Endometriosis also affects the decidua in contact with the fetal membranes during pregnancy

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STUDY QUESTION: Are the fetal membranes of women affected with endometriosis similar to those from disease-free women?

SUMMARY ANSWER: Decidua of women with endometriosis is able to generate endometriotic-like lesions in contact with the fetal membranes.

WHAT IS KNOWN ALREADY: Eutopic endometrium of women affected with endometriosis presents compromised properties. Endometrium undergoes decidualisation to accept and to further control the conceptus development during pregnancy. Decidualized endometrium is in close contact with the chorionic membrane and forms the choriodecidual layer, a major maternal–fetal interface.

STUDY DESIGN, SIZE, DURATION: This is a laboratory case–control study involving diseased versus control samples. Eleven case samples and 11 control samples were collected from women in a tertiary care/research center between November 2011 and December 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were consecutive pregnant women affected with confirmed endometriosis and disease free women, who underwent Cesarean section before labor for obstetrical indication. The choriodecidual tissues were characterized using histology, immunohistochemistry, transcriptomic and whole genome CpG methylation analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: We demonstrate for the first time the presence of endometriotic-like lesions within the decidual side of the choriodecidual of the fetal membranes from women affected with severe endometriosis. Fetal membranes from women affected with endometriosis exhibited glandular components in the choriodecidual layer surrounded by enlarged decidualized cells disseminated along the entire membrane surface. Significant deregulation (variation of expression ≥2, P-value ≤0.05) was observed for 2773 genes known to be enriched in processes involved in glandular function, endocrine and nervous system, neoangiogenesis, and autoimmune disease. CpG methylation analysis revealed 5999 differentially methylated regions with a P-value <0.05.

LIMITATIONS, REASONS FOR CAUTION: We studied women who delivered at term by Cesarean section before labor; following an uneventful pregnancy. Notwithstanding this, one cannot exclude that the presence of disseminated endometriotic lesions within the choriodecidual layer of the fetal membranes may disturb the anatomical integrity and/or the function of the membranes in some women with endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: Our results shed new light on the capability of the diseased decidua to develop lesions not only at ectopic autologous locations, but also on the semi-allogenous fetal membranes, a particularly immunotolerant environment.

STUDY FUNDING/COMPETING INTEREST(S): The authors have no competing interests to declare. The study was supported by a research grant of the AP-HP (CRC 10134).

Key words: endometriosis / decidua / pregnancy / fetal membranes
Introduction

Endometriosis is a common chronic hormone-dependent gynecologic disorder associated with persistent pelvic pain and/or infertility (de Ziegler et al., 2010). The disease affects ~15% of women of childbearing age and is characterized by the development of glandular and stromal endometrioid-like tissues, in ectopic locations (Giudice, 2010), associated with an impaired differentiation of the endometrium (Brosens et al., 2010). Several studies have demonstrated changes in the eutopic endometrium of women with endometriosis (Fowler et al., 2007; Brosens et al., 2010; Afshar et al., 2013). In normal conditions, endometrium undergoes decidualisation during the secretory phase of the menstrual cycle that persists when implantation of the blastocyst occurs. This progesterone-controlled differentiation corresponds to the transformation of stromal fibroblasts into epithelioid-like decidual cells and to an associated massive influx of immune cells (Brosens et al., 2009). During pregnancy, decidualisation serves primarily to control the implantation of the conceptus and to support a harmonious development of the embryo and its annexes (i.e., placenta and fetal membranes), while maintaining the integrity of the uterine during these processes. Compromised decidualisation of endometrial stromal fibroblasts has been described and is thought to participate as a causal factor for poor implantation rates in women affected with endometriosis (Aghajanova and Giudice, 2011; Harb et al., 2013; Lessey et al., 2013).

As pregnancy progresses, the chorionic layer of fetal membranes develops a large interface of maternal–fetal interaction where trophoblasts and decidual tissue intermingle. We reasoned that this represents the unique physiological interaction between the decidual tissue with a non-uterine soft tissue. An open question in endometriosis is the inherent capacity of endometrial tissue to form lesions in contact with non-uterine soft tissue. Furthermore, endometriosis is described as an estrogen-dependent disorder, linked to resistance to progesterone action in the endometrium (Burney et al., 2007; Brosens et al., 2012). Then, the behavior of diseased endometrium in a steroid-saturated environment, such as in pregnancy, deserves particular consideration. We designed a study to investigate the presence of endometriosis-like lesions in the fetal membranes in pregnancy with women with endometriosis in order to challenge these yet open questions. From this point of view, choriodecidua may be considered as an original in vivo model to investigate the behavior of diseased-endometrial cells within contact to non-uterine tissue under a hormonally saturated state.

Herein, we explored the changes of the choriodecidua in women with endometriosis (CDosis) through histology, immunohistochemistry (IHC), high throughput genomic and epigenomic analyses, in order to gain insight into the intrinsic molecular mechanisms involved in endometriosis.

Materials and Methods

Human choriodecidua samples

All participants signed informed consent at the Department of Obstetrics, Port Royal Maternity, Cochin University Hospital, Paris, France. The study protocol and the use of human tissue were approved by our Institutional Ethical Committee (Comité de Protection des Personnes Ile de France III, Am-5724-I-COL2991, 05/02/13). Placentas with their attached membranes were collected from women affected with severe endometriosis and from control subjects. Cases and controls were delivered by Cesarean section at term before labor for obstetrical indication following an uneventful pregnancy between 2011 and 2013 at the Port Royal Maternity. Cases, all affected with painful deep infiltrating endometriosis (DIE) (Chapron et al., 2003) were either operated before pregnancy for complete surgical resection of the endometriotic lesions (histologically confirmed for the presence of glands and stroma), or had a complete imaging workup before pregnancy that confirmed the diagnosis of endometriosis (Abrao et al., 2007; Guerriero et al., 2008; Piketty et al., 2009). Controls samples were collected from women without past history of either endometriosis or chronic pelvic pain. After delivery, collected fetal membranes were separated from the placenta in the operating room and rinsed in phosphate-buffered saline (PBS) to remove blood clots. Within 15 min of collection, biological samples were further processed in the laboratory. Choriodecidua was peeled from the amnion and appropriately prepared for RNA and DNA extractions, and IHC. This procedure yields pure specimens of choriodecidua as previously shown (Breuiller-Fouche et al., 2010). To reduce the high cost of individual transcriptome of single pool and limit the influences of individual differences among patients, three distinct pools of three series of case and control RNAs and DNAs were analyzed. The whole cohort of 11 cases and controls was used for the quantitative RT–PCR and IHC analyses.

RNA isolation

Total RNA was isolated from the choriodecidua of the fetal membranes from cases and controls, firstly lysed in Trizol (LifeTechnologies, Carlsbad, CA, USA), using the total RNA isolation Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocols. Total RNA samples were eluted in RNase-free water. Purity and concentration were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA sample concentrations were quantified using spectrophotometry by measuring absorbance at 260 nm and each sample was checked for integrity in a RNA nano-chip into Bioanalyzer System at the genomic platform of the Cochin Institute (Agilent Technologies, Santa Clara, CA, USA). Total RNA samples were eluted in RNase-free water. Purity and concentration were evaluated using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total RNA sample concentrations were quantified using spectrophotometry by measuring absorbance at 260 nm and each sample was checked for integrity in a RNA nano-chip into Bioanalyzer System at the genomic platform of the Cochin Institute.

Microarray analysis

Gene expression was assessed by hybridizing the cRNA samples to NimbleGen 12 x 135 K Microarrays (Roche NimbleGen, Roche Diagnostics, Meylan, France) at the genomic platform of the Cochin Institute.

Microarray data processing

Microarray raw data were processed using the ANAIS software (Simon and Biot, 2010). Array quality was assessed at the probe level. Robust Multi-Array analysis, background normalization, and quantile intra- and inter-array normalization were performed. Genes with signal intensities above a random threshold of 95% were chosen for further studies. Finally, the probes were tested for differential gene expression with a nominal P-value ≤0.05 considered to be significant. Among those, genes showing a variation of 2-fold were further considered in the study. The microarray data have been submitted to Gene Expression Omnibus (accession number: GSE57733).

The transcriptomic data obtained from the study of choriodecidua layer were then compared with the data from the microarray analysis of Borghese and coworkers on expression profile of endometrioma (OMA) versus eutopic endometrium (Borghese et al., 2008). Gene ontology (GO) enrichment analyses were then performed for the differentially expressed genes using GePS Genomatix software (Release 2.4.0 Genomatix Software GmbH, Munich, Germany) (Subramanian et al., 2005). A P-value <0.05 was considered significant.
Real-time PCR
The extracted RNAs were treated with deoxyribonuclease (DNase, Invitrogen Life Technologies, St Aubin, France) to remove any contaminating DNA. Total RNAs were reverse transcribed using random primers and monoloye murine leukemia virus Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. A negative control without RNA was included in each series of reverse transcription reactions. Each sample was resuspended in presence of RNaseOut (Invitrogen). Quantitative RT–PCRs were performed as previously described (Santulli et al., 2012). A set of 12 genes, including 10 target genes and 2 reference genes (internal controls), was analyzed by quantitative RT–PCR using cDNA synthesized from each sample. Primers for RT–PCR analysis were chosen using the PRIMER3 software, based on published sequences (Supplementary Table S1). The relative abundance fold changes of each target gene compared with a geometric mean of two different reference genes (ubiquitin C and Cyclophilin B, Ct = 24.0) were determined by the formula –2ΔΔCt (Livak and Schmittgen, 2001). Results were analyzed with the LightCycler software using 2nd derivation method.

Illumina 450 K methylation
DNA was extracted from the choriodecidual using the DNAEasy kit (Qiagen), according to the manufacturer’s instructions. Genomic DNA was bisulfite converted using the EZ DNA Methylation kit (Proteigene, St Marcil, France), according to the manufacturer’s protocol and purified with columns. A whole genome amplification step was followed by enzymatic end-point fragmentation and hybridization to Infinium HumanMethylation 450 BeadChip (Illumina, San Diego, CA, USA). This array includes 485 577 cytosine positions of the human genome (482 421 CpG sites (99.4%), 3091 non-CpG sites and 65 random single nucleotide polymorphisms). After the extension step and staining, the BeadChip was washed and scanned using the Illumina HiScan SQ scanner. The intensities of the images were extracted using the GenomeStudio software (v.2011.1).

Methylome data processing
Methylome array data were processed using the RnBeads R package which allows comprehensive analysis of DNA methylation data obtained with any experimental protocol that provides single-CpG resolution (Assenov et al., 2014). Probes mapping to the X and Y chromosomes or to non-CpG dinucleotides were excluded. After quality check, the microarray probes were filtered and their median foreground intensity was normalized with the “Swan” method (Maksimovic et al., 2012). The probe intensities were then converted to beta values. The probes were tested for differential methylation using the ‘limma’ method, a linear model followed by empirical Bayesian statistics for the comparisons of interest (Ritchie et al., 2006). Among the 485 577 probes present on the microarray, 465 992 were retained for further analyses after pre- and post-filtering. The interaction between CpG status (hypo- versus hyper-methylated) and CpG location with respect to the intragenic, promoter or body, or intergenic location was tested. A GO enrichment analysis was then performed for the differentially methylated regions (DMRs) with an intragenic location using the GePS Genomatix software.

Immunohistochemistry
Briefly, 4-μm thick tissue sections were cut sequentially and mounted on Super-frost pre-treated slides (Menzel-Gläser, Braunschweig, Germany). After deparaffinization in xylene, rehydration through graded ethanol concentrations, and antigen retrieval in citrate buffer at pH 6.0, the sections were incubated for 12 h at 4 °C with primary antibodies (diluted 1:100 in PBS-1% bovine serum albumin −0.1% Triton) including mouse monoclonal anti-Cytokeratin 7 (clone OV-TL 12/30, Dako, Glostrup, DK), mouse monoclonal anti-Vimentin (clone V-9, Dako), rabbit polyclonal anti-estrogen receptor (ER) (18-0174, Invitrogen, St Aubin, France), and mouse monoclonal anti-progesterone receptor (PR) (clone PgR 636, Dako) antibodies to characterize: (i) the membrane layers; amnion and decidua being Vimentin +; chorion, cytokeratin +, (ii) the epithelial or the stromal components, Cytokeratin + and Vimentin + respectively, and (iii) sex steroid hormone responsive cells, ER+ /− and/or PR+ /−, to define endometriotic-like glandular structures. To validate the genomic findings, tissue sections were incubated with primary antibodies for 12 h including rabbit polyclonal anti-c-fos (GE10, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-clun (68A2, Cell signaling technologies, Danvers, MA, USA), mouse monoclonal anti-C20 (L26, Bioprime, Kronshagen, Germany), mouse monoclonal anti-CD31 (clone JC/70A, Dako) and rabbit polyclonal anti-BDNF (SC-546, Santa Cruz Biotechnology) antibodies. The reactions were revealed using the Novolink polymer detection system (Leica, Nanterre, France) according to the manufacturer’s recommendations.

Results
Subject characteristics
The general characteristics of the subjects are reported in Supplementary Table SII. Women affected with DIE and women of the control group were comparable with respect to age, and gravidity. Parity was significantly lower in the case group, as well as neonatal birthweight of the offspring. Obstetrical indications for Cesarean section did not differ between the two groups. Seven (63.6%) of the cases were surgically treated before pregnancy while four (36.4%) of the cases had a complete imaging work up that confirmed the existence of DIE. Pregnancies of three (27.3%) cases and one (9.1%) control were obtained by assisted reproductive technologies.

Comparative gross examination
Thickening of the choriodecidual and presence of nodular lesions was observed in all of the eleven cases, in comparison to controls (Fig. 1A and B). The nodules were multiple, of different sizes, red and/or brownish in color, and randomly distributed across the fetal membrane circumference. These lesions were implanted on the decidual layer and were surrounded by small vessels. The majority of the nodular lesions showed a network of distended atrophic cystic glands, whose wall was lined by cuboidal epithelial cells, some of them developing hyperplastic papillary projections (Fig. 1C and D). In some areas, smaller size glands were also found, surrounded by myxoid stroma admixed with scattered inflammatory cellular infiltrates and fibrinoid deposits. Decidual cells surrounding the lesions were round and enlarged, with large vacuoles, when compared with control tissue (Fig. 1C and E). In addition to the atrophic cystic glands, scant isolated areas containing microscopical chorionic pseudo cysts (MCPs) filled with a homogeneous eosinophilic material were observed (Fig. 1D). These MCPs were, however, also observed in the control membranes (Fig. 1F).

Comparative immunolabelling analysis
To further characterize the presence of nodular lesions and the adjacent surrounding tissue within the fetal membranes, we focused on immunohistochemical markers: cytokeratin 7, vimentin, and steroid hormones receptors ER and PR. In cases and controls, trophoblasts were cytokeratin 7 positive, while the amnion and decidua were vimentin positive.
Specifically in CDosis, the pseudo-glandular wall lining cells exhibited intense immunoreactivity for cytokeratin 7, while the enlarged decidualized cells surrounding the glands presented a stronger vimentin staining in comparison to controls. Only in CDosis, the nucleus of decidual cells was positive for ER (Fig. 2E and F). The nucleus of the decidualized cells surrounding the glands was also strongly immunoreactive for PR antigens in CDosis, while fewer decidual cells were PR positive in controls and displayed a less intense staining (Fig. 2G and H).

**Comparative transcriptome analysis**

To gain insight in molecular processes in CDosis, we performed a comparative transcriptional analysis of the choriodecidua from cases and controls. Principal component analysis indicated that > 95% of the observed variability can be summarized in two major components: the F1 axis, which describes the common origin of the samples, i.e. choriodecidua, and the F2 axis which distinguishes cases from controls (Fig. 3A). As represented on a heatmap and a volcano plot (Fig. 3B and C), 2773 genes have a significant variation of expression ≥2 (1235 down-regulated and 1538 up-regulated genes, respectively). The list of the 2773 deregulated genes in CDosis is presented in Supplementary Table SIII. The expression level of ten genes was further investigated by RT–qPCR (Fig. 3D and Supplementary Table SIV). In CDosis, the expression level of activating transcription factor 3 (ATF3), estrogen receptor 1 (ESR1), lymphoid enhancer-binding factor 1 (LEF1), neuronal cell adhesion molecule (NRCAM) and brain-derived neurotrophic factor (BDNF) were significantly increased when compared with controls (6.7-, 16.9-, 26.5-, 3.5- and 5.3-fold, respectively; P < 0.05). In contrast, in CDosis, expression of small proline-rich protein 3 (SPRR3), interleukin...
7 receptor (IL7R), high mobility group AT-hook 1 (HMGA1), signal transducer and activator of transcription 3 (STAT3), and BARX homeobox 2 (BARX2) were significantly decreased when compared with controls (4.6-, 15.4-, 44.4-, 11.0- and 9.4-fold, respectively; P < 0.05). Among the 2773 deregulated genes, GO analyses revealed significant enrichment of GO terms related to glands, endocrine system, neurons,
autoimmunity, translation and metabolism in general, all known to be important in the development and progression of endometriosis (Table I).

We next performed a comparison between the genes deregulated in CDosis and those reported as deregulated in endometrioma (OMA) occurring in non-pregnant women (Borghese et al., 2008). Seven hundred and seventy-six genes were common ($\chi^2$, 21.739; $P$-value, $3.12 \times 10^{-6}$), with enrichment in GO terms related to immunity, nervous system, endometrium, glandular structure and estrogen activity as top processes (Fig. 4). In CDosis, 1997 genes were unique, related to blood, leucocyte, placenta, endocrine system, sensory organ and mRNA metabolic process, while OMA samples were enriched in genes related to fibrosis, vascular system and inflammation in the top processes.

**Comparative methylome analysis**

We next used Illumina’s HumanMethylation450 beadchips to compare global profile of methylation in CDosis to controls. On the 450 K DNA Illumina Methylation array, 485 577 cytosine positions of the human genome were evaluated. From these cytosine sites, 19 595 (4.03%) positions were excluded because they were located either on the X and Y chromosomes or were non-CpG dinucleotides. Density
estimations derived from the full-range of normalized beta values from each pool, shown in Figure 5A, showed an overall unidirectional shift in methylation toward hypermethylation in CDosis. To note, there is not a strict bimodal frequency distribution of methylation, but rather a sharp peak at the beta value of 0 and a large shallow peak between the values of 0.5 and 1. This most probably reflects differences in methylation within the different cells that compose the choriondecidua rather than substantial hemi-methylation. In fact, from the 465,992 CpGs dinucleotides studied, 21,468 were differentially methylated, defining 5999 DMRs that were considered as significantly deregulated (P-value < 0.05) with 257 (4.3%) hypo-methylated and 5742 (95.7%) hypermethylated regions (Fig. 5B, Median (interquartile range): 0.063 (0.04, 0.09)).

Given the tissue heterogeneity, we did not apply a cutoff for the delta values of 0.5 and 1. This most probably reflects differences in methylation at specific chromosomal hot spots (Fig. 5C). We then studied the genomic functional distribution of the 5999 significantly differentially methylated positions. Systematically we observed that whatever the genome position, hypermethylation was strikingly more represented than hypomethylation (Fig. 5D). The majority of differentially methylated loci (94.4%) were located outside of the CpG Islands. The intragenic locations corresponded to 1209 genes, in total. Non-supervised analysis of these genes revealed an enrichment in GO terms related to neurons, locations corresponded to 1209 genes, in total. Non-supervised analysis of these genes revealed an enrichment in GO terms related to neurons, endocrine system, receptor activity and immunity (MHC Class II, Brunton's disease) (Table II). We next examined whether hyper- or hypomethylation status was correlated positively or negatively to gene expression in CDosis and found no significant correlation (Fig. 5E).

Comparison of the list of genes that are deregulated in the transcriptomic array with the genes which contained differentially methylated CpG did not show any significant enrichment in common genes ($\chi^2$, 0.146; $P$, 0.70).

### Immunohistochemical validation of transcriptome and methylome data

In accordance with our transcriptomic data, c-Myc and c-Jun were overexpressed >=4-fold. We evaluated their expression by IHC and found high immunostaining for c-Myc in the cytoplasm of decidual cells (Fig. 6A and B) as well as nuclear presence of c-Jun (Fig. 6C and D) in cases when compared with control samples.

To validate at the protein level the GO terms enrichment with regards to autoimmunity, vascularization and neurons, we performed IHC experiments. We observed de novo expression of CD20 antigens, specific for lymphocytes B (Fig. 7A), CD31, specific microvascular structures (Fig. 7C) and BDNF epitopes, related to neurogenesis (Fig. 7E) in the decidual part of the CDosis. None of these three immunoreactivities were observed in control samples (Fig. 7B, D and F).

### Discussion

In this study, we report for the first time the presence of endometriotic-like lesions on the decidua layer in contact with the fetal membranes of women affected with severe endometriosis. We describe specific alterations of choriondecidua at the histological, transcriptomic and epigenomic levels.

### Histological and immunohistological aspects of CDosis resemble peritoneal endometriosis

We observed red brownish nodular lesions located in the decidual layer of CDosis, a macroscopic aspect similar to that generally seen in peritoneal endometriosis (Vercellini et al., 2014). All these structures appeared as implanted into the decidual layer. Both histological and immunohistochemical features of decidual from women with endometriosis strongly resemble the histological and IHC observations in endometriosis, including epithelial papillary projections, fibrinoid deposits and strong immunoreactivity of cells lining the cysts for cytokeratin, and in stroma for vimentin, c-Myc, c-Jun, ER and PR (Norwitz et al., 1991). Comparatively, the endometriotic-like gland histomorphology is wholly different from that of the MCPs detected in the membrane trophoblast layer. The MCPs are a common finding in normal conditions and were previously described as hypoxia-associated placental lesions (Stanek, 2013).

### Differential gene expression and methylation pattern in CDosis

The transcriptomic analysis allowed us to characterize a differential pattern of gene expression in CDosis. A significant number of these deregulated genes are also deregulated in endometriosis. This subset of genes was related to immunity, nervous system, endometrium and

<table>
<thead>
<tr>
<th>GO term</th>
<th>Observed genes</th>
<th>Expected genes</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glands</td>
<td>1939/2773</td>
<td>1640/12,087</td>
<td>2.80 x 10^{-41}</td>
</tr>
<tr>
<td>Endocrine system</td>
<td>1547/2773</td>
<td>1270/9349</td>
<td>1.29 x 10^{-32}</td>
</tr>
<tr>
<td>Placenta</td>
<td>1900/2773</td>
<td>1630/12,608</td>
<td>2.37 x 10^{-32}</td>
</tr>
<tr>
<td>Nervous system</td>
<td>2142/2773</td>
<td>1920/14,183</td>
<td>3.54 x 10^{-28}</td>
</tr>
<tr>
<td>mRNA metabolic process</td>
<td>163/2773</td>
<td>84/598</td>
<td>5.04 x 10^{-18}</td>
</tr>
<tr>
<td>Motor neuron disease</td>
<td>221/2773</td>
<td>169/1331</td>
<td>9.46 x 10^{-6}</td>
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<tr>
<td>DNA-repair deficiency disorders</td>
<td>216/2773</td>
<td>167/1312</td>
<td>2.11 x 10^{-5}</td>
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<tr>
<td>Autoimmune disease</td>
<td>49/2773</td>
<td>30/226</td>
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<tr>
<td>IL-12 mediated signaling events</td>
<td>19/2773</td>
<td>9/63</td>
<td>1.13 x 10^{-3}</td>
</tr>
</tbody>
</table>

CDosis, choriondecidua of endometriosis-affected women; GePS, Genomatix Pathway System; IL, interleukin.

*Chi-squared test.
glandular structure, confirming the endometriotic-like character of this diseased tissue at the molecular level. The genes, only significantly deregulated in CDosis, were related to placenta, recalling for the presence of trophoblast and pregnancy, and to mRNA metabolic process. In this regard, increased ribosomes have already been observed in endometriotic lesions by transmission electron microscopy, as well as overexpression of genes coding for ribosomal proteins (Arimoto et al., 2003; Jin and Zhou, 2010).

The methylation analysis showed that modifications were strongly biased toward global hypermethylation in CDosis versus controls. Likewise in the transcriptomic signature, epigenomic signature in CDosis is consistent with processes known to be causing ectopic implantation of endometriotic cells: autoimmunity, vasculogenesis and neurogenesis. Most of the DMRs (~75%) were intergenic and outside the CpG islands, which may explain why the molecular signatures of the methylome and the transcriptome did not share a significant number of genes. Moreover, most CpG sites with variable methylation seem unrelated to the variation in gene expression. DNA methylation near the transcription site of a gene has been proposed to be associated with reduced expression (Jones and Liang, 2009). However, a wide

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**Figure 4** Comparison of the transcriptome signature of choriodecidual from women with endometriosis with the transcriptome signature of endometrioma. Venn diagram representations of the significant over-represented gene ontology (GO) terms in CDosis and from endometrioma (OMA). Unsupervised global analyses were performed on the common and uniquely represented clusters of the different subsets of genes using the GePS software from Genomatix. (A) Top ten enriched GO terms in deregulated genes, uniquely in CDosis; (B) Top ten enriched GO terms in deregulated genes, common between CDosis and OMA; (C) Top ten of enriched GO terms in deregulated genes, uniquely in OMA.
variety of relationships between gene expression and DNA methylation are now described and variation in expression is best predicted using specific chromatin marks rather than position of the CpG site with respect to the gene (Wagner et al., 2014). Further analyses are thus needed to investigate the relationships between hypermethylated CpGs and regulation of gene expression in endometriotic-like lesions. Yet global methylation analyses provide biological information about the functional state of tissues and cells and point to novel genes and pathways underlying diseases. To this point of view, autoimmunity phenomena are in close relationship with the pathogenesis of endometriosis (Eisenberg et al., 2012). Autoimmune organ-specific antibodies directed against endometrial or ovarian proteins are present in peritoneal and peripheral fluids of women with endometriosis. Here we detected in CDosis unusual presence of cells positive for CD20, a marker of lymphocyte of B-lineage. The immunologic autoreactivity may derive from an inappropriate inflammation, activation of immune cells and production of cytokines. However, the role of autoantibody in endometriosis remains not well understood. Furthermore endometriotic lesions are typically characterized by a dense vascularization (Laschke et al., 2011). Neovasculogenesis is needed to provide oxygen and essential nutrient to guarantee survival and proliferation of lesions. In this regard, blood was the GO term enriched with the lowest P-value in our

Figure 5  Global CpG methylation pattern in choriodecidua from women with endometriosis versus controls. (A) Global normalized density estimation in CDosis versus normal choriodecidua. (B) Pie chart showing the number of CpG sites hypo- or hypermethylated in CDosis compared with normal choriodecidua and the number of differentially methylated regions (DMRs). (C) Pie chart showing the distribution of DMRs in CDosis on the autosomal chromosomes. (D) Pie chart showing the distribution of the DMRs in CDosis based on their genomic context, intergenic, intragenic, and promoters. (E) Scatter plot of the fold change obtained from the transcriptome array and the delta of beta values obtained from the methylome array for the genes associated with significant deregulated expression and significant differential methylation in CDosis.
Table II GO enrichment in methylome of CDosis versus Controls according to GePS.

<table>
<thead>
<tr>
<th>GO term</th>
<th>Observed genes</th>
<th>Expected genes</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseases susceptibility</td>
<td>417/1209</td>
<td>347/8347</td>
<td>$6.29 \times 10^{-8}$</td>
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<tr>
<td>Neuroectodermal tumors</td>
<td>340/1209</td>
<td>278/6699</td>
<td>$1.20 \times 10^{-6}$</td>
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<tr>
<td>Neoplasms, glandular and epithelial</td>
<td>478/1209</td>
<td>419/10075</td>
<td>$2.58 \times 10^{-6}$</td>
</tr>
<tr>
<td>Neoplasm, nerve tissues</td>
<td>342/1209</td>
<td>284/6826</td>
<td>$4.25 \times 10^{-6}$</td>
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<tr>
<td>Receptor activity</td>
<td>104/1209</td>
<td>70/1525</td>
<td>$2.44 \times 10^{-5}$</td>
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<tr>
<td>Neuroendocrine tumors</td>
<td>222/1209</td>
<td>176/4248</td>
<td>$4.28 \times 10^{-5}$</td>
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<tr>
<td>Hypertrophy</td>
<td>154/1209</td>
<td>113/2726</td>
<td>$2.64 \times 10^{-5}$</td>
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<td>MHC Class II protein complex</td>
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<td>0.7/16</td>
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<td>0.5/10</td>
<td>$7.51 \times 10^{-4}$</td>
</tr>
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<td>Bruton tyrosine kinase</td>
<td>10/1209</td>
<td>3.10/67</td>
<td>$9.64 \times 10^{-4}$</td>
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</tbody>
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*Chi-squared test.

Figure 6 IHC analysis of c-Myc and c-Jun in choriodecidua of women with endometriosis versus controls. High-magnification of fetal membranes sections collected from women with endometriosis (left panel) and from controls (right panel). Immunohistochemistry analyses encompass c-Myc (A and B) and c-Jun (C and D) staining. The immunohistochemistry reaction was revealed with DAB as a substrate, brown depots being the signal. Scale bar, 100 μm.
transcriptome analysis. Our findings indicate an increased microvessel density in CDosis, as identified with the endothelial marker CD31. Finally, neurotrophins have been previously documented in endometriosis highlighting the central role of these proteins in the genesis and modulation of pain in endometriosis (Borghese et al., 2010; Browne et al., 2012). Classically known for their participation in the development of nervous system, overexpression of neurotrophins has also been observed outside the nervous system (Yang et al., 2013). For instance, BDNF, although abundant in the nervous system, is expressed in other cell types and tissues, and BDNF mRNA is found in the majority of the human organs (Pruunsild et al., 2007). BDNF is capable of activating the adhesion, angiogenesis, apoptosis and proliferation prominently involved in reproductive physiology. In a recent cross-species study, BDNF has been found conserved and ubiquitously expressed in mammalian uterus (Wessels et al., 2014). BDNF proteins were described to be more concentrated in biopsies from eutopic endometrium of women.

Figure 7: IHC analysis of CD20, CD31 and BDNF in chorionic decida of women with endometriosis versus controls. High magnification of fetal membranes sections collected from women with endometriosis (left panel) and from controls (right panel). Immunohistochemistry analyses encompass CD20 (A and B), CD31 (C and D) and BDNF (E and F) staining. Arrow heads indicate CD20 stained cells. The immunohistochemistry reaction was revealed with DAB as a substrate, brown deops being the signal. Scale bar, 100 μm.
with endometriosis compared with controls (Browne et al., 2012). Herein, RNA expression of BDNF was increased from 6- to 8-fold in CD8+ T cells, and IHC documented the increased localization of BDNF within the decidual compartment.

Development of endometriotic-like lesions despite the high levels of steroid hormones during pregnancy

Endometriosis is described as an estrogen-dependent disorder in relation with progesterone-resistance, linked to the deregulation of a subset of progesterone-dependent genes. This is usually associated with increased expression of ER and decreased expression of PR and/or PR coactivators (Aghajanova et al., 2010). Here, we found low level of expression of ER and high expression of PR in decidual cells of endometriosis-affected women. Moreover, all these processes occurred under the saturated hormonal state in pregnancy. The hormonal-independent changes described here in the diseased choriodecidualia are similar to the recent finding of Santulli and coworkers, which highlights that hormonal treatments fail to control endometriosis progression and enhance inflammatory processes (Santulli et al., 2014). Furthermore a recent study described lower expression of ER and higher expression of PR in decidua of women with endometriosis who subsequently became pregnant within 1 year (Moberg et al., 2014). This piece of evidence points toward disparities in fertility impairment and pregnancy outcomes in women with endometriosis. It indeed remains unclear to what extent endometriosis affects pregnancy. The disease has been associated with higher risks of adverse obstetrical outcomes including miscarriage, premature birth, fetal growth retardation, preeclampsia and placental disorders (Kortelahti et al., 2012; Harb et al., 2013; Conti et al., 2013). In the present study, all the eleven case patients gave birth at term after an uneventful pregnancy. However, neonatal weight at birth was significantly lower in women affected with endometriosis when compared with the control group, suggesting a less effective placental perfusion. A larger study, which will prospectively recruit women affected with endometriosis in earlier stages of pregnancy, is warranted on this topic.

Analyses of this particular model of endometrial cells in direct contact within non-uterine tissues, the semi allogenic fetal membranes, allowed us to characterize endometriotic-like lesions, spontaneously generated on this tissue interface. The lesions in the fetal membranes from women with endometriosis are located in the decidual layer and were never observed on the fetal side of the membranes, the chorion or the amnion. This is consistent with our hypothesis and reveals the unique capacity of diseased endometrium to spontaneously form nodular endometriotic-like lesions in direct contact with non-uterine soft tissue. During normal pregnancy, the choriodecidualia is maintained in an immune-tolerant state. Notably the decidual natural killers cells (NKs) present poor cytotoxicity (Riley and Yokoyama, 2008). In non-pregnant women, uterine NKs are implicated in the clearance of endometrial cells during menstruation and of regurgitated endometrial cells in the peritoneum (Moffett and Colucci, 2014). Here, in diseased choriodecidualia, a locally altered immune environment may foster the differentiation of endometriotic lesions.

In conclusion, endometriotic-like glandular structures were identified within the fetal membranes of women with past history of severe endometriosis. The well-known hormonal ovarian blockade occurring during pregnancy was apparently not sufficient to prevent development of endometriotic lesions into the choriodecidual layer. This implies that eutopic diseased endometrium of women with endometriosis has the ability to spontaneously generate endometriotic lesions in contact with non-uterine tissue in a local immunotolerant environment. This may further point toward more general defective immune functions in the endometrium of women with endometriosis, either at a eutopic or ectopic location.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

L.M. and C.M. conceived, carried out experiments and analyzed data. J.G., C.L. and S.J. carried out experiments and analyzed the data. P.S., C.C., F.G. and D.V. provided crucial ideas, helped with design of experiments and analyzed the data. All authors were involved in writing the paper and had final approval of the submitted versions.

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Conflict of interest

None declared.

References


