1 min and then incubated with a complex of intestinal alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase antiserum (AAPA) (Dako) at a 1:50 dilution for 10 min. The signal was amplified using an additional cycle of incubations with the rabbit anti-mouse IgG antiserum and the AAPA complex with intermittent TBS washing steps. Signals were developed using freshly prepared Fast Red-containing solution (40 mg Fast Red, 18 mg levamisole and 20 mg naphtol-AS-MX-phosphate, 1 ml dimethylformamide and 40 ml propanediol buffer consisting of 50 mmol/l 2-amino-2-methyl-1,3-propanediol, pH 8.7). Following 10 min incubation, sections were washed in water and prepared for microscopy. For each histological analysis, a negative control was performed (Kireeva et al., 1997). Sections were incubated using normal rabbit serum instead of the primary antibody (rabbit anti-CYR61 antiserum) at a similar protein concentration.

Growth of human endometrial fragments in nude mice
Animals
Athymic nude mice (Han:NMRI nu/nu) were housed in a barrier unit in a well-controlled pathogen-free environment with a regulated 12:12 h light–dark cycle. All housing materials and food entering the barrier were autoclaved. Mice had free access to food and water ad libitum. All experiments were carried out in accordance with the German law for animal protection and with permission of the state.

Transplantation of fragments and hormonal treatment
Fragments (1–2 mm) of human endometrium were transplanted into the abdominal wall of cyclic female nude mice and fixed by suturing (Ethicon 6/0; Johnson and Johnson, Germany). Four to six transplants were fixed to the abdominal wall of each mouse as described previously (Grünmer et al., 2001). Mice were treated either with 17β-estradiol (3 μg/kg; Sigma, Germany) or pure anti-estrogen (1 mg/kg, ZK 191703; Schering AG, Germany) respectively.

### Table I. Oligonucleotide primers used for standard RT–PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
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<td>5'-primer</td>
<td>5'-ACCTTACACCCCATGTACG-3'</td>
<td>698</td>
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<tr>
<td>Cyr61</td>
<td>5'-primer</td>
<td>5'-CGTACCCATCTGCGAAGGTG-3'</td>
<td>197</td>
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<tr>
<td>Aromatase</td>
<td>5'-primer</td>
<td>5'-ATTCTGGCCTTGAAGGGTTG-3'</td>
<td>376</td>
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<tr>
<td>17β-HSD1</td>
<td>5'-primer</td>
<td>5'-GGACGTGCTGAAGTGTGTAAAC-3'</td>
<td>352</td>
</tr>
<tr>
<td>17β-HSD2</td>
<td>5'-primer</td>
<td>5'-GGTCGTACGTCCCTCTCATGT-3'</td>
<td>418</td>
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</table>

### Table II. Oligonucleotide primers used for real-time RT–PCR

<table>
<thead>
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<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
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<td>5'-primer</td>
<td>5'-ACCACTGGGACGACATGGAGAAA-3'</td>
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<tr>
<td>Cyr61</td>
<td>5'-primer</td>
<td>5'-TTAGCTGCGCCAAAGCAGTCAA-3'</td>
<td>223</td>
</tr>
</tbody>
</table>

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Both substances were dissolved in 100% ethanol p.a. and diluted in arachis oil at a ratio of 1:10. Animals in the control group were treated with the vehicle only. Each animal received one s.c. injection per day from the time of transplantation onwards during the course of the experiment.

After 14 days, mice were killed by cervical dislocation and the body weight was measured. The uterus and the ovaries of the mice were excised and the weights of the organs were determined. Implanted endometrial fragments were dissected by laparotomy and were frozen directly in liquid nitrogen for either RNA isolation or immunohistochemical analysis.

### Results

#### Expression profiling of endometrial samples

Using microarrays, we analysed the expression of ~12 000 genes in endometria of women with endometriosis and compared it with gene expression in endometria of women without endometriosis. We identified 95 genes that were differently regulated ≥1.5-fold (see supplementary data). A total of 64 genes were down-regulated in endometria of women with endometriosis, more than half (35 out of 64) of these genes were down-regulated in the secretory phase. Remarkably, many of the genes with decreased expression levels in the secretory phase coded for extracellular matrix proteins such as collagen IV, nidogen and fibronectin (see supplementary data).

A total of 31 genes was up-regulated in endometria of women with endometriosis compared to normal endometria (see supplementary data), 23 of them were up-regulated in the secretory phase (Table III). Many of these up-regulated genes are known to be controlled by estrogen. They include those coding for the nuclear orphan receptor TR3 (Uemura et al., 1995), and for the transcription factors early growth response protein-1 (EGR-1; Pratt et al., 1998), c-fos (Duan et al., 2002) and jun-B (Cicatiello et al., 1992). Another interesting up-regulated group was formed by genes involved in angiogenesis such as vascular endothelial growth factor (VEGF), Cyr61 and CTGF. EGR-1, which regulates the expression of several pro-angiogenic factors, can also be allocated to this group. Indeed, the most markedly up-regulated gene was the Cyr61 gene. Its expression was enhanced between endometria of women with and without endometriosis in 18 out of 20 comparisons. The average fold change was one of the highest observed in our study (5.0-fold).

### Increased expression of Cyr61 in endometrium of women with endometriosis

To confirm the finding that Cyr61 was up-regulated in the endometrium of patients with endometriosis during the secretory phase, real-time RT–PCR was performed. We found a significant up-regulation (~14-fold) of Cyr61 expression in 11 out of 15 (70%) endometria of women with endometriosis (n = 15) compared to the median expression level of Cyr61 in the endometria of women without endometriosis of the proliferative phase (n = 16) (Figure 1A). In the secretory phase, Cyr61 expression was increased (~12-fold) in six out of 10 (60%) comparisons between endometria of women with endometriosis and the median expression level of Cyr61 in normal endometria (n = 8) (Figure 1B). In three cases, no difference in expression levels between endometria of women with and without endometriosis was found. When comparing the Cyr61 expression levels in mild and severe endometriosis and in patients with different ethnic backgrounds, no significant differences were observed.

To test whether Cyr61 expression was regulated during the menstrual cycle, the results of real-time RT–PCR experiments were used to compare proliferative phase with secretory phase endometria (Figure 2). The expression level of Cyr61 was significantly higher in

#### Table III. Up-regulation of genes in the endometria of patients with endometriosis compared to normal endometria of the secretory phase

<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Gene description</th>
<th>Secretory phase:</th>
<th>Average fold change</th>
</tr>
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<tbody>
<tr>
<td>U62015</td>
<td>Cyr61 mRNA</td>
<td>18/0</td>
<td>5.0</td>
</tr>
<tr>
<td>M92843</td>
<td>Zinc finger transcriptional regulator</td>
<td>16/0</td>
<td>4.0</td>
</tr>
<tr>
<td>D90097</td>
<td>AMY2B gene for alpha-amylase</td>
<td>16/0</td>
<td>2.7</td>
</tr>
<tr>
<td>L13740</td>
<td>TR3 orphan receptor mRNA</td>
<td>16/0</td>
<td>2.2</td>
</tr>
<tr>
<td>X53587</td>
<td>mRNA for integrin beta 4</td>
<td>15/0</td>
<td>2.1</td>
</tr>
<tr>
<td>J05428</td>
<td>3,4-Catechol estrogen UDP-glucuronosyltransferase</td>
<td>15/0</td>
<td>2.4</td>
</tr>
<tr>
<td>X68277</td>
<td>CL 100 mRNA for protein tyrosine phosphatase</td>
<td>14/0</td>
<td>4.8</td>
</tr>
<tr>
<td>M92934</td>
<td>Connective tissue growth factor (CTGF)</td>
<td>13/0</td>
<td>1.8</td>
</tr>
<tr>
<td>L11329</td>
<td>Protein tyrosine phosphatase (PAC-1)</td>
<td>13/1</td>
<td>6.3</td>
</tr>
<tr>
<td>U20734</td>
<td>Transcription factor junB (junB) gene</td>
<td>13/1</td>
<td>3.7</td>
</tr>
<tr>
<td>U82319</td>
<td>Clone YDD19 mRNA sequence</td>
<td>13/1</td>
<td>2.4</td>
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<tr>
<td>U48296</td>
<td>Protein tyrosine phosphatase PTPCAAX1 (hPTPCAAX1)</td>
<td>13/1</td>
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<td></td>
<td>Biliary Glycoprotein, Alt. Splice 5, A</td>
<td>13/1</td>
<td>1.8</td>
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<tr>
<td>X52541</td>
<td>mRNA for early growth response protein 1 (hEGR1)</td>
<td>12/0</td>
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<tr>
<td>V01512</td>
<td>Cellular oncogene c-fos</td>
<td>12/0</td>
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<td>X62048</td>
<td>Wee1 hu gene</td>
<td>12/0</td>
<td>2.0</td>
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<tr>
<td>X51345</td>
<td>jun-B mRNA for JUN-B protein</td>
<td>12/1</td>
<td>2.5</td>
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<td>S78825</td>
<td>Id1</td>
<td>11/0</td>
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<tr>
<td>US9914</td>
<td>Chromosome 15 Mad homolog Smad6 mRNA</td>
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</tr>
<tr>
<td>AF010193</td>
<td>MAD-related gene SMAD7 (SMAD7) mRNA</td>
<td>11/0</td>
<td>1.6</td>
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<tr>
<td></td>
<td>Oncogene Aml1-Evi-1, Fusion Activated</td>
<td>11/0</td>
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<tr>
<td>L13720</td>
<td>Growth-arrest-specific protein (gas) mRNA</td>
<td>10/0</td>
<td>2.5</td>
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<tr>
<td>M27281</td>
<td>Vascular permeability factor mRNA</td>
<td>10/0</td>
<td>2.0</td>
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<tr>
<td>X01703</td>
<td>Gene for alpha-tubulin (b alpha 1)</td>
<td>10/0</td>
<td>2.0</td>
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</table>

The genes were classified according to the number of pairwise comparisons in which expression up-regulation was observed. Only genes with up-regulated expression in ≥50% of comparisons are shown. The threshold was set to 1.5. The junB gene is represented by two different probe sets.
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Figure 1. Real-time RT-PCR of Cyr61 expression in eutopic endometria. Expression of Cyr61 is up-regulated in endometria of women with endometriosis compared to healthy women. In the proliferative phase, an increase is seen in 70% of the comparisons between different endometria of women with endometriosis and the median expression level of Cyr61 in normal endometria (A), whereas in the secretory phase, Cyr61 expression is up-regulated in 60% of the comparisons (B).

Figure 2. Real-time RT-PCR of Cyr61 expression in eutopic endometria during the menstrual cycle. In the proliferative phase Cyr61 expression is up-regulated in ~80% of the cases compared to the secretory phase independent from the disease. \(*P<0.05.\)

~80% of the endometria of the proliferative phase compared to the expression levels of endometria of the secretory phase independent from the disease (Figure 2).

We also investigated the expression of Cyr61 in endometriotic lesions and found a marked up-regulation. In the proliferative phase, Cyr61 was up-regulated in four out of five endometriotic lesions (80%) compared to the median expression level of Cyr61 in the endometria of women with endometriosis (\(n=15\)) (Figure 3A). In the secretory phase, Cyr61 expression was increased in all endometriotic lesions (\(n=3\)) compared to the median expression level in the endometria of women with endometriosis (\(n=10\)) (Figure 3B).

Localization of Cyr61

In order to determine in which cell types of the endometrium and endometriotic lesions Cyr61 was expressed, immunohistochemical analysis was performed. Cyr61 was localized in glandular epithelial cells in eutopic endometrial tissue of women with endometriosis (Figure 4A) as well as in endometriotic lesions (Figure 4B). Enhanced staining was observed in the apical portion of the epithelial cells. Furthermore, secreted Cyr61 was observed within the glandular lumen (Figure 4A and B).

Regulation of Cyr61 by estrogen in human endometrium transplanted into nude mice

Since Cyr61 expression varied in the human endometrium during the menstrual cycle, possibly due to regulation by ovarian hormones, we investigated its estrogen dependence using a nude mouse model (GruÈmmer et al., 2001). Human endometrial tissue samples were transplanted into the abdominal wall of cyclic female nude mice which were substituted with 17β-estradiol or treated with the anti-estrogen ZK 191703 for 14 days.

In order to control the effect of the applied compounds, the ovarian and uterine weights of the treated mice were determined. The uterine but not the ovarian weights of mice were significantly regulated by the applied hormones in all study groups (data not shown).

The possibility for estrogen responsiveness was given by the presence of estrogen receptor (ER) up to 14 days in the transplanted human endometrial fragments (GruÈmmer et al., 2001) and present results identified this receptor as ERα (data not shown).

The expression level of the Cyr61 gene was significantly down-regulated in human endometrial fragments grown in anti-estrogen-treated mice, as compared to the vehicle control (Figure 5). High variations of Cyr61 expression were observed in the human endometrial fragments transplanted into cyclic mice substituted with 17β-estradiol (Figure 5).

The decrease of Cyr61 expression following anti-estrogen treatment was furthermore demonstrated using immunohistochemistry. In animals treated with anti-estrogen, Cyr61 protein expression was nearly undetectable in the transplanted fragments (data not shown).

Expression of estrogen-converting enzymes in eutopic and ectopic endometrium

It has been described recently that estrogen is produced locally in endometriotic lesions independently of the ovarian estrogen synthesis. Aromatase and 17β-HSD1 and 2 are three enzymes involved in controlling local estrogen synthesis (Zeitoun and Bulun, 1999). Aromatase and 17β-HSD1 increase whereas 17β-HSD2 decreases local estrogen concentrations. To address this point, eutopic endometrium of women with and without endometriosis as well as endometriotic lesions from the proliferative and the secretory phase were examined using RT-PCR.

No expression of aromatase was detected either in endometria of women with or without endometriosis in the proliferative (\(n=4\)) or in the secretory phase (\(n=6\)) nor in endometriotic lesions of the proliferative (\(n=5\)) or the secretory (\(n=3\)) phase. Results of one representative experiment are shown in Figure 6.

Concerning 17β-HSD1 expression, only very low transcript levels were detected in all tissue samples. No differences were noted between endometria of women with endometriosis (\(n=4\) in the proliferative phase, \(n=6\) in the secretory phase) and without endometriosis (\(n=4\) in the proliferative phase, \(n=6\) in the secretory phase) (Figure 7A and B). In endometriotic lesions of the secretory phase (\(n=3\), expression of 17β-HSD1 was slightly increased compared to the corresponding eutopic endometria (Figure 7B), whereas in the proliferative phase, expression of 17β-HSD1 was
significantly down-regulated in endometriotic lesions ($n = 5$) compared to the eutopic endometria (Figure 7A).

No obvious differences in the expression levels of $17\beta$-HSD2 were detectable between endometria of women with endometriosis ($n = 4$ in the proliferative phase, $n = 6$ in the secretory phase) and without endometriosis ($n = 4$ in the proliferative phase, $n = 6$ in the secretory phase) (Figure 7C and D). In normal as well as in endometriotic tissue there was a higher expression level of $17\beta$-HSD2 in the secretory phase than in the proliferative phase. However, in endometriotic lesions of both cyclic phases ($n = 7$ in the proliferative phase, $n = 3$ in the secretory phase) expression of $17\beta$-HSD2 was hardly detectable and significantly down-regulated compared to the corresponding eutopic endometria (Figure 7C and D).

In order to determine whether the additionally enhanced expression of Cyr61 in endometriotic lesions was due to an imbalance in $17\beta$-HSD1 and 2 expression, we compared endometriotic lesions of the proliferative phase ($n = 5$) and secretory phase ($n = 4$). In addition, endometriotic lesions of women whose ovarian estrogen synthesis had been suppressed by GnRH analogues for 6 months ($n = 3$) were tested using real-time RT-PCR. Differences in Cyr61 expression were not statistically significant between lesions of the different groups, due to the high intra-group variations (Figure 8).

Discussion

Although some molecular mechanisms which may be involved in the development of endometriosis have been described in recent years (Gaetje et al., 1995; Beliard et al., 1997), the aetiology of the disease is still poorly understood. The concept of using microarrays was therefore introduced to explore the molecular mechanisms of endometriosis in a hypothesis-free manner and to identify genes abnormally expressed and possibly responsible for the development of this disease (Taylor et al., 2002).

The microarray analysis presented in our study revealed differences in gene regulation between eutopic endometria from normal and endometriotic cases of both phases of the menstrual cycle. A number of up-regulated genes was identified in the secretory phase, including several genes which are under the control of estrogen such as the TR3 orphan receptor (Uemura et al., 1995), EGR-1 (Pratt et al., 1998), as well as c-fos (Duan et al., 2002) and jun-B (Cicatiello et al., 1992). Similar results have been described by Taylor et al. (2002) and Lundeen et al. (2000) who also investigated eutopic endometrium of women with endometriosis versus normal endometrium. Similar to
our work, these groups found an up-regulation of estrogen-responsive genes such as EGR-1 in the proliferative phase.

Normal endometrium undergoes important structural modifications combined with changes of the specific functions of its cells in response to the cyclic variations of estrogen and progesterone during the menstrual cycle. Since estrogen serum levels are not different in patients with endometriosis compared to healthy women, an inappropriate response of the endometrium of women with endometriosis to estrogen may be inferred. Our gene expression profiling experiments suggest that the endometrium of women with endometriosis is in an abnormally prolonged proliferative phase. This may lead to failure of an adequate transformation of the endometrium into a receptive one and provide an explanation for the observed reduced fertility in these women (Barnhart et al., 2002; Garrido et al., 2002). Furthermore, this prolonged activity during the secretory phase may be a precondition for the shed fragments to establish lesions in the peritoneal cavity.

Among the estrogen-dependent genes, Cyr61 was found to display the most significant up-regulation in endometria of women with endometriosis compared to healthy women during the secretory phase. A high, cycle-independent increase in expression was observed in endometriotic lesions, suggesting that Cyr61 represents an important player in the development and/or survival of the ectopic endometrium. It has been demonstrated earlier that Cyr61 expression is regulated by 17β-estradiol in the uterus of mature ovariectomized rats in a time-dependent fashion (Rivera-Gonzalez et al., 1998). In our study we showed that Cyr61 was regulated by estrogen in the human endometrium as well. First, higher expression was observed in the proliferative than in the secretory phase. Second, in endometrial fragments grafted into nude mice, a sharp drop in Cyr61 expression was observed following treatment with a pure anti-estrogen compound. When supplementing these mice with estrogen, large variations in Cyr61 expression of the grafted human endometrium were noted. This gives a hint that in principle Cyr61 is regulated by estrogen. However, it remains to be clarified how these high levels of Cyr61 can be achieved in the ectopic endometriotic lesions. Additional mediators, for example a variety of chemokines identified in the peritoneal fluid of endometriotic patients, have to be taken into account to modulate Cyr61 expression not only in the ectopic endometriotic lesions but also in the eutopic endometrium (Khorram et al., 1993; Akoum et al., 1996).

Previous studies have reported high aromatase activity in the stromal cell compartment of endometriotic tissue, whereas aromatase expression is barely detectable or even absent in eutopic endometrium (Noble et al., 1996, 1997). We were not able to detect any aromatase expression in human endometrial tissue or in endometriotic lesions. Other important enzymes controlling local estrogen synthesis are the 17β-HSD. 17β-HSD1 is responsible for converting estrone into the more potent 17β-estradiol and significant levels have been determined in human endometrium as well as in endometriotic lesions during both phases.
the proliferative and the secretory phase (Zeitoun et al., 1998). Conversely, 17β-HSD2 inactivates estrogen and is expressed during the secretory phase (Casey et al., 1994; Mustonen et al., 1998). Our results revealed an imbalance of the converting enzymes 17β-HSD1 and 17β-HSD2 during the secretory phase which may lead to elevated estrogen synthesis in endometriotic lesions. This imbalance could be responsible for the additional elevation of Cyr61 expression observed in the endometriotic lesions. However, this overexpression was independent of the cyclic phase and not affected by prolonged treatment with a GnRH agonist. The role of estrogen-independent mechanisms in the pathology of these lesions is supported by clinical observations showing that chemical suppression of ovarian steroid production by GnRH analogues does not lead to a complete cure (Waller and Shaw, 1993). Furthermore, medication with GnRH analogues for 6 months can reduce endometriosis scores as defined by the American Fertility Society but only by a half (Lindheim, 1999).

The high expression of Cyr61 in endometriotic lesions may be linked to its function as a pro-angiogenic factor (Lau and Lam, 1999). Cyr61-deficient mice die in utero due to defects in chorio-allantoic fusion or to vascular pathologies such as impaired allantoic vessel bifurcation in the placenta and compromised vascular integrity in embryonic arteries (Mo et al., 2002). Furthermore, Cyr61 is thought to augment adhesion and migration of endothelial cells, probably by interacting with integrin αvβ3 (Babic et al., 1998). Many members of the integrin family are expressed in the endometrium during the menstrual cycle. It has been reported that integrin α2, α4 and β3 subunits show spatial and temporal variations in glandular and luminal epithelial expression during the menstrual cycle (Lessey et al., 1992; Tabibzadeh, 1992). In addition, the endometrium of women with endometriosis has significantly higher vascular expression of integrin αvβ3 than control endometrium (Hii and Rogers, 1998). Increased expression of Cyr61 in the endometrium of women with endometriosis may therefore promote the development of endometriotic lesions by enhancing cell adhesion and angiogenesis, an effect exacerbated by the elevated integrin αvβ3 protein levels. Importantly, we found that expression of CTGF, another member of the CCN family which interacts with integrins and has pro-angiogenic properties (Lau and Lam, 1999), was also up-regulated in endometria of women suffering from endometriosis (Table III).

In summary, Cyr61 is highly up-regulated in endometria of women with endometriosis and an additional up-regulation can be observed in endometriotic lesions. Even though Cyr61 is regulated by estrogen in the eutopic endometrium, the further increase in expression in the ectopic endometrium seems to originate from other presently unknown mediators. We hypothesize that Cyr61 is probably a useful marker gene for endometriosis that may also be causally implicated in the development and persistence of endometriotic lesions by facilitating adhesion and angiogenesis.

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