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Ovarian-like differentiation in eutopic and ectopic endometrioses with aberrant FSH receptor, INSL3 and GATA4/6 expression

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ABSTRACT

Endometriosis, the hormone-dependent extrauterine dissemination of endometrial tissue outside the uterus, affects 5–15% of women of reproductive age. Pathogenesis remains poorly understood as well as the estrogen production by endometriotic tissue yielding autocrine growth. Estrogens (E2) are normally produced by the ovaries. We investigated whether aberrant "ovarian-like" differentiation occurred in endometriosis.
69 women, with ($n = 38$) and without ($n = 31$) histologically proven endometriosis were recruited. Comparative RT-qPCR was performed on 20 genes in paired eutopic and ectopic lesions, together with immunohistochemistry. Functional studies were performed in primary cultures of epithelial endometriotic cells (EEC).
A broaden ovarian-like differentiation was found in half eutopic and all ectopic endometriosis with aberrant expression of transcripts and protein for the transcription factors GATA4 and GATA6 triggering ovarian differentiation, for the FSH receptor (FSHR) and the ovarian hormone INSL3. Like in ovaries the FSHR induced aromatase, the key enzyme in E2 production, and vascular factors in EEC. The LH receptor (LHR) was also aberrantly expressed in a subset of ectopic endometriosis (21%) and induced strongly androgen-synthesizing enzymes and INSL3 in EEC, as in ovaries, as well as endometriotic cell growth. The ERK pathway mediates signaling by both hormones. A positive feedback loop occurred through FSHR and LHR-dependent induction of GATA4/6 in EEC, as in ovaries, enhancing the production of the steroidogenic cascade.
This work highlights a novel pathophysiological mechanism with a broadly ovarian pattern of differentiation in half eutopic and all ectopic endometriosis. This study provides new tools that might improve the diagnosis of endometriosis in the future.
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1. Introduction

Endometriosis, characterized by the extrauterine dissemination of endometrial tissue, is a major public health problem affecting 5–15% of women of reproductive age. It has an incidence of up to 50% in women seeking treatment for pain and infertility, places a large economic burden on countries throughout the world and is functionally debilitating for affected women. The pathogenesis of endometriosis is still poorly understood but it is known to be a hormone-dependent disease. The survival and proliferation of lesions is dependent on estrogens (E2) produced by endometriotic implants which acquire aberrant endocrine function. E2 induces autocrine and paracrine growth pathways. This chronic disease is often diagnosed late, about 7–10 years after the apparition of the first symptoms during surgery [1–4] when lesions have already disseminated in the pelvis. There is no early non-invasive marker predicting the development of the disease. Endometriosis is considered benign disease, but has features common with cancers, such as progressive estrogen-dependent proliferation, peritoneal and pelvic invasion, associated with chronic inflammation and angiogenesis [1,5]. The primary mechanism of action of hormone therapy for endometriosis is the inhibition of estrogen production [2,3,6].

The mechanisms underlying the abnormal production of estrogens by endometriotic tissue are poorly understood. Inflammation is thought to be responsible for overexpression of the Steroidogenic Factor-1 (SF1) transcription factor and in situ E2 production in endometriosis [1]. However, E2 is normally produced by the ovary. The FSH receptor (FSHR) and LH receptor (LHR) play a critical role in this process by inducing respectively aromatase, which converts androgens into

Abbreviations: CYP11A1, Cytochrome P450 Family 11 Subfamily A Member 1; CYP17, Cytochrome P450 Family 17 Subfamily A Member 1; CYP19A1, Cytochrome P450 Family 19 Subfamily A Member 1; EEC, Epithelial Endometriotic Cells; EGVEGF, Endocrine Gland-derived vascular endothelial growth factor; GATA4/6, GATA binding protein 4/6; INSL3, Insulin Like 3; LHR, Luteinizing Hormone Receptor; FSHR, Follicle Stimulating Hormone Receptor; PTGER, Prostaglandin E Receptor; PTGS2, Prostaglandin-Endoperoxide Synthase 2; RT-qPCR, Reverse Transcription quantitative Polymerase Chain Reaction; SF1, Steroidogenic Factor-1; VEGF, Vascular Endothelial Growth Factor.

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estrogens, and steroidogenic enzymes responsible for the synthesis of androgens, which serve as substrates for aromatase [7]. This raises questions about the role of the FSHR and LHR in controlling endometriotic steroidogenesis and whether more general aberrant "ovarian-like" differentiation occurs in endometriosis.

Angiogenesis of the endometriotic cells in implanted places appears to be essential for the survival and development of endometriotic cells, as already reported for tumor growth [2]. FSHR is also expressed in tumor blood vessels [8] and most metastases [9]. It is thought to stimulate angiogenesis, thereby favoring tumorigenesis.

We investigate a possible "ovarian-like" differentiation in endometriosis. We thus studied FSHR and LHR expression in paired eutopic endometrium and ectopic lesions of patients with endometriosis by RT-qPCR and immunohistochemistry using monoclonal antibodies. To understand the aberrant expression of receptors we also studied the expression of other markers of ovarian differentiation and transcription factors necessary for gonadotropin receptor onset in the ovary. We also perform functional studies in primary cultures of epithelial endometriotic cells (EEC).

2. Materials and methods

2.1. Patients and study design

All the patients studied in this work have been described in a previous study [10]. The cohort recruited in this study consisted of 69 women with ($n = 38$) and without ($n = 31$) macroscopic endometriotic lesions already described [10]. The patients and controls groups were homogenous for age, gestity, parity, and phase of the menstrual cycle with the same number of patients being in proliferative or secretory phases [10]. All samples were histologically characterized for patients and controls. Patients with endometriosis provided both eutopic and ectopic endometrium. The samples were selected from 38 patients afflicted with painful endometriosis and operated for complete surgical exeresis of all endometriotic lesions. Endometriosis was categorized according to a previously published surgical classification based on the location of the worst endometriotic lesion location: superficial (SUP), endometrioma (OMA) ($n = 19$), and deeply infiltrating endometriosis (DIE) ($n = 19$). Endometriosis was scored according to the revised American Fertility Society classification [10]. Control endometrial specimens were collected from 31 patients without any macroscopic endometriotic lesion as checked during a thorough surgical examination of the abdominopelvic cavity. Indications for surgery in controls were the following: tubal infertility, non endometriotic ovarian cysts, or uterine myoma. The local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale of Paris- Cochin) approved the study protocol. All patients gave an informed written consent.

2.2. Total RNA extraction, reverse transcription and RT-qPCR

We used 47 tissue samples (32 paired eutopic and ectopic endometrioses, and 15 controls) for RT-qPCR on 17 genes of interest and 3 control genes. The characteristics of these selected patients have been described [10]. After surgical resection, samples of disease-free control biopsy specimens and eutopic or ectopic endometrium biopsy specimens were immediately frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA quality was checked by agarose gel electrophoresis and spectrophotometry. We generated cDNA by reverse transcribing 4 μg of total RNA with random primers and M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. We treated the cDNA with RNase before RT-PCR. The primers used for RT-PCR analysis were selected with PRIMER3 software, on the basis of published sequences. The primers were produced by Eurogentec (Angers, France). Quantitative PCR was carried out on a 96-well Light Cycler 480 machine (Roche Diagnostics, Manheim, Germany), with a Light Cycler 480 SYBR Green I Master kit (Roche). Results were analyzed with Light Cycler software, by the three-fit point method. Relative abundances in endometriotic tissues were calculated by the $-2\Delta\Delta Ct$ method with respect to the mean for the three control genes (mean for 3 housekeeping genes SDHA, HPRT and HISTH1A genes) and normalization with the mean value for a pool of genes from control endometria. We also studied FSHR and LHR expression in 12 additional patients with endometriosis and six supplementary controls from the same cohort already described [10]. Sequences of primers are available on request.

2.3. Immunohistochemistry

Immunohistochemistry was performed on 10 matched ectopic endometriosis and 10 control disease-free endometria (for each 5 in proliferative phase and 5 in secretory phase). The patients belong to a cohort already described [10]. A previously described antigen retrieval method was used [11]. Briefly, the samples were embedded in paraffin and 4-μm sections were cut sequentially and mounted on SuperFrost treated slides (Menzel-Glasse, Braunschweig, Germany). The slides were allowed to dry overnight at 37 °C. The paraffin was then eliminated by immersion in xylene and the sections were rehydrated in a graded series of ethanol solutions. For epitope retrieval, the slides were immersed in citrate buffer pH 6.0, in a water bath at 95 °C to 99 °C for 60 min. The slides were then allowed to cool in the buffer for 20 min at room temperature. H_2O_2 (0.3%) was added to the slides, which were then incubated at room temperature for 30 min. They were then incubated with the primary antibodies for 2 h. The NovolLink max Polymer Detection System (Newcastle, UK) was used for the subsequent steps, according to the manufacturer's instructions. Diaminobenzidine-hydrogen peroxide or aminoethyl carbazole (Clinisciences,FR) were used for chromogenic development. The slides were lightly counterstained with hematoxylin, dehydrated, and covered with a coverslip. For control IgG, replacement of the specific primary antibody was performed on serial sections incubated with corresponding pre-immune immunoglobulins (mouse and rabbit IgG, Sigma Aldrich) at the same concentration used for the specific primary antibody.

We controlled also for nonspecific binding of the primary antibody preparation, by replacing the first layer of serial sections with non specific slides. Normal human ovarian granulosa cells and/or theca cells served as positive controls. The following antibodies were used: monoclonal antibody against LHR (clone 29, dilution: 1/200) [12], monoclonal antibody against FSHR (clone 323, dilution: 1/300) [12], and polyclonal antibodies against aromatase (H4, dilution: 1/50) (Acris Antibodies, San Diego, USA), CYP 17 *A*1 (M-80-SC/66850, dilution: 1/100) (Santa Cruz Biotechnology Inc., USA), CYP11A1 (13363-1-AP, dilution 1/100) (Proteintech, USA) , INSL3 (ab65981, dilution 1/100) (abcam, FRA), GATA6 (ab22600, dilution 1/100) (abcam, FRA), and GATA 4 (SC:25310, dilution: 1/50) (Santa Cruz Biotechnology Inc., USA).

2.4. Primary cultures

Primary cultures of epithelial endometriotic cells (EEC) from women with and without endometriosis were obtained from 12 consecutive patients, as previously described [10,13]. All cells were cultured and treated with hCG or rFSH as described [13–15]. Briefly, for each of these patients, a surgical biopsy was performed from DIE. Specimens were collected under sterile conditions and transferred, in DMEM (Dulbecco's modified Eagle medium, Gibco Invitrogen, Cergy Pontoise, France), to the laboratory, where they were prepared for cell extraction or in vivo experiments within 30 min. Biopsy specimens were rinsed, minced into small pieces, digested with dispase and collagenase (2 mg/ml, Gibco Invitrogen, Cergy Pontoise, France) for 1 h at 37 °C and separated by serial filtration. Red blood cells were removed by

hypotonic lysis (0.15 mol/l NH_4Cl, 1 mmol/l $KHCO_3$, 0.1 mmol/l Na_2EDTA). Debris was removed by passage through sieves with 100-μm pores. Epithelial cells were retained on sieves with 40-μm pores, whereas stromal cells remained in the filtrate. Both cell types were plated in Primaria 75 cm^2 flasks (Becton Dickinson Labware, Le Pont de Claix, France) and cultured in DMEM with 10% FBS (fetal bovine serum). The purity of the stromal and epithelial cell preparations was confirmed by fluorescence staining in a previous study [12].

2.5. Treatments of cells and immunoblotting of cell lysates

For all in vitro experiments, cells were prepared as follows. Each cell type was cultured in its specific medium to 90% confluence. Cells were rendered quiescent by two washes with ice-cold PBS (Invitrogen) and incubated with serum-free medium for 12–16 h. The cells were then left untreated or were treated with hCG or rFSH, as indicated in the figure legends, in fresh serum-free medium. Doses and exposure times for hCG (Gonadotrophine Chorionique Endo®, 5000 IU/ml, Schering-Plough) were as described elsewhere [15] or were determined in preliminary experiments, as for the doses and exposure times for rFSH (GONAL-F Follitropin Alfa, 450 IU/0.75 ml, MERCK). For each treatment, the controls contained an equivalent volume of the carrier solution and were conducted in parallel. After treatment, cells were rinsed with ice-cold PBS and treated with trypsin. Cells were lysed in ice-cold RIPA buffer (10 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate) supplemented with 25 mmol/l sodium fluoride, 1% protease inhibitor and 0.5 mmol/l sodium orthovanadate. For RNA extraction after treatment, cells were rinsed with ice-cold PBS and lysed with Trizol (Invirogen). Equal amounts of proteins from cell lysates were subjected to SDS-PAGE in 10% polyacrylamide gels. The bands obtained were transferred onto nitrocellulose membranes, which were blocked and incubated overnight at 4 °C with a 1:200 dilution of a goat *anti*-human pERK1/2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Incubation with the primary antibody was followed by incubation with the corresponding horseradish peroxidase-labeled secondary antibody at a 1:1000 dilution for 1 h at room temperature. Immunocomplexes were visualized by enhanced chemiluminescence (Pierce Perbio Sciences, Brebières, France). All membranes were reprobed with rabbit anti-human antibodies against ERK1/2 (1:200), as a loading control. ERK1/2 and pERK1/2 levels were explored in all of the cell lines extracted from the six patients with endometriosis. The values shown are means ± SEM (*P < 0.05).

2.6. In vitro cell proliferation and viability assays

We assessed the proliferative effect of hCG on control endometrial cells and EEC, by treating the cells with various concentrations of hCG (0, 2.5, 5, 10, 20 IU/ml) for 48 h and assessing cell proliferation by measuring [^3H]thymidine incorporation during the last 16 h of culture. Results are expressed as the ratio of cpm for treated cells versus cpm for untreated cells in three experiments. The red dotted line corresponds to untreated cells. ($n = 3$ independent experiments; *P < 0.05; **P < 0.01).

2.7. Statistical analysis

All statistical analyses were performed with XLSTAT software. Parametric analyses were carried out as the data for all experiments were normally distributed. We used Student's *t*-test for quantitative variables and Pearson's χ^2 or Fisher's test for qualitative variables, as appropriate. A one-way ANOVA was conducted for comparisons of more than two groups. If group means were significantly different on one-way ANOVA (P < 0.05), we carried out pairwise comparisons with Student-Newman-Keuls post hoc tests. A two-way ANOVA was used when two or more datasets were compared for different factors. Results are means ± SD. P < 0.05 was considered significant (*P < 0.05; **P < 0.01).

3. Results

All patients of this study population have been described elsewhere [10] and consisted of 69 patients originating from the same cohort, including 38 with endometriosis and 31 disease-free control women, the same number of patients being in proliferative and in secretory phases for all experiments. The same results were obtained when OMA or DIE were studied.

3.1. Aberrant expression of the FSHR in half of eutopic and all ectopic endometriosis

The FSHR plays a key role in the ovary, by inducing aromatase, which converts androgens into estrogens. We used reverse transcription-quantitative PCR (RT-qPCR) on controls and eutopic or ectopic endometriotic tissues [10] to analyzed FSHR mRNA levels. We performed individual comparative studies on paired eutopic and ectopic lesions.

In half of eutopic endometrioses studied, aberrant FSHR mRNA levels were detected (6 times higher than in control women; P ≤ 0.05) (Fig. 1A). We defined this eutopic FSHR positive group of patients as group A, and the other half devoid of eutopic FSHR as group B patients (Fig. 1A).

In all the ectopic endometriotic tissues studied, FSHR mRNA levels were highly upregulated. They were much higher in ectopic lesions from group A than from group B patients (239 and 29 times higher than control levels, respectively (Fig. 1B). We confirmed the expression of the FSHR protein in tissue samples by immunohistochemistry using a monoclonal antibody. The FSHR staining was weakly detected in the epithelial cells of control patients, but much stronger in ectopic endometriotic lesions (Fig. 1C). Stromal cells were also labeled, but less strongly. The FSHR protein was also detected in the endometrial blood vessels of ectopic endometriotic tissues, but not in control blood vessels (Fig. 1C).

3.2. Aberrant expression of other markers of ovarian differentiation, the LHR and INSL3 in endometriosis

To determine whether FSHR expression in endometriosis was accompanied by a broader pattern of inappropriate “ovarian-like” differentiation, we studied other specific ovarian genes which might be concomitantly aberrantly expressed in eutopic or ectopic endometriotic tissues. In ovaries, the LHR is critical for androgen synthesis, which serves as substrates for estrogen production by aromatase. In control endometria and eutopic endometriotic tissues, RT-qPCR detected very low levels of LHR transcripts with no significant differences between groups (Fig. 1A). Interestingly, ectopic endometriotic lesions of a subset of patients (6/28 (21%)) had much higher LHR mRNA levels than controls (>10,000-fold induction (P ≤ 0.05)). We defined these LHR and FSHR positive ectopic endometrioses as group C (Fig. 1B). Immunohistochemistry using a monoclonal antibody revealed a faint LHR labeling in control epithelial cells with a strong labeling detected in the epithelial cells of ectopic endometriotic tissue from a representative patient (Fig. 1C). Stromal cells were weakly labeled.

The ovarian hormone INSL3 plays an important role in maintaining androgen production by ovarian theca cells by inducing CYP17 steroidogenic enzyme [16] and modulates both LH and FSH actions [16,17]. INSL3 is regulated by LH in ovarian theca cells [18]. We found that INSL3 was aberrantly expressed in group A eutopic endometriosis (Fig. 1A) and all ectopic lesions (Fig. 1B) together with CYP17 transcripts. The highest levels of INSL3 (290–fold; P ≤ 0.05) transcripts were found in ectopic group C endometriosis also aberrantly expressing LHR (Fig. 1B). No immunohistochemical labeling of INSL3 protein was detected in control endometria. By contrast, INSL3 protein levels were abnormally high in the epithelial cells of ectopic endometriotic tissues (Fig. 1C). Stromal cells were not labeled.

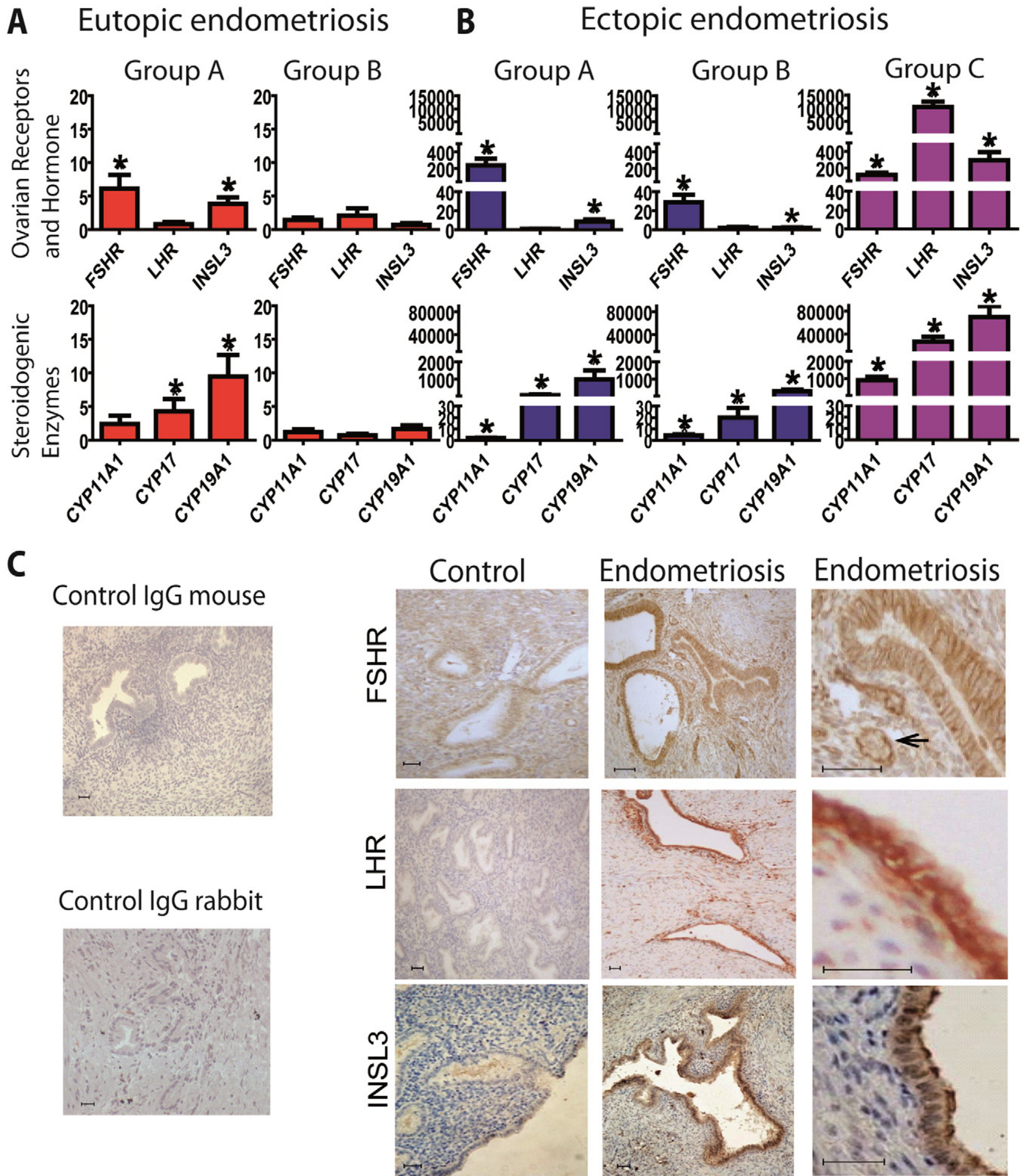

Fig. 1. Aberrant expression of the FSHR and the LHR in a broad ovarian-like differentiation in endometriosis. A and B. RT-qPCR analysis of FSHR, LHR, INSL3 and steroidogenic enzymes CYP11, CYP17, CYP19 transcripts from eutopic ($n = 16$) (A) and ectopic ($n = 16$) (B) endometriotic tissues. Y axis: mRNA expression relative to control. The values shown are means ± SEM (*P < 0.05). C. Left: pre-immune IgG were used as controls. Right: immunohistochemical staining of FSHR, LHR and INSL3 with specific antibodies in control (disease-free) endometria ($n = 10$) and ectopic endometriotic tissues ($n = 10$). The arrow indicates a vessel. Illustrative pictures shown are representative of the median level of staining for each protein studied except for the LHR for which an aberrant expression of proteins is detected in only 21% of patients. All scale bars indicate 100 μm.

3.3. The FSHR and LHR are functional in endometriotic cells, inducing the same steroidogenic cascade as in ovaries, and their signaling involves the ERK 1/2 pathway

To verify the functionality of FSHR and LHR in endometriotic cells, we used primary cultures of epithelial cells derived from ectopic endometriotic tissue obtained from DIE ($n = 12$) or from control women ($n = 6$) [13]. We first study the signaling of these receptors in EEC after stimulation of each receptor by the cognate hormone. In ovaries, FSHR and LHR signaling involve mainly the cAMP pathway [7]. However no stimulation of the PKA-cAMP pathway by rFSH or hCG was observed in endometriotic cells (data not shown). It has been

previously shown in endometrial epithelial cells from baboon and human endometrial HES cells that the immediate signal transduction pathways induced by hCG are cAMP independent and stimulate phosphorylation of ERK 1/2 [15]. EEC treated with hCG or rFSH had higher levels of phosphorylated ERK (pERK) 1/2 with maximal effect after 10 min (respectively 5.91-fold and 3.23-fold increase in pERK/ERK 1/2 ratio (P ≤ 0.05)) (Fig. 2A). We can conclude that the ERK 1/2 pathway mediates signaling by both hormones in EECs.

FSHR and LHR function in the ovary is dependent on the induction of steroidogenic enzymes. In all endometriotic tissues aberrantly expressing the FSHR, aromatase (CYP19A1) mRNA levels were concomitantly upregulated. In eutopic endometrioses of group A, but not group B, CYP19A1 mRNA levels were upregulated (9.5 fold higher than controls (P ≤ 0.05)) (Fig. 1A). In all ectopic lesions the FSHR was upregulated to an even greater extent, and CYP19A1 transcript levels were very high, particularly in group A patients (980-fold in ectopic group A, 310-fold in ectopic group B (P ≤ 0.05)) (Fig. 1B). The parallel increase in FSHR and CYP19A1 transcript levels suggests that the FSHR induces aromatase in endometriosis, as in ovaries. CYP17 transcripts were also upregulated in eutopic endometrioses from group A whereas CYP11A1 transcripts were not (Fig. 1A). Thus, the enzymatic machinery in eutopic endometriosis may be able to convert exogenous circulating androgens into estrogens.

In ectopic endometriosis CYP17 transcript levels were highly upregulated (79 and 19-fold in groups A and B, P ≤ 0.05), associated with a moderate but significant increased expression of CYP11A1 transcripts. Thus, mRNAs for androgen-synthesizing enzymes are present in ectopic lesions, together with aromatase, potentially yielding complete in situ steroidogenesis. In an interesting way, when the LHR was expressed (group C) the strongest induction of transcripts encoding the complete steroidogenic cascade was detected (69,792-fold for CYP19A1, 26,519-fold for CYP17 and 917-fold for CYP11A1 (P ≤ 0.05)) (Fig. 1B).

To know whether the signaling pathway of gonadotropins in EEC was functional, we studied the induction by FSH or hCG of the complete steroidogenic cascade and ovarian steroidogenic factors. We found that recombinant FSH (rFSH) induced the accumulation of aromatase transcripts (3.5-fold, $p \leq 0.05$) in EEC as in ovaries (Fig. 2B). hCG upregulated transcripts encoding androgen-synthesizing enzymes CYP11A1, CYP17 (1.8 and 3.5-fold; P ≤ 0.05; Fig. 2C) and INSL3 (5.7-fold, P ≤ 0.05), a potent stimulator of CYP17, as in ovaries [18]. Interestingly hCG also induced FSHR transcripts (5.8-fold) as in ovaries [7] (Fig. 2B).

ERK 1/2 belongs to the MAPK family proteins which are keys regulator of many processes including cellular growth, differentiation, cell survival or immune response [19]. In endometrial cancer cells, it has been shown that the LHR controls cell proliferation and could represent a prognostic factor and a new therapeutic target in endometrial cancer [20,21]. Endometriosis shares features common with cancers [1,5]. We thus aim to study comparatively the effect of hCG on the proliferation of EEC. We show that hCG yielded a dose-dependent increase in the proliferation of EEC but not of control endometrial cells from disease-free women. The maximum increase in proliferation relative to untreated cells was 44% (P < 0.05) (Fig. 2C). This experiment highlights the functional proliferative effect of the LHR in EEC, similarly to what was shown in endometrial cancer cells [20,21].

Altogether these experiments show that the FSHR and LHR are functional in EEC. Their signaling involves the ERK 1/2 pathway, the LHR induces EEC proliferation and both receptors induce the expression of the complete steroidogenic cascade and ovarian steroidogenic factors transcripts also induced by the same receptors in ovaries.

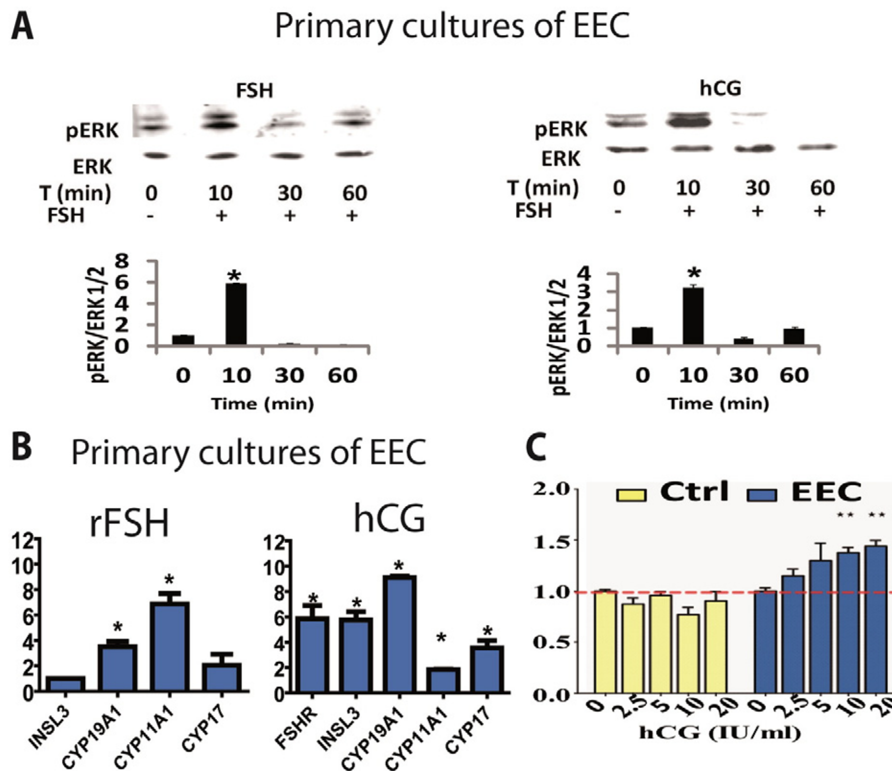

Fig. 2. The FSHR and LHR are functional in endometriosis inducing the same steroidogenic cascade as in ovaries. A. Kinetics of phospho-ERK 1/2 induction in primary cultures of epithelial endometriotic cells (EECs) stimulated by rFSH ($n = 6$) or hCG ($n = 6$). ERK 1/2 phosphorylation was quantified by densitometry with the ImageJ software (pERK/ERK 1/2 fold induction) (*P < 0.05). B. RT-qPCR analysis of EECs stimulated with rFSH ($n = 6$) or hCG ($n = 6$). Y axis: mRNA expression relative to control. Values are means ± SEM (*P < 0.05). C. Proliferative response to hCG in EECs and control epithelial endometrial cells ($n = 6$ for each cell type). Cells were treated with various concentrations of hCG (0, 2.5, 5, 10, 20 IU/ml) for 48 h and cell proliferation was assessed by measuring [^3H]thymidine incorporation during the last 16 h of culture. Results are expressed as the ratio of cpm for treated cells versus cpm for untreated cells. Y axis: proliferation relative to untreated cells. The red dotted line corresponds to untreated cells. ($n = 3$ independent experiments; *$P < 0.05$; **$P < 0.01$).

3.4. Expression of GATA4 and GATA6 transcription factors in half of eutopic and all ectopic endometriosis

To investigate the mechanism underlying this aberrant expression of the FSHR in endometriosis, we analyze the expression of GATA4 and GATA6 transcription factors, which are involved in gonadal differentiation and in the onset of FSHR and LHR genes expression in the ovaries [22].

Eutopic endometriotic tissues from group A but not from group B patients, displayed aberrant GATA6/4 expression (respectively 12.4 and 2.2 fold stronger than in controls; P ≤ 0.05; Fig. 3A). All ectopic endometriotic tissues had very high levels of FSHR expression, together

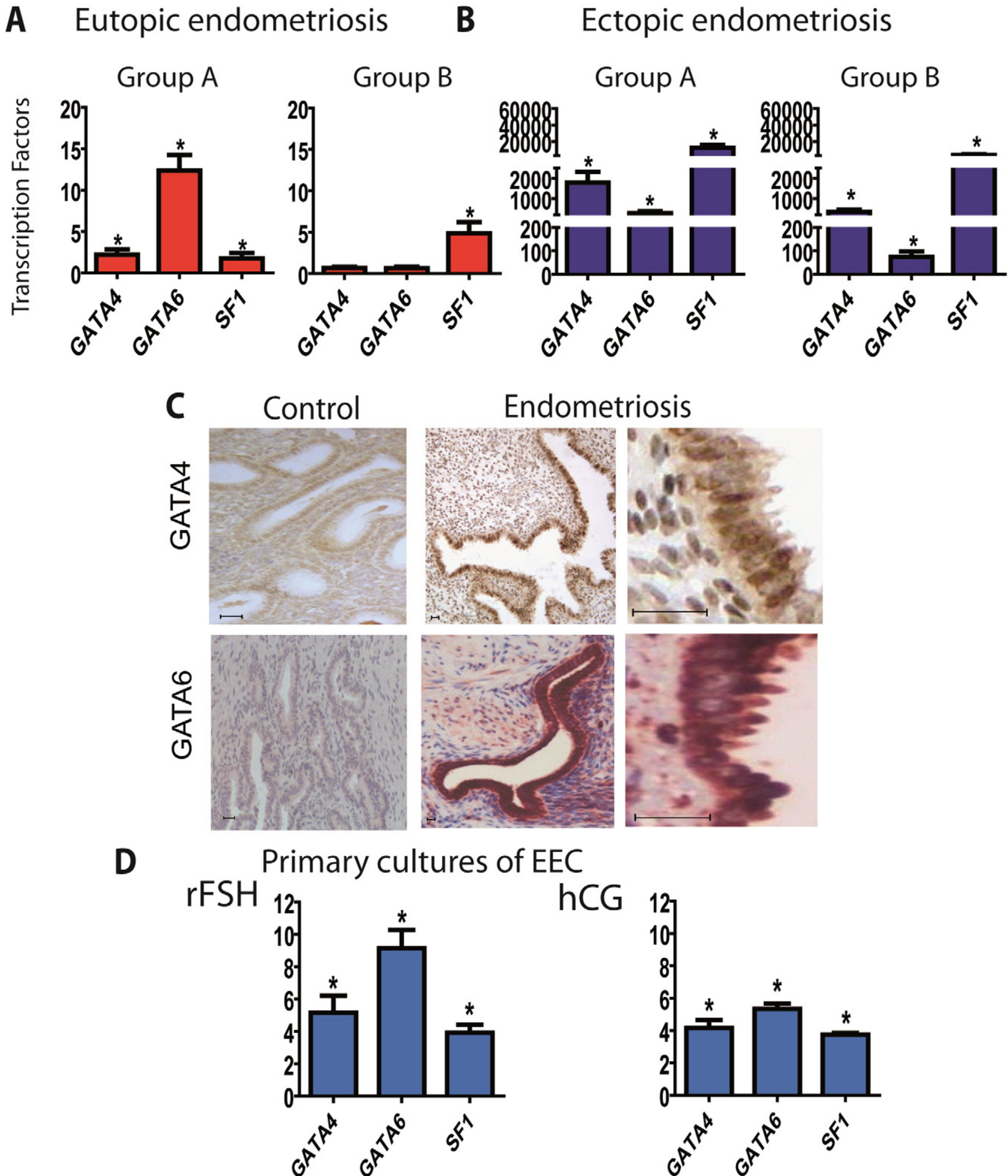

Fig. 3. Ovarian transcription factors are aberrantly expressed in endometriosis and induced by gonadotropin receptors. A and B. RT-qPCR analysis of GATA4 and GATA6 transcripts from eutopic (A) and ectopic (B) endometric tissues. Y axis : mRNA expression relative to control. The values shown are means ± SEM (*P < 0.05). C. Immunohistochemical staining with specific antibodies of GATA-4/6 in control endometria ($n = 10$) and ectopic endometriotic tissues ($n = 10$). All scale bars indicate 100 μm. D. RT-qPCR analysis in EEC stimulated with rFSH ($n = 6$) or hCG ($n = 6$). Y axis : mRNA expression relative to control. Values are means ± SEM (*P < 0.05).

with dramatic induction of GATA4 and GATA6 mRNAs, higher in group A patients (respectively 1779- and 293-fold induction in ectopic group A; and 356- and 74-fold in ectopic group B; ($P \leq 0.05$) Fig. 3B). No difference in the accumulation of transcripts for GATA 4/6 is found between patients from ectopic groups A and C, so we merged both groups in group A.

On immunohistochemistry labeling for GATA4 was weak and no labeling for GATA6 was observed in control endometria, whereas labeling for both GATA4 and GATA6 was intense especially in the nucleus of epithelial endometriotic cells (Fig. 3C). Stromal cells were also labeled, but less strongly.

In EEC both rFSH and hCG significantly increased GATA4/6 (by 5.1-, 9.1 -fold, respectively, for rFSH; and by 4.1-, 5.3- fold for hCG; ($P \leq 0.05$)) (Fig. 3D). These results suggest that a positive autoregulatory loop, similar to that in ovaries [23] operates in endometriotic cells, favoring estrogen-dependent disease progression. FSH and LH also induced at a lesser extent SF1 transcript levels (Fig. 3D).

3.5. FSHR and LHR regulate vascular and inflammatory factors in endometriotic cells

Angiogenesis and inflammation are important part in the physiopathology of endometriosis, dissemination of endometriotic lesions, and in the development of pain [1,2]. It has been shown previously that FSH could play a role in angiogenesis and hCG in inflammation in different cell types. Apart from induction of aromatase, rFSH induces VEGF mRNA levels in human granulosa cells [24]. FSH promotes angiogenesis in placental endothelial cells [25]. FSHR is also widely present in tumor blood vessels [9]. It is thought to be angiogenic, favoring tumorigenesis [8,9]. Endothelial FSHR expression in breast cancer is associated with vascular remodeling at the tumor periphery [26]. hCG induces accumulation of inflammatory factors transcripts in different cell types. In endometrial epithelial cells hCG increases PGE2 release as the possible result of cyclooxygenase-2 activation [15,27]. Studies of genetically modified LHR-knockout mice have shown that LH or hCG can activate PTGS2 gene which plays an essential role in implantation [28]. It has been shown that human decidual luteal and endometrial macrophages contain LHR [29].

We thus wondered whether these receptors could contribute to the pathogenesis of endometriosis not only through the induction of estrogen-dependent proliferative effect but also through inflammation and angiogenesis. We observe an upregulation of vascular factors transcripts (VEGFC, VEGFD and EGVEGF) in eutopic endometriosis even more enhanced in ectopic endometriosis when the FSHR is more strongly up-regulated. There is also an up-regulation of inflammatory factors and their receptors transcripts (PTGS2 and PTGER2, 3 and 4) in eutopic and ectopic endometrioses (Fig. 4 A and B).

No difference in the accumulation of transcripts for vascular or inflammatory factors is found between patients from ectopic groups A and C, so we merged both groups in group A.

We also studied the induction of angiogenic and inflammatory factors by FSH and hCG in primary culture of epithelial endometriotic cells. In EEC, rFSH induced a strong increase in mRNA levels for the angiogenic factors VEGFD and EGVEGF (7.85- and 5.18-fold induction; $P \leq 0.05$) as in ovarian cells [24] (Fig. 4C).

These findings suggest that FSH might play a novel angiogenic role in endometriotic cells similar to that proposed for tumor cells [8,9]. We found that hCG induced accumulation of inflammatory factors transcripts PTGS2, PTGER2, PTGER3 and PTGER4 (2.85-, 10.59-, 18.8-, and 7.37-fold induction respectively, $P \leq 0.05$) in EEC (Fig. 4C). This should lead to an increase in prostaglandin production, contributing to inflammation.

We show that in EEC rFSH induces mainly vascular while hCG induces inflammatory factors. These observations in EEC support a contributive role for FSH and LH receptors in the angiogenesis and inflammation found in endometriosis in addition to their hormonal role (Figs. 4 and 5).

Altogether our results suggest an alternative physiopathological mechanism of endometriosis, involving a broad ovarian-like differentiation with expression of GATA4/6 transcription factors, INSL3, and aberrant FSHR and LHR expression and function in a subset of endometriotic tissues leading to in situ production of E2 (Fig. 5).

4. Discussion

Endometriosis is a major public health problem due to the infertility and/or chronic pelvic pain it causes [1–4]. There is currently no biological markers for early diagnosis which is delayed about 7–10 years after the apparition of the first symptoms during surgery. Improvement of our understanding of the pathophysiology of endometriosis is therefore required, for the development of novel methods for early diagnosis. This work highlights a novel pathophysiological mechanism in endometriosis with a global ovarian pattern of differentiation in a subset of eutopic and all ectopic endometrioses.

The mechanism of estrogen production by endometriotic tissue is poorly understood. One possible mechanism of E2 production is ovarian-like differentiation of the endometriotic tissue, with abnormal gonadotropin receptors expression and function. Indeed aberrant FSHR expression was detected in half eutopic endometria studied (group A). In group A patients, there is a concomitant aberrant expression of other genes normally expressed in ovaries especially aromatase, INSL3 and transcription factors GATA4/6. Eutopic group A is thus called "ovarian-like differentiated" while group B patients do not exhibit eutopic ovarian-like differentiation. In eutopic endometriotic tissues from group A patients, circulating androgens may therefore be converted to estrogens in situ by aromatase, at early stages of disease. In all disseminated endometriosis, strong FSHR expression and high levels of aromatase transcripts are observed. The FSHR is functional, as FSH upregulates aromatase transcripts in EEC, consistent with concomitant upregulation of aromatase and FSHR during endometriosis, as in ovaries. Recently, a faint expression of the FSHR protein was detected in ectopic endometriotic glandular epithelial or stromal cells in a low proportion of patients with endometriosis (≤ 25%) [30]. Another recent study reports functional expression of the FSHR in ectopic endometriotic lesions inducing aromatase [31]. However both reports did not study other ovarian markers allowing complete in situ steroidogenesis in endometriosis as well as the expression of transcription factors triggering endocrine function in ovaries [30,31]. Eutopic endometrium was not studied in these reports. In group A patients, an ovarian-like differentiation is detected before dissemination of endometriotic lesions. This observation could be useful to improve early diagnosis of endometiosis.

We found that eutopic tissues from patients with endometrioses displayed no upregulation of LHR transcripts [32]. However a high expression of the LHR was detected in a susbet of ectopic endometriosis (~21%, group C patients) together with a dramatic increase in androgen-synthesizing enzymes transcripts. The LHR is also functional, hCG upregulates androgen-synthesizing enzymes transcripts in EEC, consistent with concomitant upregulation of CYP11A1, CYP17 and the LHR during endometriosis, as in ovaries.

Altogether these results show that both FSHR and LHR are functional in endometriotic cells, inducing the same target genes as in the ovaries, yielding a full steroidogenic cascade able to synthesize in situ androgens and estrogens necessary for autocrine proliferation and growth.

The aberrant expression of the ovarian steroidogenic factor INSL3 also parallels the increased expression of the FSHR in endometriotic lesions. INSL3 is a circulating peptide hormone of the relaxin-insulin family involved in tumor cell growth, differentiation, invasion and neovascularization [33]. INSL3 induces CYP17 expression in the ovary, maintaining androgen production [16]. The highest levels of INSL3 transcripts were found in group C endometriosis also aberrantly expressing

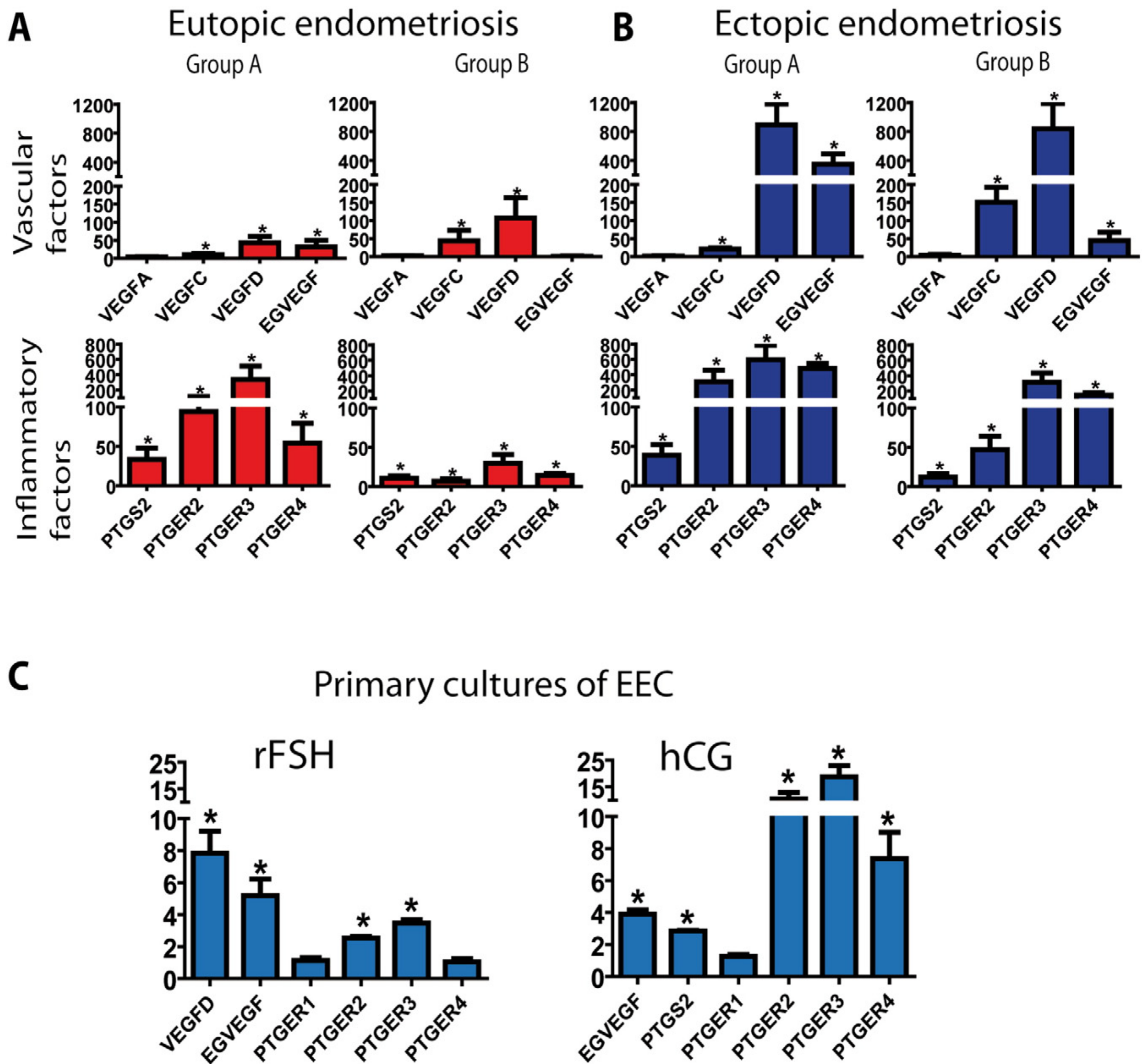

Fig. 4. FSHR induces vascular and LHR induces inflammatory factors in endometriotic cells. A and B. RT-qPCR analysis from eutopic ($n = 16$) (A) and ectopic ($n = 16$) (B) endometric tissues. Y axis: mRNA expression relative to control. The values shown are means ± SEM (*P < 0.05). C. RT-qPCR analysis in EECs stimulated with rFSH ($n = 6$) or hCG ($n = 6$). Y axis: mRNA expression relative to control. Values are means ± SEM (*P < 0.05).

the LHR, together with the strongest induction of the complete steroidogenic cascade, involving CYP17 and CYP11. We found that hCG upregulated INSL3 transcripts in EEC. These observations in EECs are concordant with what is known in ovarian theca cells where INSL3 is induced by LH (13) and induces CYP17 expression [16]. Thus it seems that ovarian regulations of INSL3 (by LH) and of androgen synthesizing enzymes (by INSL3 and LH) could be aberrantly reproduced in endometriosis.

GATA4 and 6 are transcription factors involved in gonadal differentiation and in the regulation of FSHR and LHR genes expression in the ovaries [22]. The loss of GATA4/6 from granulosa cells blocks FSHR expression, leading to female infertility [34]. Women lacking FSHR have low levels of GATA4 expression [22]. In mice lacking GATA4 or GATA4/6, expression levels of LHR and also CYP17, CYP19A1 and CYP11A1 are significantly lower than in controls, and FSHR is undetectable [34]. The onset of ectopic adrenal GATA4 production coincides with LHR expression and the occurrence of adrenocortical tumors [23]. Genome-wide DNA methylation analysis predicted an epigenetic switch for GATA factor expression in cultured ectopic endometriotic stromal cells [35]. There is also evidence that FSH and LH regulate gonadal GATA gene expression [22]. Thus, the parallel increase in GATA4/6 transcription factors and gonadotropin receptors levels strongly suggests that these transcription factors regulate FSHR and LHR expression in endometriosis, as in ovaries [22]. GATA 4 and GATA6 are induced by the FSHR and LHR in EEC. These results suggest that a positive autoregulatory loop similar to that in ovaries operates in endometriotic cells, favoring estrogen-dependent disease progression [23].

5. Conclusions

This work highlights a novel pathophysiological mechanism in endometriosis. A broad ovarian-like differentiation is observed in half eutopic tissues and all disseminated ectopic lesions of endometriosis with the aberrant expression of transcription factors GATA4 and GATA6 triggering ovarian differentiation and gonadotropin receptors expression in the ovary. A positive feedback loop probably occurs

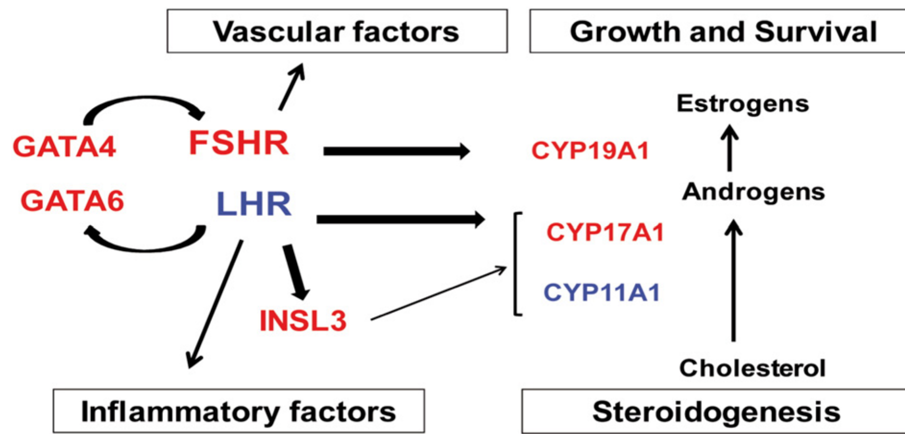

Fig. 5. Model of ovarian-like differentiation in endometriosis. In red, genes upregulated in eutopic endometriosis; in blue, additional genes upregulated in ectopic endometriosis. The aberrant production of GATA4/6 factors may trigger FSHR expression at the start of the disease in eutopic tissues, in a subset of patients (group A), and during disease progression in all ectopic lesions (groups A and B). Together with INSL3, these molecules may induce partial steroidogenesis in situ especially through aromatase induction. In a subset of ectopic lesions, strong GATA4/6 expression also induces aberrant LHR upregulation (group C), resulting in the strongest induction of the complete steroidogenic cascade and the highest levels of INSL3 transcripts. The upregulation of GATA4/6 factors by FSHR and LHR in endometriotic cells may result in an autoregulatory loop favoring estrogen-dependent disease progression. FSHR and LHR also have angiogenic and inflammatory properties, respectively.

through FSHR and LHR-dependent induction of GATA4/6 in EEC, as in ovaries, enhancing the production of the steroidogenic cascade. The FSHR and the ovarian hormone INSL3 which modulates LH and FSH action in ovaries are aberrantly expressed concomitantly with GATA 4/6. FSH induces aromatase in endometriotic cells. Partial steroidogenesis with aromatization of circulating androgens into estrogens can therefore occur. In a subset of disseminated lesions, the LHR is also highly expressed and induces androgen synthesizing enzymes and INSL3, yielding complete in situ steroidogenesis, as in ovaries. FSH induces vascular while hCG iduces inflammatory factors in EEC. Apart from their hormonal role the FSHR and LHR might also contribute to the pathogenesis of endometriosis through inflammation and angiogenesis.

The ovarian-specific markers highlighted in this study could open new avenues, in the future, in the development of an early non-invasive diagnostic method for a subset of patients with endometriosis. Further studies in a larger population of patients are required to establish the proportion of patients with an eutopic “ovarian-like phenotype” at early stage of the disease, characterized by the expression of the ovarian genes highlighted here. RT-qPCR and immunohistochemical staining revealed high expression of FSHR, INSL3, GATA4/6 in all ectopic endometrioses, suggesting that this pattern might give new tools for the diagnosis of the disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contribution

M.M designed research; B.F, P.S and JC.N performed research; B.F, JC.N and M.M analyzed data; BF and M.M wrote the paper.

Transparency document

The Transparency document associated with this article can be found, in online version.

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