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High-throughput mRNA sequencing of stromal cells from endometriomas and endometrium

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Abstract

The aetiology of endometriosis is still unclear and to find mechanisms behind the disease development, it is important to study each cell type from endometrium and ectopic lesions independently. The objective of this study was to uncover complete mRNA profiles in uncultured stromal cells from paired samples of endometriomas and eutopic endometrium. High-throughput mRNA sequencing revealed over 1300 dysregulated genes in stromal cells from ectopic lesions, including several novel genes in the context of endometriosis. Functional annotation analysis of differentially expressed genes highlighted pathways related to cell adhesion, extracellular matrix–receptor interaction and complement and coagulation cascade. Most importantly, we found a simultaneous upregulation of complement system components and inhibitors, indicating major imbalances in complement regulation in ectopic stromal cells. We also performed *in vitro* experiments to evaluate the effect of endometriosis patients' peritoneal fluid (PF) on complement system gene expression levels, but no significant impact of PF on *C3*, *CD55* and *CFH* levels was observed. In conclusion, the use of isolated stromal cells enables to determine gene expression levels without the background interference of other cell types. In the future, a new standard design studying all cell types from endometriotic lesions separately should be applied to reveal novel mechanisms behind endometriosis pathogenesis.

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Introduction

Endometrial tissue outside the uterine cavity can form lesions on ovaries and other peritoneal organs, resulting in endometriosis. As the molecular aetiology of endometriosis is still unclear, it is important to detect gene expression alterations in endometriotic lesions and eutopic endometrium of endometriosis patients. Three major types of endometriotic lesions are known, peritoneal lesions, ovarian endometriomas and deep infiltrating lesions (Nisolle & Donnez 1997), that are distinct clinical entities with unique histopathogeneses accompanied by specific expression profiles, depending on the anatomical location of lesions (Meola *et al.* 2010, Ballester *et al.* 2012, Filippi *et al.* 2016). Therefore, it seems reasonable to study endometriomas and lesions from other locations separately.

Several transcriptomic studies have been conducted to find molecular alterations in endometrial cells lining the inner surface of endometriomas (reviewed in Sanchez *et al.* 2015). However, despite hundreds to thousands of genes and specific pathways that have been revealed in these studies, the results remain inconclusive and no common molecular markers or pathways linked to endometriosis have been described. The most probable reason for these discordances could be the tissue heterogeneity of whole-lesion biopsies, as in addition to endometrial cells the endometrioma wall includes various proportions of ovarian stroma, follicles and fibrous tissue that prevent the detection of gene expression alterations characteristic to ectopic endometrial cells (reviewed in Sanchez *et al.* 2014). There are only a few studies utilizing pure cell populations for exploring gene expression in endometriotic lesions. The first study applied laser capture microdissection to gather epithelial cells from eu- and ectopic endometrial tissues (Wu *et al.* 2006), while another study analysed cultured immortalized stromal cells from ovarian endometriosis

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(Kobayashi *et al.* 2012). The studies investigating gene expression alterations in purified and uncultured stromal cells from eu- and ectopic endometrial tissues are still missing, but could be a helpful source of information to understand the disease pathogenesis.

Therefore, the aim of the current study was to reveal the full transcriptome of uncultured endometrial stromal cells from paired samples of endometriomas and endometrium of the same woman.

Materials and methods

Patient characteristics and sample collection

The study protocol was approved by Research Ethics Committee of the University of Tartu (Tartu, Estonia). Patients were enrolled in the study from Tartu University Hospital's Women's Clinic after signing a written informed consent form. Endometriomas and eutopic endometrial tissue samples were obtained from 10 women to isolate stromal cells by fluorescence-activated cell sorting (FACS, Table 1). From nine patients (six with and three without endometriosis), eutopic endometrial samples were collected to establish primary cell cultures (Table 1). All tissue samples were collected during laparoscopy for suspected endometriosis or infertility.

Table 1 General characteristics of the study participants.

Patient ID	Age (years)	BMI (kg/m^2)	Menstrual cycle phase[c]	Endometriosis stage	Infertility
CD10$^+$ stromal cell study					
E213[a,b]	43	24.0	Proliferative	III	No
E218[a,b]	32	28.6	Proliferative	III	Yes, primary
E238[a,b]	32	20.0	Proliferative	IV	Yes, secondary
E244[a,b]	28	17.8	Proliferative	III	Yes, secondary
E160[b]	31	19.7	Proliferative	IV	No
E230[b]	35	17.6	Proliferative	IV	No
E270[b]	33	21.5	Proliferative	III	Yes, secondary
E302[b]	40	25.4	Proliferative	III	Yes, primary
E097[b]	25	17.9	Proliferative	III	Yes, primary
E224[b]	30	21.1	Proliferative	II	No
Cell culture study					
E305*	30	29.1	Proliferative	NA	Yes, primary
E339*	32	21.8	Proliferative	NA	Yes, secondary
E324*	26	21.0	Proliferative	NA	Yes, primary
E279	22	21.4	Proliferative	I	Yes, primary
E316	28	19.0	Proliferative	I	No
E306	25	19.1	Proliferative	I	Yes, secondary
E142	31	20.1	Proliferative	IV	Yes, primary
E302	40	25.4	Proliferative	III	Yes, primary
E343	26	20.5	Proliferative	I	Yes, primary
Peritoneal fluid pool					
E314	44	22.2	Secretory	III	No
E316	28	19.0	Proliferative	I	No
E327	35	20.2	Proliferative	IV	No
E330	35	24.5	Secretory	III	Yes, secondary
E331	30	23.4	Proliferative	II	No
E319	30	19.6	Secretory	III	Yes, primary

[a]mRNA sequencing, [b]validation, [c]based on the date of last menstruation reported by patient and *patients without endometriosis.

Endometrial samples were obtained using an endometrial suction catheter (Pipelle, Laboratoire CCD, Paris, France). Recruited women had not received hormonal medications at least three months before surgery. All collected tissue samples were immediately placed into the cryopreservation medium containing 1× Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific), 30% fetal bovine serum (FBS, Biowest, Riverside, MO, USA) and 7.5% Dimethyl Sulfoxide Hybri-Max (Sigma-Aldrich), placed into Nalgene Cryo 1°C 'Mr Frosty' Freezing Container (Thermo Scientific) and deposited into a −80°C freezer overnight. The frozen biopsies were stored in liquid nitrogen until further use. A proportion of the freshly collected endometrioma samples was placed into formalin for histopathological evaluation, and endometriosis diagnosis was verified on all endometrioma samples.

From a subset of patients (Table 1), peritoneal fluid (PF) was aspirated into a sterile 15 mL polystyrene tube and transported to the laboratory on ice within one hour. Blood-contaminated PFs were not collected. The PF was centrifuged (15 min, 1200 ***g***, at 4°C), filtered through a 0.22 µm pore size membrane (Millex GV filter, Merck Millipore) and stored at −80°C until further use.

Stromal cell isolation from eu- and ectopic endometria

Stromal cells were isolated from endometrial or endometrioma biopsies using FACS as described previously (Krjutskov *et al.* 2016) with minor modifications for ectopic endometria. Briefly, endometrioma samples were rapidly thawed (<1 min) in a 37°C water bath, rinsed with medium, minced with sterile scalpel and then enzymatically digested with collagenase (Sigma-Aldrich) for up to 1 h. Erythrocytes were removed with ACK lysing buffer (Gibco, Thermo Fisher Scientific), and cell suspensions were filtered through 50 µm and 35 µm strainer (Cell Strainer Cap, BD Falcon, San Jose, CA, USA) to separate cells from undigested tissue fragments. Isolated cells from eu- and ectopic endometria were stained with phycoerythrin-conjugated mouse anti-human CD10 antibody (1:20 dilution, clone HI10a, BD Pharmingen, USA) and prior to FACS, DAPI (1 mg/mL, 1:2000 dilution, Invitrogen) was added to the suspension to exclude dead cells from the analysis. On average, the viability of cells was 76.4% (ranging from 71.8 to 81.2%) and 76.4% (ranging from 72.4 to 83.2%) for endometrial and endometrioma biopsies, respectively. Alive CD10+ cells were sorted directly to QIAzol Lysis Reagent (Qiagen). Total RNA was isolated immediately using RNeasy Micro kit (Qiagen).

RNA sequencing

Before library construction, RNA was treated with DNA-free DNA Removal Kit (Invitrogen, Thermo Fisher Scientific), and the quality of the total RNA was analysed on Bioanalyzer 2100 using RNA 6000 Nano chips (Agilent Technologies). Library construction and sequencing were performed at the Estonian Genome Center Core Facility (Tartu, Estonia). 100 bp paired-end mRNA libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) as per manufacturer's instructions and were sequenced on Illumina HiSeq2500.

Sequencing data analysis

Raw sequencing reads were quality controlled using FastQC version 0.9.5 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and cutadapt version 1.8.1 (Martin 2011). Quality-controlled reads were aligned to the human genome (NCBI build 37, hg19) using STAR (version 2.4.0j) alignment tool (Dobin *et al.* 2013). Gene and transcript level quantification of read counts was performed using HTSeq version 0.6.1 (Anders *et al.* 2015). Differentially expressed genes (DEGs) between stromal cells from eutopic and ectopic endometria were identified using the edgeR (Robinson *et al.* 2010), DE*Seq2* (Love *et al.* 2014) and baySeq (v2) (Hardcastle 2016) packages. DEGs were defined as follows: (a) with adjusted *P* value ≤0.05, (b) with at least 2-fold difference between compared samples and (c) recognized by at least two out of three differential expression detection methods used. Biological mechanisms underlying DEGs were investigated using g:Profiler (Reimand *et al.* 2016).

Experiments with peritoneal fluid (PF)

Six PF samples collected from endometriosis patients (Table 1) were thawed, pooled together in equal amounts and the same PF pool was used in all experiments. Preconditioning experiments to optimize the supplemental PF concentration for endometrial cell cultures were performed with increasing amounts of PF pool (0, 10 or 25% of final concentration). As also previously demonstrated (Braza-Boils *et al.* 2015), 25% of final PF concentration was not toxic to cells (data not shown), and was used for further experiments.

Primary cultures of human endometrial stromal cells were prepared from frozen endometrial biopsies collected from nine patients (four with I–II stage endometriosis, two with III–IV stage endometriosis and three without endometriosis, Table 1). Single-cell suspensions of endometrial stromal cells for primary culture were isolated as described previously (Kasvandik *et al.* 2016), isolated cells were seeded into the 24-well cell culture plate and cultured at 37°C in 5% CO_2 until 90% of confluency was reached. Then medium was removed, and cells were washed twice with Mg^{2+}- and Ca^{2+}-free PBS. After washing, cells were treated either with medium containing PF (25% of final concentration) or without PF (0% of final concentration), supplemented with 100 pM oestradiol (Sigma-Aldrich) and incubated for 6, 12 and 24 at 37°C, 5% CO_2 (Fig. 1A). After the exposure time, cells were washed twice with Mg^{2+}- and Ca^{2+}-free PBS, detached using 0.25% trypsin/0.02% EDTA (Gibco, Thermo Fisher Scientific) at 37°C, 15 min. For trypsin inactivation, the full media of DMEM/F12 with 10% FBS serum was added to the cell suspension and collected to low-binding tubes (Eppendorf, Hamburg, Germany). Cells were pelleted at 300 ***g*** for 5 min at room temperature and lysed with QIAzol Lysis Reagent for subsequent RNA extraction. Total RNA was isolated using RNeasy Micro kit (Qiagen).

Real-time PCR

The relative expression of complement cascade genes (*C3*, *CD55*, *CFH*, *A2M* and *SERPINA5*), cell adhesion-related genes (*SELP*, *ESAM*, *CLDN1* and *CLDN11*) and *SERPINE2* was verified in uncultured stromal cells isolated by FACS from nine eutopic and 10 ectopic endometrial tissues (Table 1). Complement cascade genes *C3*, *CD55* and *CFH* were also quantified from primary cultures of endometrial stromal cells conditioned with or without PF. cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), and real-time PCR was performed using 1× HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia).

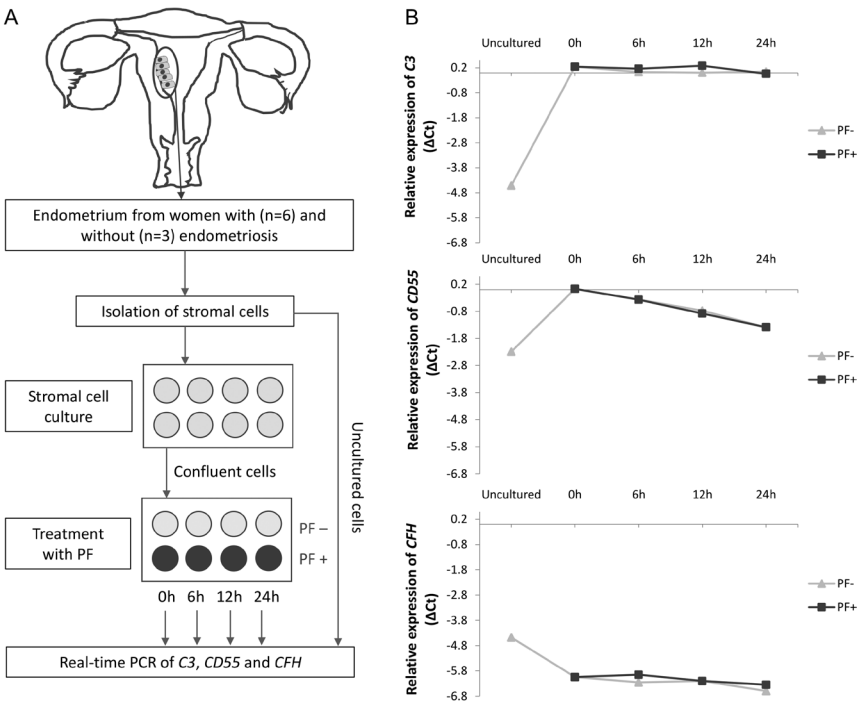

Figure 1 (A) Experimental design of PF study; (B) average relative expression of complement cascade genes in PF experiments. The relative expression of *C3*, *CD55* and *CFH* was measured from cells before cultivation (uncultured), after achieving confluency but before treatment with PF (0 h), and 6, 12 and 24 h after adding PF. In parallel, untreated cells from the same biopsies were cultured and analysed at the same time points; PF, peritoneal fluid.

Table 2 Primer sequences used in the study.

Gene name	Strand	Primer sequence 5′→3′
ESAM	Forward	CCTTTCGTCTTCCATGGCTG
	Reverse	AGCAACCCCAGTCCAACC
A2M	Forward	AGTCTGGTTCTTCTCCTCTTGG
	Reverse	CCCTGACAGACTCCAAGGAA
SERPINA5	Forward	GAACCTCGATAGCAATGCGG
	Reverse	AGTGATACTGATCCTCGCGG
SELP	Forward	GTCAGCTACTCCACCAACCT
	Reverse	ACTGCTGTCCATTGTCCTGA
CLDN1	Forward	GTGCGATATTTCTTCTTGCAGGTC
	Reverse	TTCGTACCTGGCATTGACTGG
CLDN11	Forward	GTACCACTGCAAGCCCCT
	Reverse	TCAGCAGCAGTAAAATGGCC
SERPINE2	Forward	AATGAAACCAGGGATATGATTGAC
	Reverse	TTGCAAGATATGAGAAACATGGAG
C3	Forward	GACTCCATCACCACGTGGGA
	Reverse	CCTGCATTACTGTGACCTCGAA
CD55	Forward	TCCTGGCGAGAAGGACTCAGTGA
	Reverse	AGCCTTGTTGGCACCTCGCA
CFH	Forward	TTGCACACAAGATGGATGGT
	Reverse	GGATGCATCTGGGAGTAGGA
SDHA	Forward	TGGGAACAAGAGGGCATCTG
	Reverse	CCACCACTGCATCAAATTCATG

SDHA was used as a reference gene. Primer sequences are listed in Table 2. Fold change (FC) was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Studied mRNA differences between eu- and ectopic endometrial stromal cells were analysed with two-tailed Student's *t*-test, and *P* value ≤0.05 was considered as significant. Relative expression levels in studied PF experiment groups at different time points were compared using ANOVA and Tukey–Kramer *post hoc* test.

Results

Gene expression profile of eu- and ectopic endometrial stromal cells

To identify DEGs between eutopic endometria and ovarian endometriomas, mRNA sequencing was performed on stromal cells isolated from four paired tissue samples. On average, 7.6 million reads (between 7.0 and 8.6 million per sample) were obtained using Illumina HiSeq2500 platform of which 93% had high-quality scores (≥Q30). Data analysis revealed 1395 DEGs (FC ≥2, adjusted *P* values <0.05) from which 339 and 1056 genes were more abundantly expressed in eutopic and ectopic endometrium, respectively (Supplementary Table 1, see section on supplementary data given at the end of this article). The top 15 most significantly down- and up-regulated genes are listed in Table 3.

Functional annotation analysis of all DEGs utilizing g:Profiler software revealed 'Cell adhesion molecules' (26 genes, *P* value 8.2E-08), 'ECM-receptor interaction' (17 genes, *P* value 3.0E-05) and 'Complement and coagulation cascades' (16 genes, *P* value 5.6E-04) as the most enriched KEGG pathways (Supplementary Table 2). Among other cell adhesion molecules, *CLDN1*, *-5* and *-11*, cadherins and several major histocompatibility complex genes were present. 'Complement and coagulation cascade' pathway included highly upregulated genes in endometriomas, such as *C3*, *C7* and *SERPINA5* (Table 3), and several integrin genes (*ITGA4*, *ITGA7*, *ITGA10*, *ITGA11* and *ITGB4*) from 'ECM-receptor interaction' pathway were differentially expressed in studied cells (Supplementary Table 2). In addition, various other pathways were highlighted by g:Profiler software where 'Calcium signalling pathway' and 'PI3K-Akt signalling pathway' had the highest number of DEGs involved (29 and 43 genes, respectively; Supplementary Table 2).

Validation of selected DEGs

Several genes from the complement cascade, cell adhesion pathways and *SERPINE2* were selected according to sequencing data for further validation in stromal cells from eu- and ectopic endometrial tissues. Real-time PCR analyses confirmed differential expression of all studied genes (Fig. 2), showing upregulation in ectopic endometrial stroma.

Complement pathway genes in endometrial stromal cells are not regulated by PF from endometriosis patients

Next, in order to find out whether the higher expression of complement pathway genes in lesions could be caused by PF composition of endometriosis patients, primary eutopic endometrial stromal cells from nine women (three women without endometriosis and six women with the disease) were cultured and treated with cell culture medium containing 25% of PF collected from endometriosis patients. To avoid bias from individual differences in PF composition, pooled PF from six endometriosis patients with different stages of the disease was used. The relative expression of *C3*, *CD55* and *CFH* was measured from cells before cultivation (uncultured), after achieving confluency but before treatment with PF (0 h), and 6, 12 and 24 h after adding PF. In parallel, untreated cells from the same biopsies were cultured and analysed at the same time points (Fig. 1A).

The uncultured stromal cells showed gene expression levels comparable to FACS-isolated stromal cells used for determining the mRNA differences between eu- and ectopic samples. The studied expression levels in endometrial stromal cells of endometriosis-free women and patients with the disease were similar and were grouped together for further analysis. No significant impact of PF on *C3*, *CD55* and *CFH* levels in stromal cells were observed during the 24-h treatment time compared to untreated cells (Fig. 1B; all $P > 0.05$);

Table 3 Top 15 up- and downregulated genes between stromal cells from endometriomas and endometrium.

Gene	Description	Log2 fold change	Adjusted *P* value (BaySeq)
Upregulated in endometriomas			
C3	Complement component 3	7.9	6.4E-11
HP	Haptoglobin	8.7	1.7E-05
SERPINA5	Serpin family A member 5	6.7	3.6E-05
FMO2	Flavin-containing monooxygenase 2	6.4	5.7E-05
TCF23	Transcription factor 23	5.9	7.8E-05
S100A10	S100 calcium-binding protein A10	6.2	1.3E-04
C7	Complement component 7	7.6	1.6E-04
NR5A1	Nuclear receptor subfamily 5 group A member 1	8.8	2.2E-04
BST2	Bone marrow stromal cell antigen 2	5.0	3.5E-04
GPX3	Glutathione peroxidase 3	6.4	3.9E-04
DAPK1	Death-associated protein kinase 1	4.4	4.4E-04
SLC19A3	Solute carrier family 19 member 3	6.5	4.8E-04
CLDN11	Claudin 11	6.5	6.3E-04
ARHGEF28	Rho guanine nucleotide exchange factor 28	3.9	6.9E-04
SCN7A	Sodium voltage-gated channel alpha subunit 7	8.5	8.7E-04
Downregulated in endometriomas			
KIAA1210	KIAA1210	−5.2	5.7E-06
C8orf31	Chromosome 8 open reading frame 31	−4.5	1.0E-04
SDK2	Sidekick cell adhesion molecule 2	−4.4	1.9E-04
DOK7	Docking protein 7	−5.4	2.6E-04
LRFN5	Leucine-rich repeat and fibronectin type III domain containing 5	−4.1	3.0E-04
TMEM120B	Transmembrane protein 120B	−2.6	5.3E-04
GUCY1A2	Guanylate cyclase 1, soluble, alpha 2	−2.7	7.5E-04
PPP2R2C	Protein phosphatase 2 regulatory subunit Bgamma	−5.8	8.1E-04
ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif 16	−4.5	1.0E-03
RCOR2	REST corepressor 2	−3.2	1.2E-03
TMEM132B	Transmembrane protein 132B	−4.2	1.4E-03
HSD11B2	Hydroxysteroid 11-beta dehydrogenase 2	−5.1	1.8E-03
ARSJ	Arylsulfatase family member J	−3.2	2.0E-03
GDF7	Growth differentiation factor 7	−5.2	2.2E-03
DUSP15	Dual specificity phosphatase 15	−2.2	2.3E-03

however, considerable increase in *C3* and *CD55*, and decrease in *CFH* levels were detected in cultured (0, 6, 12 and 24 h) vs uncultured cells (Fig. 1B; all $P<0.05$), indicating that cell culturing *per se* had larger effect on complement gene expression levels compared to the influence of PF.

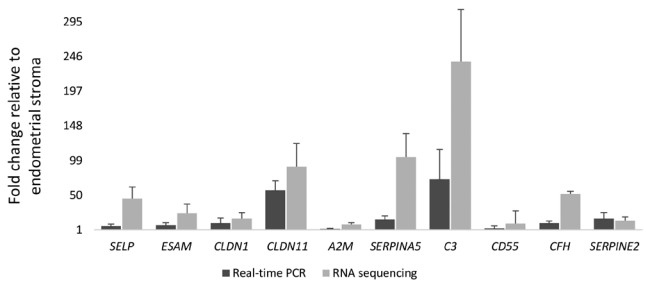

Figure 2 Gene expression level differences between stromal cells from endometrium and endometriomas studied by RNA sequencing and quantitative real-time PCR. Fold change values are given relative to endometrial stroma. All studied gene expression differences were statistically significant ($P<0.05$). The error bars denote S.E.M. (standard error of the mean).

Discussion

Endometriotic lesion biopsies typically contain only a small proportion of endometrial stromal and epithelial cells in addition to various other cell types and surrounding tissue that impedes the detection of gene expression alterations in endometriotic cells (Sanchez *et al.* 2015). In addition, molecular profiles correlate to the anatomical location of lesions (Meola *et al.* 2010, Ballester *et al.* 2012, Filippi *et al.* 2016) and therefore specific cell populations from the selected type of lesions should be studied. For the first time to our knowledge, we used high-throughput mRNA sequencing to reveal transcriptome changes in uncultured endometrial stromal cells from endometriomas and paired samples of eutopic endometrium. Our analysis revealed over 1300 DEGs, and confirmed associations between endometriosis and numerous pathways, including cell adhesion along with complement and coagulation cascade.

It is widely accepted that the impairment of innate immune system via the complement system plays a crucial part in the pathogenesis of endometriosis. Higher expression of complement components, e.g. C3, C7, CFH and CFD, in endometriomas has been

demonstrated (Kobayashi *et al.* 2012, Suryawanshi *et al.* 2014, Ahn *et al.* 2016) and, in our study, *C3* showed the most significantly altered levels between eu- and ectopic endometrial stromal cells (Table 3). Moreover, in our study, complement central components (*C3*, *C7*, *C1QA* and *C1QB*) as well as complement inhibitors (*CD55*, *CFH*, *C4BPB*, *SERPING1* and *CLU*) were upregulated at the same time. Complement regulatory proteins (including CD55, CFH and C4BP) prevent the complement system from being activated when it is not needed (Ferreira *et al.* 2010, Toomey *et al.* 2014). Several tumour cells express complement regulatory proteins at very high levels to protect the tissue from complement-dependent cytotoxicity (Morgan *et al.* 2002). For example, in endometrial tumours, the overexpression of complement regulatory proteins CD55 and protectin has been linked to high resistance to complement-mediated cell destruction (Bellone *et al.* 2012). Therefore, we can speculate that the imbalances in complement activation and inhibition in endometriotic cells result in inflammation and, on the other hand, protect the cells at extrauterine location.

It has been hypothesised that one of the factors affecting complement gene expression levels in endometrial cells outside the normal environment is PF (Tao *et al.* 1997). PF is in direct contact with all pelvic surfaces where endometriotic lesions are most often located and seems to play a pivotal role in the pathogenesis of endometriosis by influencing the anatomical distribution of lesions (Bricou *et al.* 2008). In addition, PF of endometriosis patients has an altered composition (Gilabert-Estelles *et al.* 2007, Berkes *et al.* 2014, Liu *et al.* 2016) that can modify miRNA expression of endometrial stromal cells (Braza-Boils *et al.* 2015). Moreover, the PF of endometriosis patients contains increased levels of cytokines (Taketani *et al.* 1992, Khorram *et al.* 1993, Pizzo *et al.* 2002) that can cause gene expression changes in endometrial stromal cells, including complement cascade genes (Chalpe 2015). Although an upregulation of several complement components in endometriomas has been previously demonstrated (Eyster *et al.* 2007, Hever *et al.* 2007, Borghese *et al.* 2008, Suryawanshi *et al.* 2014, Ahn *et al.* 2016), we were the first who examined the influence of endometriosis PF on the expression level of complement components in endometrial stromal cells. Nevertheless, no differences in *C3*, *CD55* and *CFH* levels were detected in cultured endometrial stromal cells treated with PF from endometriosis patients compared to untreated cultured stromal cells from the same patients. Thus, some factors other than the altered composition of PF must be involved in the complement gene regulation in endometriosis. However, differences in complement cascade gene expression levels were observed between cultured and uncultured stromal cells indicating that cell culturing has a significant impact on gene expression, an issue previously demonstrated by Barragan *et al.* (2016).

We also observed a rise in cell adhesion molecule (CAM) pathway gene levels in ectopic endometrial stromal cells. Increased levels of CAMs, including claudins, selectins, integrins and cadherins have been linked to the development and persistence of endometriotic lesions in peritoneal environment (Witz 2003). CAMs take part in intercellular adhesion and interaction with the extracellular matrix. Through this mechanism, CAMs facilitate the binding of endometrial cells to ectopic sites. Besides cell-to-cell interactions, CAMs mediate various immune and inflammatory processes (Malik & Lo 1996), an attribute very commonly described in endometriosis. Aside from previously described CAMs, our sequencing data revealed several novel cell adhesion pathway genes including *ESAM*, *CD6*, *CDH3*, *MAG*, *LRRC4B*, *NFASC*, *NLGN1* and *NRXN1* that have not been described in relation to endometriosis before (Supplementary Table 1). Thereby, our study results confirmed the relevance of CAMs in the pathogenesis of endometriosis and added several novel candidates to the list of endometriosis-associated CAM-genes.

Several pathways highlighted in our study have also been linked to tumorigenesis (focal adhesion, ECM–receptor interaction, cytokine–cytokine receptor interaction and PI3K-Akt signalling pathway). Endometriosis and ovarian cancer share numerous common features including high oestrogen and cytokine concentration, oxidative stress, etc. that may activate these pathways; however, the association between endometriosis and ovarian cancer is still unclear, and it is generally accepted that endometriosis does not increase cancer incidence (Dunselman *et al.* 2014).

On protein level, our previous study revealed that the protein with the highest difference (FC 19.2) between cultured ectopic and eutopic stromal cells was glia-derived nexin (encoded by *SERPINE2* gene) (Kasvandik *et al.* 2016). In the current study, we also demonstrated an overexpression of *SERPINE2* mRNA (FC 17.1) in uncultured ectopic stromal cells. In cancers, the main functions of *SERPINE2* are enhancement of cellular invasiveness and extracellular matrix production (Buchholz *et al.* 2003). Therefore, our results collectively hint that *SERPINE2* could play a pivotal role in the pathogenesis of endometriosis by altering stromal cell invasive capabilities.

Despite the small number of samples in our mRNA sequencing analysis, we detected substantial differences in gene expression levels between eu- and ectopic endometrial stroma. The number of false positive associations was diminished using three differential expression analysis packages, and DEGs were considered true if confirmed by at least two analysis methods. The verification of selected targets by real-time PCR confirmed the results of mRNA sequencing. The validity of our results is further supported by several previous studies reporting similar findings

(Eyster *et al*. 2007, Hever *et al*. 2007, Borghese *et al*. 2008, Sohler *et al*. 2013, Suryawanshi *et al*. 2014, Ahn *et al*. 2016), suggesting that if a certain gene is highly upregulated in endometrial cells from ectopic location, the surrounding tissue does not hinder the detection of this gene. In addition, as we analysed the specific population of stromal cells from endometrioma and endometrium, we were able to identify several new genes previously not described in endometriosis studies. Therefore, studying specific cell populations has clear advantages over analysing whole tissue biopsies, as this approach enables to reveal changes that occur in certain endometrial cells. For example, an increased expression of genes associated with leukocytes (e.g. *CD4*, *CD45R0*, *CD8A*, *CD3D* and *CD48*) has been observed in endometriotic lesions (Ahn *et al*. 2016). In our study, we did not detect higher expression of these genes in stromal cells from lesions compared to eutopic endometrium, demonstrating that exploring specific cell populations enables to study changes that have occurred particularly in endometrial cells from ectopic locations.

In conclusion, this is the first study utilizing uncultured $CD10^+$ stromal cells to discover large-scale alterations from eu- and ectopic endometria of endometriosis patients. Our data confirmed the upregulation of several cell adhesion molecules and complement components in endometriotic stromal cells and added new molecular candidates for further studies in pathogenesis of endometriosis. The benefits of the current study in identifying novel genes in endometriosis provide proof that in the future, all cell types of endometriotic lesions should be examined separately to uncover mechanisms for the disease development that have thus far remained hidden.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0092.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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