Identification of novel antigens in blood vessels in rectovaginal endometriosis

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To identify specific markers of rectovaginal endometriotic nodule vasculature, highly enriched preparations of vascular endothelial cells and pericytes were obtained from endometriotic nodules and control endometrial and myometrial tissue by laser capture microdissection (LCM), and gene expression profiles were screened by microarray analysis. Of the 18 400 transcripts on the arrays, 734 were significantly overexpressed in vessels from fibromuscular tissue and 923 in vessels from stromal tissue of endometriotic nodules, compared with vessels dissected from control tissues. The most frequently expressed transcripts included known endothelial cell-associated genes, as well as transcripts with little or no previous association with vascular cells. The higher expression in blood vessels was further corroborated by immunohistochemical staining of six potential markers, five of which showed strong expression in pericytes. The most promising marker was matrix Gla protein, which was found to be present in both glandular epithelial cells and vascular endothelial cells of endometriotic lesions, although it was barely expressed at all in normal endometrium. LCM, combined with microarray analysis, constitutes a powerful tool for mapping the transcriptome of vascular cells. After immunohistochemical validation, markers of vascular endothelial and perivascular cells from endometriotic nodules could be identified, which may provide targets to improve early diagnosis or to selectively deliver therapeutic agents.

Keywords: angiogenesis; rectovaginal endometriosis; laser capture microdissection; microarray; vascular targeting

Introduction

Rectovaginal endometriotic nodules are strongly associated with pelvic pain and dysmenorrhea in 95% of cases, rectal dyschezia in 25% of cases and infertility (Donnez and Squifflet, 2004). They are characterized by a nodular aggregate of smooth muscle cells, with islets or strands of endometrial-type stroma and glandular epithelium, showing striking histological similarities to adenomyomas (Donnez et al., 1996; Donnez and Squifflet, 2004).

Their etiology is still a matter of debate. The notion that rectovaginal endometriosis and peritoneal endometriosis are two distinct entities was first proposed by Donnez et al. (1996). Recent evidence indicates that endometriotic nodules, being anchored to the cervix, may originate from the rectovaginal space, as reported by Donnez and Squifflet (2004), whereas Konincx and Martin (1992) suggested that endometriotic nodules localized in the rectovaginal space are a severe form of deep endometriosis, resulting from the natural evolution of peritoneal endometriosis. Surgery is the gold standard for the treatment of rectovaginal endometriotic nodules (Donnez and Squifflet, 2004), but treatment has proved so far relatively ineffective, highlighting the importance of developing new treatment strategies.

Antiangiogenic agents have yielded promising results for the treatment of both malignant diseases (Hajitou et al., 2006) and chronic inflammatory diseases, such as endometriosis, as suggested by experimental studies on murine models (Dabrosin et al., 2002; Hull et al., 2003; Nap et al., 2004a; Laschke et al., 2006). Neovascularization of newly formed endometriotic lesions is likely to play a key role in their development and persistence, by providing them with nutrients and growth factors and promoting the recruitment of leukocytes (Donnez et al., 1998; Taylor et al., 2002; Groothuis et al., 2005). However, antiangiogenic approaches cannot target mature vessels surrounded by pericytes (Benjamin et al., 1998). This is supported by the observation that angiotastic agents induced a regression of neovessels, but not smooth muscle cell-protected vessels, in endometriotic lesions established in nude mice (Nap et al., 2004a). This limitation may prove particularly critical for the treatment of rectovaginal endometriotic nodules, which contain a high proportion of mature vessels, as observed by α-smooth muscle actin (ASMA) immunostaining (Groothuis et al., 2005). Moreover, current antiangiogenic molecules may have side effects, targeting physiologically normal angiogenesis in the reproductive system. In a study by Dabrosin et al. (2002), angiostatin gene transfer was effective for the treatment of endometriosis, but also affected angiogenesis in the ovary and uterus.

Identification of tissue-specific and disease-specific endothelial cell markers has opened up new perspectives for the targeted delivery of therapeutics to the vasculature. In addition, disease- or tissue-specific antigens can be employed to improve diagnosis. Vascular targeting has several advantages: (i) the endothelium is easily accessible; (ii) endothelial cells are genetically stable, which reduces the possibility

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of developing resistance and (iii) local delivery decreases side effects. Ligand-directed vascular targeting agents have proved their antitumoral efficacy in animal models (Arap et al., 2002; Hajitou et al., 2006).

Global proteomic and microarray approaches have revealed extensive heterogeneity of the endothelium in vivo (Arap et al., 2002; Chi et al., 2003). Differences in endothelial cell phenotypes may arise from two mechanisms (Minami and Aird, 2005). Early lineage determination is mediated by epigenetic modification of DNA, whereas site-specific properties of capillary endothelium are reversibly governed by signals residing in the microenvironment (Aird et al., 1997). It is therefore likely that endothelial cells in endometriotic lesions display different antigens on their surface than those in the local environment or eutopic endometrium.

The aim of the present study was to carry out genomic profiling of the vasculature of fibromuscular and stromal tissue of endometriotic nodules of the rectovaginal septum in order to identify novel antigens that can be used as potential targets in the design of more specific diagnostic and treatment tools. To this end, vessels were collected by laser capture microdissection (LCM) from frozen sections of (i) fibromuscular and (ii) stromal tissue of rectovaginal endometriotic nodules, as well as (iii) endometrial and (iv) myometrial tissue. The RNA was isolated, amplified and used for gene expression profiling. Array data were further validated using real-time reverse transcriptase polymerase chain reaction (qRT–PCR) and immunohistochemical staining to confirm differences at the protein level.

Materials and Methods

Collection and preparation of tissue

The use of human tissues for this study was approved by the Institutional Review Board of the Université Catholique de Louvain and by the Medical Ethics Committee of the Academic Hospital of Maastricht.

Rectovaginal endometriotic nodules and vaginal tissue were collected from nine young patients with endometriosis (mean age: 29.7 years) not receiving hormonal treatment. All of them had severe endometriosis (stage IV according to the revised classification of the American Fertility Society, 1985). Samples of healthy uterine tissue, including the endometrium and myometrium (with no histological evidence of endometrial pathology, as assessed by a pathologist) were collected from 5 of these patients and were used as control of Uterine biopsies were taken at different phases of the menstrual cycle to account for differences in vascular cell expression associated with proliferation, regeneration and differentiation of the endometrium (Punyadeera et al., 2006). Endometrial biopsies were dated according to the beginning of the last menstrual cycle and adjusted according to the criteria of Noyes et al. (1950).

Biopsies were cut into pieces of ~0.5–1 cm³, embedded with Tissue-Teck OCT® (Sakura, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen and stored frozen at −80°C. All procedures were performed with extreme caution to avoid RNAse contamination.

In addition to the biopsies used for LCM and gene expression profiling, biopsies of rectovaginal endometriosis and eutopic endometrium were collected from four additional patients for validation of the array data by qRT–PCR analysis. For immunohistochemical evaluation of gene expression at the protein level, 15 patients with a surgical and histological diagnosis of deep-infiltrating endometriosis, who were operated on between 1998 and 2004 at the University Hospital of Maastricht, were selected. Deep-infiltrating endometriosis was defined as the presence of one or more deep-infiltrating lesions in the rectovaginal septum, bowel wall, vaginal wall and/or bladder wall. Eutopic endometrium/ myometrium and endometriotic tissue were collected from all patients.

Matrix metalloproteinase-3 (MMP-3) and tissue inhibitor of MMP-3 (TIMP-3) immunostaining were previously performed in normal endometrium (n = 20) and rectovaginal endometriosis (n = 5) in an earlier study by Nap et al. (2004b), but vascular expression was not evaluated.

Preparation of tissue for LCM

Frozen tissue was cut into 5 μm serial sections at −23°C with a cryotome (2800 Frigocut E, Reichert-Jung, Heidelberg, Germany). Sections were mounted on autoclaved-Star Frosted glass slides (Knittel Glaser, Braunschweig, Germany) and kept frozen on dry ice. They were stored at −80°C in tightly sealed boxes containing silica gel (Sigma-Aldrich Corp., St Louis, MO, USA) as a desiccant for a maximum of 2 months until LCM. Half of the sections were used for immunohistochemical staining to identify stroma and glands in endometriotic tissue and to highlight endothelial cells; the other half were used for LCM.

The first section was double-stained with two monoclonal antibodies: one against CD10 (1:50, clone 56C6, Monosan, Uden, The Netherlands), an immunohistochemical marker of endometrial stroma (Tokí et al., 2002), and the other against cytokeratin (1:200, clone MNF116, Dako, Glostrup, Denmark), a marker of epithelial cells. The next serial section was stained for CD31 [platelet endothelial cell adhesion molecule-1 (PECAM-1), 1:200, clone JC70A, Dako], a marker of blood vessels in endometrium and endometriotic tissue (Van Langendonckt et al., 2004). The two remaining serially cut sections were used for LCM.

For immunohistochemical characterization, sections were fixed in 70% ethanol followed by 100% ice cold acetone and then incubated for 20 min in 0.3% H₂O₂ in methanol to inactivate endogenous peroxidases. After 30 min incubation with the primary antibodies, antibody binding was visualized with the ChemMate EnVision detection kit, according to the manufacturer’s instructions (Dako).

Sections prepared for LCM were fixed and stained with hematoxylin according to a slightly modified protocol from the National Institute of Health Laser Capture Microdissection Website. Sections were thawed for 30 s at room temperature, fixed for 15 s in 70% ethanol (Merck, Darmstadt, Germany) followed by 5 min in ice cold acetone (Merck), rinsed for 15 s in diethyl-pyrocarbonate (DEPC, Sigma-Aldrich Corp.) treated water, stained for 15 s in hematoxylin (Klinipath, Duiven, The Netherlands), rinsed in DEPC-treated water for 15 s and dehydrated: 30 s in 70% ethanol, 30 s in 96% ethanol, 30 s in 100% ethanol rendered anhydrous with molecular sieves (type 3A, Sigma-Aldrich Corp.) and 2 × 30 s in 100% xylene (Prolabo, Fontenay-sous-Bois, France). Sections were air-dried for 3 min, stored in a vacuum desiccator containing silica gel and used for LCM within 1 h.

LCM procedure

LCM was performed on a PixCell II-LCM instrument (Arcturus Bioscience Corporation, CA, USA) using high-sensitivity (HS) caps (CapSure LCM caps, Arcturus Bioscience Corporation). Loose material was blotted from the section prior to capture using the preparation strip provided with the HS caps. For microdissection of vessels, the laser was set to a 7.5 μm spot size, 55–70 mW power and a 1 ms pulse duration, corresponding to the lowest possible settings for LCM. A larger, 15 μm laser spot size was applied to collect surrounding tissue. Between 50 and 100 laser shots were fired at each section to collect vessels; five to six sections were used per tissue type and patient. Captured cells were recovered from the caps by incubating for 30 min at 42°C with RNA extraction buffer (PicoPure RNA isolation kit, Arcturus Bioscience Corporation).

Blood vessels and tissue were collected by LCM from nine rectovaginal endometriotic nodules, five endometriotic biopsies, five myometrial biopsies and five vaginal biopsies. Vessels were selectively microdissected from the stromal and fibromuscular tissue of endometriotic nodules. Immunostaining with anti-CD10 and anti-cytokeratin antibodies allowed us to discriminate between these two cell types. In all nine nodules examined, the surface area of glands and stroma represented <10% of the total nodule surface area and, in four nodules, the stromal surface area proved too small for LCM of vessels (fewer than 10 endothelial cells per section). A crucial aspect of successful microdissection of vessels appeared to be adequate dehydration of sections (Minami and Aird, 2005). Early lineage determination is mediated by epigenetic modification of DNA, whereas site-specific properties of capillary endothelium are reversibly governed by signals residing in the microenvironment (Aird et al., 1997). It is therefore likely that endothelial cells in endometriotic lesions display different antigens on their surface than those in the local environment or eutopic endometrium.

Preparation of tissue for LCM

Frozen tissue was cut into 5 μm serial sections at −23°C with a cryotome (2800 Frigocut E, Reichert-Jung, Heidelberg, Germany). Sections were
Purified RNA was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

RNA was amplified linearly using a standard T7 polymerase amplification procedure (Picopure RNA amplification kit, Arcturus Bioscience Corporation).

**Enrichment of vascular cells after LCM**

After LCM, enrichment of blood vessels and pericyte markers was assessed by qRT–PCR as described below. ASMA was used as a pericyte marker, PECAM-1 as an endothelial cell marker and CD45 as a leukocyte marker (Fig. 1). Expression levels were assessed in RNA isolated from vessels collected by LCM and in RNA isolated from surrounding whole tissue, also collected by LCM.

Mean enrichment of mRNA encoding PECAM-1 in captured cells compared with whole tissue was 50.3-fold (range 9.3- to 83.3-fold; n = 5) in stromal tissue from nodules, 22.1-fold (range 4.3- to 62.3-fold; n = 9) in fibromuscular tissue from nodules, 45.3-fold (range 10.3- to 83-fold; n = 5) in endometrium and 27.7-fold (range 6.3- to 65.0-fold; n = 5) in myometrium.

Enrichment of ASMA mRNA was 69.0-fold in stromal tissue from nodules (range 12.3- to 80.2-fold; n = 5), 41.9-fold (range 18- to 76.4-fold; n = 9) in fibromuscular tissue from nodules, 46.0-fold (range 9.3- to 76.5-fold; n = 5) in endometrium and 34.3-fold (range 10.2- to 80-fold; n = 5) in myometrium.

Expression of CD45, a leukocyte marker, was not detected in RNA extracted from captured vessels in any of the tissue.

Only RNA samples showing a minimum enrichment of 10-fold for genes associated with endothelial cells were included in the study and further

**Figure 1:** LCM selectively enriches pericyte and endothelial cell RNA, as assessed by quantification of mRNA transcript levels of cell-specific markers

Mean levels (relative to cyclophilin) of the endothelium specific marker PECAM-1 (A) and pericyte marker ASMA (B) were evaluated in RNA extracted from LCM-captured vessels, and compared with RNA extracted from whole tissue collected by LCM from the stromal and fibromuscular components of endometriotic nodules, endometrium and myometrium.
amplified. This condition was met by 4/5 samples collected from stromal tissue of nodules, 6/9 from fibromuscular tissue of nodules, 5/5 from endometrium and 3/5 from myometrial tissue.

**RNA quality controls and pooling of samples for microarray analysis**

After one round of amplification, between 5.5 and 17.9 ng/μl of aRNA was obtained. The quality of the aRNA samples was assessed using the eukaryotic total RNA pico assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Good quality aRNA appears as a broad peak, ranging from 200 to 2000 bases in length. All the aRNA amplified from samples corresponding to fewer than 200 laser shots was of poor quality. Amplified RNA samples of good quality designed for microarray analysis were pooled as follows: (i) vessels collected from the stroma of an endometriotic nodule (n = 1); (ii) vessels captured from the fibromuscular tissue of endometriotic nodules (secretory phase n = 2; proliferative phase n = 2); (iii) vessels isolated from the endometrium and myometrium of endometriosis patients (secretory endometrium n = 1, proliferative endometrium n = 1 and myometrium n = 1) and (iv) vaginal, endometrial and myometrial tissue from endometriosis patients (vaginal tissue n = 1, proliferative endometrium n = 1, secretory endometrium n = 1, menstrual endometrium n = 1 and myometrium n = 1).

All the technical complications encountered in the course of these studies eventually resulted in the exclusion of a large number of LCM samples. In order to be able to perform hybridizations under optimal conditions, it was necessary to pool samples. Control pools are particularly important, as elimination of genes that are also expressed in normal tissue decreases the chances of false positive results.

A second round of linear amplification was performed to obtain a sufficient amount of RNA for array analysis. Average amplification of 500-fold was achieved during this step. The eukaryotic total RNA nano assay was used for quality control (Agilent Technologies).

The quality of aRNA obtained after two rounds of amplification from all four pools included in the study was good, as indicated by the presence of a broad smear, ranging from ~100 to 2000 bp.

The experiments were performed according to MIAME standards and the primary data made available on the Array Express database (accession number E-MEXP-1251).

**Affymetrix gene chip microarrays**

cDNA was generated from the aRNA pools (1 μg/μl) and labeled according to the Affymetrix GeneChip labeling procedure. Subsequently, the cDNA was hybridized in triplicate to the Affymetrix HG-U133A_2.0 chip, analyzing the expression levels of 18 400 transcripts and variants, including 14 500 well-characterized human genes. After washing, the chips were scanned using the GeneChip Scanner 3000, designed for high-resolution scanning and analyzed using the Affymetrix GeneChip Microarray Analysis System. Resolver uses the Affymetrix Gene Chip error model to transfer by FTP to the server housing the Rosetta Resolver Gene Expression Data Analysis System. Resolver uses the Affymetrix Gene Chip error model to transform raw data into a processed form that can be used for various expression analyses. Sample data on triplicate hybridizations were normalized using one-way analysis of variance (ANOVA), as detailed in www.rosettabio.com/tech/Data_processing_and_analysis_methods.pdf. After data transformation, scatter plots of log-ratio relative intensities of all detected gene transcripts had slopes of ~1, indicating that hybridization efficiency was similar between the groups and that array comparison could be performed. By combining the fold change or log-ratios and the P-value, a so-called significance code was generated, which simplified the selection and extraction of genes of interest, especially when analyzing various groups. The significance code assigned to the genes was based on ANOVA-returned P-values. Data were then exported from Rosetta Resolver to Spotfire Decision Site 7.1 (Spotfire, Göteborg, Sweden), in which gene sets of interest were selected. Using this strategy, genes that were over- or underexpressed in experimental, compared with control, pools could easily be identified. Array data were analyzed in order to identify transcripts overexpressed in vessels in the fibromuscular and stromal tissue of rectovaginal endometriotic lesions.

**Validation of gene expression with qRT–PCR**

qRT–PCR was performed to assess the degree of enrichment of vascular cells isolated by LCM and to validate the findings of the array hybridizations. All individual RNA samples of good quality (integrity and enrichment) were analyzed by qRT–PCR to assess the enrichment of blood vessels and pericyte markers.

Only vessels from endometrium and endometriotic lesions were used for validation. Samples used for qRT–PCR validation included non-amplified samples from the microarray pools, including vessels from two control endometrium (P1 = proliferative and S1 = secretory), vessels from the fibromuscular tissue of four endometriosis samples (FM tissue, P4, P5 = proliferative and S4, S5 = secretory; Table IV) and endometrial vessels from an additional four control samples (P2, P3 = proliferative and S2, S3 = secretory; Table IV) and two endometriosis samples (FM tissue, P6 = proliferative and S6 = secretory; Table IV). To evaluate differences in expression, a reference pool was generated containing equal amounts of RNA from all control endometrium (P1, P2, P3, S1, S2 and S3). The whole tissue control pool consisted of RNA isolated from the remaining endometrial tissue after LCM of vessels from P1, P2, P3, S1, S2 and S3.

Reverse transcription was performed as described by Punyadeera et al. (2005). RNA was incubated with 0.1 μg/ml oligo dT primers (Promega, Leiden, The Netherlands) at 70°C for 10 min. A reverse transcriptase mix consisting of 5 × RT-buffer (4 μl), 10 mM dNTP mix (1 μl) (Pharmacia, Uppsala, Sweden), 0.1 M dithiothreitol (2 μl) (Invitrogen, CA, USA) and superscript II reverse transcriptase (200 U/ml, Invitrogen) was added and the samples were incubated at 42°C for 1 h, followed by a 5 min incubation at 95°C to deactivate reverse transcriptase.

Real-time PCR was performed on an ABI 7700 machine (Applied Biosystems) using TaqMan Universal PCR Master Mix (ref 436441, Applied Biosystems) and predeveloped TaqMan Assay-on-Demand primers and probes, according to the manufacturer’s instructions. Thermal cycling conditions comprised an initial decontamination step at 50°C for 2 min, a denaturation step at 95°C for 10 min and 40 15 s cycles at 95°C, followed by 1 min at 60°C. The following primer/probe sets were selected to determine enrichment in pericyte [ASMA (Hs00426835)], endothelial [PECAM-1 (Hs00169777)] and leukocyte [CD45 (HS00174541)] markers after LCM of vessels. To further validate some of the genes differentially expressed, the following primer/probe sets were used: retinoic acid receptor responder-1 (Hs00161204), gastrin-releasing peptide (GRP) (Hs00181852), cholinergic receptor muscarinic 3 (CHR3) (Hs00922646), Purkinje cell protein 4 (PCP4) (Hs01113637), calderin 19 (Hs00253534), granulysin (GLY) (Hs01120097) and dickkopf homolog 1 (DDK1) (Hs00183740). For all qRT–PCR reactions, human cyclophilin A (Hs99999990) was included as a control to correct for differences in the amount of total RNA added to each reaction.

The ΔCT value was determined by subtracting the cyclophilin A CT value from the target gene CT value. Relative quantitative evaluation (Applied Biosystems User Bulletin Number 2) of target gene levels was performed by comparing ΔCT and calculating 2-ΔΔCT. For validation tests, qRT–PCR analysis was performed twice in duplicate.

**Validation of gene expression by immunohistochemistry**

A subset of markers was also validated at the protein level by immunohistochemistry. Paraffin sections were prepared from endometrium/myometrium and endometriotic nodules collected from the same patients (n = 15). The following genes were selected according to their level of expression, biological relevance, novelty and/or antibody availability: matrix GlA protein (MGP) (active form and all forms), desmin, desmuslin and PCP4 (PEP-19). The desmuslin antibody was kindly provided by Dr Y. Mizuno (Department of Anatomy, Gunma University, Gunma, Japan), the PEP-19 antibody by Prof. J. Morgan (Developmental Neurobiology, St Jude Children’s Research Hospital, Memphis, TN, USA), the antibodies against the MGP by Dr L. Schurgers (Department of Biochemistry, University Maastricht, Maastricht, The Netherlands) and the polyDES antibody by Prof. F. Ramaekers (Department of Molecular Cell Biology, University Maastricht, Maastricht, The Netherlands). Immunoreactivity of MMP-3 and TIMP-3 was performed as described by Nap et al. (2004b). Paraffin sections were deparaffinized in xylene and rehydrated in an alcohol series. After 20 min incubation in methanol/0.3% hydrogen peroxide to inhibit
endogenous peroxidase activity, the sections were subjected to an antigen retrieval step. The antigen retrieval steps, antibody dilutions and incubation conditions are summarized in Table I.

Antibody binding was visualized with the ChemMateTM DAKO EnVisionTM Detection Kit (DAKO). This reagent is a peroxidase-conjugated polymer, which also carries antibodies to rabbit and mouse immunoglobulins. Negative controls included sections incubated with normal mouse IgGs (DAKO) of the same isotype and concentration as the primary antibodies.

Statistical analysis of qRT–PCR data
Differences in gene expression (normalized to cyclophilin) between the groups were tested with the non-parametric Mann–Whitney U-test. A level of 0.05 or less was considered statistically significant. All statistical analyses were performed using the SPSS 11.5 for Windows package (SPSS Inc., Chicago, IL, USA).

Results
Array hybridization
After control of RNA quality (enrichment and integrity) as detailed above, the following amplified RNA pools were included in the microarray analyses: (i) vessels collected from the stroma of an endometriotic nodule (n = 1); (ii) vessels captured from the fibromuscular tissue of endometriotic nodules (secretory phase n = 2; proliferative phase n = 2); (iii) vessels isolated from control tissues (secretory endometrium n = 1, proliferative endometrium n = 1 and myometrium n = 1) and (iv) tissue control pool (vaginal tissue n = 1, proliferative endometrium n = 1, secretory endometrium n = 1, menstrual endometrium n = 1 and myometrium n = 1).

Hybridization efficiency was similar in all four pools, as the scatter plots of log relative intensities of all detected gene transcripts had slopes of ~1. The percentage of probe sets present ranged from 40% to 44%.

Of the 18 400 transcripts evaluated by the arrays, 1690 were significantly enriched compared with whole tissue controls in the vascular cell fraction collected by LCM from fibromuscular tissue of endometriotic nodules, 1808 in vessels collected from the stroma of endometriotic nodules and 1689 in vessels from endometrium and myometrium. Among these genes, many endothelial cell-associated genes were found, including E-selectin, von Willebrand factor, PECAM-1, VEGF, thrombomodulin, angiopoietin 1, as well as genes involved in signaling pathways associated with vascular development and angiogenesis, such as frizzled 4, jagged 1 and protein C receptor (Table II). This further confirms that the LCM procedure was successful for the enrichment of vascular cells. When evaluating
gene expression levels in the three vascular cell populations isolated by LCM, 734 transcripts were found to be significantly overexpressed in vessels from fibromuscular tissue of endometriotic nodules, compared with the vessel control pool. In vessels from endometrial stroma nodules, 923 genes were overexpressed compared with the vessel control pool. Among these transcripts, 68% showed similar levels of expression in vessels from both the stroma and the fibromuscular tissue of nodules. The 90 transcripts with the highest levels of overexpression relative to the vessel control pool are listed in Table III. They include known endothelial cell-associated genes, as well as transcripts with little or no previous association with endothelial cells. Strong overexpression was found for several angiogenic factors (angiopoietin 1, VEGF-4, angiogenin, neuropilin-2), extracellular matrix components (cadherin 19, collagen Iva4, MGP), surface glycoproteins (stanniocalcin, periforin), mediators of integrin signaling (integrin alpha X, thrombospondin), receptors (tumor necrosis factor receptor 11b, insulin-like growth factor binding protein 1, interleukin 2 receptor, CHRM3), mediators of immune response (E-selectin, T cell receptor delta, killer cell immunoglobulin-like receptor, killer cell lectin-like receptor c3) and transcription factors (AP-4, jagged 1, ephrin A3 and A4, sprouty homolog 1).

Validation of microarray data by qRT–PCR

Expression of a subset of overexpressed genes was validated by qRT–PCR (Table IV). The following gene transcripts were selected: two gene transcripts overexpressed more than 20-fold ($P < 0.01$) in vessels collected from the fibromuscular tissue of nodules (cadherin 19 type 2, CDH19; and retinoic acid receptor responder, RARRES), three transcripts overexpressed more than 20-fold ($P < 0.01$) in vessels collected from the stroma of nodules (GRP, GNLY and DKK1) and three transcripts overexpressed more than 20-fold ($P < 0.01$) in vessels collected from both the fibromuscular tissue and the stroma of nodules (CHRM3, PCP4 and tyrosinase-related protein 1, TYRP1). Expression of all transcripts was normalized using cyclophilin A as a reference. All the transcripts analyzed by qRT–PCR showed lower expression ($P < 0.05$) in the whole tissue control pool than in vessels collected from control tissue or the stromal and fibromuscular tissue of nodules, except for GRP (Table IV). Analysis of qRT–PCR data confirms that CHRM3, TYRP1, RARRES, GRP and GNLY are significantly overexpressed in vessels collected from the stroma of nodules compared with control vessels, whereas a similar level of expression was found for PCP4 and DKK1 in both vessel types. In vessels collected from the fibromuscular tissue of nodules, overexpression was confirmed for CHRM3, PCP4, TYRP1, RARRES, GRP and CDH19, compared with the vessel control pool ($P < 0.05$), but not for DKK1 (Table IV). For the two transcripts whose overexpression was not confirmed by qRT–PCR, the level of expression was very low (array signal $< 500$ intensity units and a CT value $> 35$ in the qRT–PCR). qRT–PCR data confirmed that the overexpression of CHRM3, PCP4, TYRP1, RARRES, GRP and CDH19 was specific to vessels from lesions and not dependent on the phase of the cycle. As shown in Table IV, expression of all the genes analyzed by qRT–PCR was low in vessels collected from control tissue from proliferative and secretory endometrium, whereas in vessels collected from the fibromuscular tissue of nodules, gene expression was significantly higher in 5/6 samples during both the secretory and the proliferative phases.

Immunohistochemical validation of endothelial cell expression

A subset of biologically relevant genes that were strongly expressed in vessels isolated from endometriotic lesions compared with control pools was used for validation at the protein level by immunohistochemistry: MGP (active form and all forms), desmin, desmuslin, PEP-19, MMP-3 and TIMP-3. Immunohistochemical staining clearly demonstrated expression of all these markers in the endothelial and/or perivascular cells of endometrium and endometriosis (Figs 2–7). It is also evident that most of these markers are expressed in the smooth muscle cells of vessels and myometrium, or in the epithelial and/or stromal cells of eutopic and ectopic endometrium, as detailed below.

As illustrated in Fig. 2, desmin immunostaining was observed in endothelial and perivascular cells in ectopic endometrium, but not in vessels from eutopic endometrium or myometrium. Strong desmin expression was also found in the smooth muscle cells of the myometrium and rectovaginal space.

Desmuslin expression was detected in vessels recovered from eutopic endometrium and myometrium, and was strong in vessels from endometriotic lesions (Fig. 3). As shown in Fig. 4, low PCP4 expression was observed in vessels in both vessels from eutopic endometrial stroma and myometrium, and those found in and near ectopic endometrial tissue. In some lesions, PCP4 expression was evidenced in epithelial cells.

MMP-3 expression was strong in the vascular and perivascular cells of stromal and fibromuscular tissue of endometriotic lesions (Fig. 5). MMP-3 immunostaining was also found in stromal tissue from eutopic and ectopic endometrium.

TIMP-3 was mainly expressed in glandular epithelium and both vascular and perivascular cells in endometrium and endometriosis (Fig. 6). It is interesting to note that MMP-3 and TIMP-3 were differentially expressed in ectopic endometrial tissue. Expression in the stromal cell compartment was high for MMP-3 and low for TIMP-3, which is consistent with the higher proteolytic activity and increased invasiveness of ectopic endometrial stromal cells.

The most pronounced marker of endometriotic vessels was the MGP whose antibodies recognize both its active and inactive forms. MGP expression was consistently higher in vessels of ectopic endometrium and fibromuscular tissue, compared with matched eutopic endometrium (Fig. 7). Strong MGP immunostaining was also found in ectopic endometrial epithelial cells, whereas expression was weak in the epithelium of corresponding eutopic endometrium.

Discussion

A growing number of studies have shown that gene expression in vascular cells is altered by the environment in which the blood vessels reside (Aird et al., 1997). In a diseased state especially, novel antigens have been evidenced on the surface of endothelial cells, which can be used for both diagnostic purpose and to monitor disease progression (Stevens et al., 2001; Hajitou et al., 2006). In rectovaginal endometriotic nodules, differential gene expression was demonstrated in vessels isolated from fibromuscular tissue and stroma, compared with vessels dissected from endometrium and myometrium. Mapping the transcriptome of vascular cells and preliminary validation of a subset of these genes showed that we were able to selectively isolate transcripts from blood vessels. It also demonstrated that differences in gene expression profiles between vessels from eutopic and ectopic endometrium clearly exist in vivo, justifying our approach. However, these candidate targets need to be investigated further for their diagnostic applicability and should preferably also be validated in a larger clinical study.

We developed methods to achieve selective capture of vascular endothelial cells and pericytes from rectovaginal endometriotic nodules, endometrium and myometrium by LCM and obtain sufficient
Table III. The 90 most up-regulated gene transcripts in vessels collected by LCM from the fibromuscular tissue and stroma of endometriotic nodules, compared with vessels collected from control endometrial and myometrial tissues, as assessed by microarray analysis.

| Gene title | GenBank No. | Fold increase FM tissue vessels | P-value | Fold increase stromal vessels | P-value |
|------------|-------------|---------------------------------|---------|-------------------------------|---------|
| Cholinergic receptor, muscarinic 3 | NM_000740 | 98.7 | 0.05 | 33.7 | 0.05 |
| Purkinje cell protein 4 (PEP 19) | NM_006198 | 79 | 0.05 | 24.3 | 0.05 |
| Cadherin 19, type 2 | NM_021153 | 49.2 | 0.05 | 7.9 | 0.05 |
| Tyrosinase-related protein 1 | NM_000850 | 32.6 | 0.05 | 49.6 | 0.05 |
| Immunoglobulin superfamily, member 4B | NM_021189 | 40.3 | 0.05 | 15.4 | 0.05 |
| Hypothetical protein DKFZp434F0318 | NM_030817 | 35.7 | 0.05 | 3.4 | 0.05 |
| Collagen, type IV, alpha 4 | NM_000092 | 34.4 | 0.05 | 15.6 | 0.05 |
| S100 calcium binding protein, beta (neural) | NM_006272 | 28.8 | 0.05 | 7.2 | 0.05 |
| Titin immunoglobulin domain protein (myotilin) | NM_006790 | 28 | 0.05 | 11.5 | 0.05 |
| Proprotein convertase subtilisin/kexin type 1 | NM_000439 | 27 | 0.05 | 36.2 | 0.05 |
| Desmin | NM_015286 | 26.8 | 0.05 | 7.7 | 0.05 |
| Retinoic acid receptor responder tazarotene induced | NM_002888 | 22.4 | 0.05 | 7 | 0.05 |
| Major histocompatibility complex, class II beta 4 | NM_021983 | 18.5 | 0.05 | 32.8 | 0.05 |
| Calsequestrin 2 | NM_001232 | 17.6 | 0.05 | 4.4 | 0.05 |
| KIAA0146 protein | NM_015931 | 14.3 | 0.05 | 1.9 | 0.05 |
| Actin binding LIM protein family, member 3 | NM_014945 | 14.9 | 0.05 | 9.1 | 0.05 |
| Tumor necrosis factor receptor, member 11b | NM_002546 | 14.8 | 0.05 | 23.1 | 0.05 |
| Desmin | NM_006457 | 13.7 | 0.05 | 7.1 | 0.05 |
| Neurotrimin | NM_006422 | 13 | 0.05 | 9.9 | 0.05 |
| Matrix metalloproteinase 3 | NM_000389 | 12.1 | 0.05 | 2.2 | 0.05 |
| Tangerin | NM_000356 | 10.9 | 0.05 | 2.2 | 0.05 |
| Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | NM_000361 | 10.5 | 0.05 | 2.3 | 0.05 |
| LIM domain binding 3 | NM_006457 | 10 | 0.05 | 2.3 | 0.05 |
| Hepaptomin | NM_000601 | 9.4 | 0.05 | 8.7 | 0.05 |
| Transcription factor AP-4 | NM_003223 | 9.3 | 0.05 | 3.1 | 0.05 |
| Actin-dependent regulator of chromatin, a1 | NM_000369 | 9.1 | 0.05 | 4 | 0.05 |
| Leukocyte immunoglobulin-like receptor B1 | NM_000669 | 9 | 0.05 | 5.1 | 0.05 |
| H factor (complement)-like 1 | NM_002113 | 11 | 0.05 | 5.6 | 0.05 |
| Early B-cell factor 2 | NM_002269 | 10.9 | 0.05 | 1 | 0.05 |
| Tachykinin, precursor 1 | NM_003182 | 10.9 | 0.05 | 20.8 | 0.05 |
| Thrombomodulin | NM_000361 | 10.5 | 0.05 | 2.2 | 0.05 |
| LIM protein | NM_006457 | 10 | 0.05 | 2.3 | 0.05 |
| Hepaptomin | NM_000601 | 9.4 | 0.05 | 8.7 | 0.05 |
| Transcription factor AP-4 | NM_003223 | 9.3 | 0.05 | 3.1 | 0.05 |
| Actin-dependent regulator of chromatin, a1 | NM_000369 | 9.1 | 0.05 | 4 | 0.05 |
| Leukocyte immunoglobulin-like receptor B1 | NM_000669 | 9 | 0.05 | 5.1 | 0.05 |
| H factor (complement) | NM_000186 | 8.7 | 0.05 | 4.9 | 0.05 |
| Metallothionein 1E | NM_014244 | 8.6 | 0.05 | 2.3 | 0.05 |
| 1, 2 matrix, Gla protein | NM_000900 | 8.6 | 0.05 | 5.9 | 0.05 |
| Complement component 4A | NM_007293 | 4.1 | 0.05 | 20.8 | 0.05 |
| T cell receptor delta locus | NM_000362 | 2.6 | 0.05 | 57 | 0.05 |
| Granulysin | NM_006433 | 2.7 | 0.05 | 42.3 | 0.05 |
| Granzyme B | NM_004131 | 2 | 0.05 | 25.5 | 0.05 |
| Dickkopf homolog 1 | NM_012242 | 2.7 | 0.05 | 21.3 | 0.05 |
| D component of complement (adipsin) | NM_001928 | 5.8 | 0.05 | 12.5 | 0.05 |
| Gamma-amino butyric acid A receptor beta 1 | NM_000812 | 5.6 | 0.05 | 11.5 | 0.05 |
| Sorting nexin 10 | NM_013322 | 3 | 0.05 | 11.3 | 0.05 |
| Carboxypeptidase A3 | NM_001870 | 4.2 | 0.05 | 10.5 | 0.05 |
| Ubiquitin-conjugating enzyme E2I | NM_003345 | 6.1 | 0.05 | 9.9 | 0.05 |
| Leukocyte immunoglobulin-like receptor subfamily B2 | NM_005874 | 6.7 | 0.05 | 9.5 | 0.05 |
| Thrombospondin type 1 motif, 2 | NM_014244 | 3.3 | 0.05 | 8.8 | 0.05 |
| Scleroderma autoantigen 1 | NM_006396 | 2.9 | 0.05 | 8.8 | 0.05 |
| Tissue inhibitor of metalloproteinase 3 | NM_000362 | 2.6 | 0.05 | 5.1 | 0.05 |

Overexpressed in vessels from stromal tissue

| gastrin-releasing peptidea | NM_002091 | 4.2 | 0.15 | 57.3 | 0.05 |
| Steroidogenic acute regulator | NM_003349 | 2.2 | 0.15 | 38.1 | 0.05 |
| Killer cell lectin-like receptor subfamily C1 | NM_002259 | 1.5 | 0.3 | 21.8 | 0.05 |
| Perforin 1 | NM_005041 | 1.3 | 0.15 | 20.2 | 0.05 |
| Cathepsin W | NM_001335 | 0.5 | 0.85 | 18.5 | 0.05 |
| Tryptase beta 2 | NM_024164 | 5.4 | 0.15 | 16.8 | 0.05 |
| Granzyme A | NM_006144 | 0.9 | 0.55 | 16.7 | 0.05 |
| Insulin-like growth factor binding protein 1 | NM_000596 | 2.6 | 0.15 | 16.5 | 0.05 |
| Insulin cell immunoglobulin-like receptor | NM_014511 | 3.2 | 0.55 | 15.8 | 0.05 |

Continued
amounts of RNA from these cells to apply a global microarray profiling approach.

Isolation of vascular cells is crucial to the investigation of gene expression in endothelial cells and pericytes, as vascular cells represent only a minor fraction of the total cell population (Ball et al., 2002). Innovative proteomic and genomic approaches have been described to investigate vascular diversity, including LCM (Kinnecom and Pichter, 2005), immunomagnetic isolation of endothelial cells (Lacroce et al., 2004) or fluorescence-activated cell sorting after in vivo endothelial cell labeling (Favre et al., 2003) and in vivo screening by phage display libraries (Arap et al., 2002).

The LCM procedure applied here offers several advantages: (i) it allows recovery of vascular cells from human tissue biopsies, unlike in vivo selection approaches, which are restricted to animal models;

| Gene title | GenBank No. | Fold increase FM tissue vessels | P-value | Fold increase stromal vessels | P-value |
|------------|-------------|--------------------------------|---------|-------------------------------|---------|
| Killer cell lectin-like receptor C3 | NM_002261 | 1.2 | 0.55 | 15.3 | 0.05 |
| Tryptase beta 2 | NM_024164 | 1.4 | 0.55 | 13.2 | 0.05 |
| Matrix metalloproteinase 14 | NM_004995 | 2.9 | 0.15 | 13.1 | 0.05 |
| Interleukin 2 receptor beta | NM_000878 | 1 | 0.85 | 12.8 | 0.05 |
| Plasminogen activator inhibitor type 1 | NM_000602 | 2.8 | 0.15 | 11.9 | 0.05 |
| ATPase, Ca++ transporting plasma membrane 2 | NM_001683 | 5.7 | 0.3 | 11.6 | 0.05 |
| Integrin, alpha X | NM_000887 | 0.9 | 0.85 | 10.8 | 0.05 |
| Hippocalcin-like 1 | NM_002149 | 2.9 | 0.55 | 10.7 | 0.05 |
| Hydroxysteroid (17-beta) dehydrogenase 3 | NM_000197 | 13.8 | 0.05 | 3.4 | 0.15 |
| Cyclin-dependent kinase inhibitor 2A | NM_000878 | 11 | 0.05 | 3.8 | 0.15 |
| Coronin, actin binding protein, 1A | NM_000887 | 10.6 | 0.05 | 3.4 | 0.15 |
| Insulin-like growth factor 2 | NM_000887 | 9.4 | 0.05 | 2.5 | 0.55 |
| Tumor necrosis factor (ligand) superfamily, member 11 | NM_003701 | 9.3 | 0.05 | 10.9 | 0.15 |
| CREB binding protein | NM_003701 | 9.3 | 0.05 | 10.9 | 0.15 |
| N-acysphingosine amidohydrolase 1 | NM_003431 | 9.3 | 0.05 | 10.9 | 0.15 |

Overexpressed in vessels from FM tissue

Myosin, heavy polypeptide 11, smooth muscle | NM_002474 | 20.5 | 0.05 | 1.6 | 0.15 |
| Coagulation factor C homolog, cochin | NM_004086 | 14.2 | 0.05 | 2.7 | 0.15 |
| L1 cell adhesion molecule | NM_000425 | 13.8 | 0.05 | 2.2 | 0.3 |
| Protein tyrosine phosphatase, receptor type T | NM_000705 | 11 | 0.05 | 3.8 | 0.85 |
| Sodium channel, voltage-gated, type VII, alpha | NM_001297 | 10.6 | 0.05 | 3.4 | 0.15 |
| Regulator of G-protein signaling 5 | NM_003617 | 9.4 | 0.05 | 2.5 | 0.55 |
| Phosphatidylinositol binding clathrin assembly protein | NM_003617 | 9.3 | 0.05 | 10.9 | 0.15 |
| Hypothetical protein FLJ11535 | NM_024888 | 9.2 | 0.05 | 2.7 | 0.15 |
| Endothelin receptor type B | NM_000115 | 8.6 | 0.05 | 3.9 | 0.15 |
| Metallothionein 2A | NM_005953 | 8.4 | 0.05 | 1.4 | 0.15 |

aGene selected for qRT–PCR validation.
bGene selected for immunohistochemical validation.

Table IV. Validation of array data by real-time RT–PCR quantification.

| Gene title | GenBank no. | Vessels from eutopic endometrium | Vessels from endometriotic nodules FM tissue | Whole tissue controls |
|------------|-------------|---------------------------------|------------------------------------------|----------------------|
|            |             | Reference pool | Proliferative | Secretary | Proliferative | Secretary | Proliferative | Secretary | Proliferative | Secretary | Proliferative | Secretary | Proliferative | Secretary |
| CHRM3      | NM_000740   | n = 6            | P1 | P2 | P3 | S1 | S2 | S3 | P4 | P5 | P6 | S4 | S5 | S6 |
| PCP4       | NM_006198   | 1.53 | 0.12 | 0.02 | 0.14 | 0.5 | 1.02 | 3.89 | 14.07 | 1.11 | 7.52 | 17.51 | 15.08 | 2.87 | 0.24 |
| TYRP1      | NM_000550   | 0.14 | 1.43 | ND | 0.53 | 0.8 | 1.24 | 1.59 | 24.27 | 43.27 | 1.73 | 5.43 | 11.63 | 35.46 | 0.07 |
| RARRES     | NM_002888   | 1 ND | 0.71 | 0.96 | 0.6 | 8.47 | 9.3 | 0.05 | 2.5 | 0.08 | 1.24 | 1.59 | 24.27 | 43.27 | 0.07 |
| GRP        | NM_002091   | 0.03 | 0.12 | 0.71 | 1.23 | 0.87 | 1.45 | 88.65 | 0.04 | 0.12 | 0.71 | 1.23 | 0.87 | 1.45 | 88.65 | 0.04 |
| DKK1       | NM_001242   | 1 ND | 0.33 | 0.11 | 2.34 | ND | 0.76 | 1.71 | 0.9 | 0.25 | 0.74 | 0.02 | 1.65 | 1.12 |
| CDH19      | NM_021553   | 1 ND | 0.10 | ND | 0.89 | ND | 1.34 | 1.44 | 64.17 | 31.30 | 2.08 | 8.90 | 43.76 | 13.45 | ND |
| Overexpressed in stromal vessels
| GNLY       | NM_006433   | 0.03 | ND | 0.78 | ND | 1.97 | 0.34 | 9.22 | 0.54 | 0.72 | 0.03 | 0.75 | ND | 0.03 | 0.22 |

Results were normalized using cyclophilin as a control and expressed as fold increase relative to the vessel control pool. ND, not detected; CHRM3, cholinergic receptor muscarinic 3; PCP4, Purkinje cell protein 4; TYRP1, tyrosinase-related protein 1; RARRES, retinoic acid receptor responder; GRP, gastrin releasing peptide; DKK1, dickkopf homolog; CDH19, cadherin 19, type 2; GNLY, granulysin.
aControl pool consists in vessels collected by LCM from proliferative (n = 3) and secretory (n = 3) endometria.
bSamples included in the microarray pools.
Significantly overexpressed compared with the vessel control pool (P < 0.05).
Desmin expression was mainly found in the smooth muscle cells of the myometrium (B) and rectovaginal space (C–F). Staining was also observed in the endothelial and perivascular cells in the stromal (C, E) and fibromuscular tissue (D, F) of ectopic endometrium (arrows). Original magnification ×200 (A–D) and ×800 (E, F).

(ii) tissue can be fixed immediately after collection, avoiding lengthy isolation procedures that may alter gene expression; (iii) collection can be precisely targeted at distinct areas (stroma and fibromuscular tissue) of nodules. However, extensive pre- and post-LCM controls are essential to monitor the integrity of microdissected material and confirm the contemplated enrichment, since LCM collection is operator-dependent and hard to standardize. Pre-LCM immunostaining with anti-CD31, anti-cytokeratin and anti-CD10 antibodies allowed us to identify lesions and differentiate blood vessels from epithelial glands.

Figure 2: Desmin immunostaining in normal endometrium and myometrium, and in the stromal and fibromuscular tissue of an endometriotic lesion. Desmin staining was observed in the stromal and fibromuscular tissue of an endometriotic lesion. Desmin immunostaining in normal endometrium and myometrium, and in the stromal and fibromuscular tissue of an endometriotic lesion (arrows). Original magnification ×200 (A–D) and ×800 (E, F).

(ii) tissue can be fixed immediately after collection, avoiding lengthy isolation procedures that may alter gene expression; (iii) collection can be precisely targeted at distinct areas (stroma and fibromuscular tissue) of nodules. However, extensive pre- and post-LCM controls are essential to monitor the integrity of microdissected material and confirm the contemplated enrichment, since LCM collection is operator-dependent and hard to standardize. Pre-LCM immunostaining with anti-CD31, anti-cytokeratin and anti-CD10 antibodies allowed us to identify lesions and differentiate blood vessels from epithelial glands.

After LCM, measuring levels of endothelial cell (PECAM-1) and pericyte (α-ASMA) markers proved to be a useful way of determining the purity of the captured population (Ball et al., 2002). One of the limitations of the LCM approach was that vascular endothelial cells and pericytes could not be collected separately, especially when targeting longitudinally sectioned vessels possibly due to insufficient resolution of the laser beam (~7 µm). This process may also be hindered by the fact that the two cell types are closely connected by adhesion molecules, integrins, gap junctions and extracellular matrix components (reviewed by Hirschi and D’Amore, 1996). However, since recent evidence has shown both endothelial cells and pericytes to be relevant targets for vascular therapy in diseased states (Bagley et al., 2006; Hajitou et al., 2006; Ozerdem, 2006), we did not attempt to separate the two.

Expression profiles were generated from the captured cells. Two rounds of linear RNA amplification were performed using a standard T7 polymerase-based RNA linear amplification protocol to ensure sufficient amounts of RNA for microarray analyses. Previous studies have demonstrated that as many as four rounds of amplification can be performed reliably without significantly altering array results (Xiang et al., 2003). In the present study, reliable RNA amplification was only achieved for samples containing at least 200 captured cells.

We identified a large number of vascular transcripts differentially expressed in vessels collected from fibromuscular and stromal tissue of endometriotic nodules, compared with endometrial and myometrial vessels. Of the 18 400 transcripts included in the arrays, 734 were significantly overexpressed in vessels from the fibromuscular tissue and 923 in vessels from stromal tissue of endometriotic nodules, compared with vessels recovered from control tissues. We catalogued the most frequently expressed transcripts. These included known endothelial cell-associated genes, as well as transcripts with little or no previous association with endothelial cells. The validity of our approach is supported by the fact that transcripts encoding typical endothelial markers, such as von Willebrand factor, PECAM-1, VCAM-1, as well as factors from the VEGF family, were strongly overexpressed in vessels collected from nodules and control tissues, compared with the whole tissue control pool. Further validation was achieved by confirming, by qRT–PCR, overexpression of six out of eight transcripts in the pooled samples used for microarray analysis. The two transcripts that could not be confirmed appeared to be expressed at very low levels. qRT–PCR analyses confirmed overexpression of the six transcripts in vessels collected from the fibromuscular tissue of nodules during both the proliferative and the secretory phases, suggesting that the higher level of expression in endometriotic vessels is not cycle-dependent. However, further studies are needed to investigate whether expression of these genes is regulated by steroids in vascular cells.

A subset of biologically relevant genes, including MGP (active form and all forms), desmin, desmuslin, PEP-19, MMP-3 and TIMP-3, was used for validation at the protein level by immunohistochemistry. Expression levels of these genes were clearly higher in blood vessels than their immediate environment, which further corroborates the validity of our gene expression analysis. Unexpectedly, however, many of these genes were particularly expressed in vascular smooth muscle cells, rather than endothelium. This once again illustrates the difficulty of separating pericytes from endothelial cells. One exception was the MGP, which was mostly found in the extracellular matrix and in endothelial cells of vessels from endometriotic lesions.

Despite strong expression in vessels from endometriotic nodules, PEP-19, desmuslin, desmin, TIMP-3 and MMP-3 do not appear to be markers specific for endometriotic vessels, as they were also expressed in other cell types. These observations strongly suggest that only verification at the protein level can distinguish biologically relevant targets.

Among the proteins analyzed, MGP looks to be one of the most promising marker for targeting ectopic endometrial vessels. It was strongly expressed in vascular endothelial cells and in epithelial cells from endometriotic nodules whereas it was barely expressed in normal endometrium, suggesting the presence of local signals specifically enhancing MGP expression in this tissue. The fact that matrix Gla protein is expressed in endometriotic glands does not preclude its use as a potential vascular marker for endometriotic nodule vascular lus- tature as intravenously administered agents targeting the matrix Gla protein are not likely to reach the glandular epithelium of endometriotic nodules. The MCP is an extracellular matrix protein containing five carboxyglutamate (Gla) residues. It is considered to be an inhibitor of cardiovascular calcification, based on the extensive arterial calcification observed in MGP null mice (Luo et al., 1997; Spronk et al., 2001). More recently, MGP expression was reported in vascular endothelium, where it was shown to enhance VEGF expression by increasing the activity of transforming growth factor-ß1 (Bostrom et al.,...
In turn, MGP is also a TGFB-responsive gene (Shao et al., 2006), suggesting that its up-regulation may be related to a chronic inflammatory environment.

Conclusions and Perspectives

Current treatment modalities, including ovarian suppression therapy and surgical resection of affected tissue, are associated with high morbidity and recurring disease (Donnez and Squifflet, 2004; Emmanuel and Davis, 2005). New options for the diagnosis and treatment of rectovaginal endometriosis are clearly needed. Identification of markers differentially expressed in endometriotic nodules opens up new perspectives for the development of more specific and efficient therapeutic tools (Blumenthal et al., 2001; Matsuzaki et al., 2004).

Vascular and perivascular cell surface markers are attractive targets, directly accessible via the circulation. Ligand-directed vascular targeting agents have indeed proved their efficacy in the treatment of cancer and cardiovascular disease in several preclinical animal models (Hajitou et al., 2006).

LCM of vessels, combined with microarray analysis, constitutes a powerful tool for mapping the transcriptome of vascular cells in rectovaginal endometriotic nodules. Vascular cells in endometriosis...
Currently, it takes overexpress a wide range of transcripts, compared with those collected from control endometrial and myometrial tissues. Resident pericyte populations complicate the identification of endothelial cell-specific antigens. Verification at the protein level is essential to determine biologically relevant molecules, as evidenced by the fact that expression of only one gene validated at the protein level mimicked the difference observed at the mRNA level.

Fusing immunomodulatory, procoagulant or proapoptotic molecules to ligands that bind markers on endothelium (Arap et al., 2002; Hajitou et al., 2006) or pericytes in diseased tissue ligands (Ozerdem, 2006) may allow selective destruction of endometriotic lesions, providing an alternative to surgical resection and preventing recurrence.

Alternatively, combining vascular targeting with contrast-enhancing agents, near-infrared fluorochromes or radioisotopes may also prove useful for the non-invasive detection of endometriosis. Currently, it takes \( \sim 6-8 \) years for endometriosis to be diagnosed (Matsuzaki et al., 2006), during which time the patient typically suffers from severe pain and/or subfertility. Moreover, patients presenting with symptoms often have advanced disease, which is difficult to treat. Improved diagnosis will lead to better treatment, resulting in enhanced quality of life (Squifflet et al., 2002).

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