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G-Protein-Coupled Receptor CXCR7 Is Overexpressed in Human and Murine Endometriosis

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Abstract

Endometriosis is a chronic inflammatory disease. Dysfunctional regulation of chemokines and chemokine receptors is a crucial aspect of endometriosis pathogenesis. Chemokine G-protein-coupled receptors (GPCRs) are important drug targets that regulate inflammation and immunity. Recently, *CXCR7, a* C-X-C motif containing GPCR, has been identified as a receptor for chemokine ligand *CXCL12*, one of the best characterized chemokines for cell trafficking, angiogenesis, and cell proliferation in cancer and inflammation. Here, we investigated the expression and localization of *CXCR7* in human endometriosis and a murine model of the disease. Normal endometrial epithelium and stroma showed undetectable or very low expression of *CXCR7*, without any significant changes across phases of the menstrual cycle in humans. *CXCR7* is significantly upregulated in endometriosis, showing higher staining in glands and in associated vessels. The mouse model recapitulated the human findings. In conclusion, overexpression of *CXCR7* in different cellular populations of endometriosis microenvironment may play a role in the pathogenesis and represent a novel target for treatment.

Keywords

endometriosis, CXCR7, CXCL12, human, murine, microenvironment

Introduction

Endometriosis is a common gynecologic disorder characterized by the presence of hormonally responsive ectopic implants of endometrial epithelium and stroma dispersed in extra-uterine locations.[1-4] Although the etiology is not fully characterized, endometriosis is a multifactorial disorder, resulting from retrograde menstruation,[5] ectopic differentiation of stem cells,[6-8] dysregulation of immune/inflammatory cells,[9] and abnormal hormonal response.[10-12] Chemokines are a group of small chemotactic cytokine proteins (<15 kDa) secreted by various cell types after induction or which may be constitutively expressed. They are divided into 4 groups: CXC, CC, C, and CX3C based on the number and position of conserved cysteine residues (cysteine motif) in the N-terminal region of the protein.[13-15] They exert functional effects by binding to their corresponding receptors.[16] *CXCL12*, also known as stromal cell-derived factor 1 (SDF-1), is a chemokine ligand and one of the best characterized chemokines in mobilization of BM-derived stem cells in cancer and inflammation.[17] In the endometriosis niche, *CXCL12* participates in epithelial/glandular cell proliferation via autocrine/paracrine mechanisms,[18,19] vasculogenesis, and angiogenesis.[20]

CXCR4 is a G-protein-coupled receptor (GPCR) for CXCL12 and expressed on bone marrow-derived mesenchymal stem cells, solid tumors, and endometriosis implants. *CXCL12-CXCR4* signaling is upregulated in women with endometriosis[21-24] and has been established as a crucial signal for bone marrow-derived cells (BMDCs) migration to endometriosis.[25] Recently, *CXCR7*, another GPCR, has been identified as a second receptor for *CXCL12*.[26,27] *CXCR7*, which is phylogenetically closely related to other chemokine receptors, binds SDF-1 with high affinity and the alternative ligand *CXCL11* with low affinity;[28] however, it fails to couple to G-proteins to induce typical chemokine receptor-mediated cellular responses.[29] *CXCR7* has been demonstrated to regulate cell migration and survival through several pathways that include ligand scavenging, direct signal transduction, and direct interaction with *CXCR4*.[28,30,31] *CXCR7* expression is enhanced during pathological inflammation and tumor development, and *CXCR7* mediates transforming growth factor β1 (TGFβ1)-

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promoted epithelial–mesenchymal transition.[32] Emerging data suggest that this receptor is an attractive therapeutic target for autoimmune diseases and cancer.[33] Although human endometriosis implants have higher *CXCL12* and *CXCR4* expression,[21,25] no information is currently available on *CXCR7*. Here, we report the expression of *CXCR7* in human endometriosis and in a murine model of abdominal endometriosis.

Materials and Methods

Human Sample Collection and Analysis

Tissue samples consisting of peritoneal lesions were collected from 11 patients (after excluding criteria) with severe endometriosis [American Fertility Society (AFS) stage IV] at the time of surgery and following a Human Investigation Committee protocol approved by Yale University. Fresh endometrial tissue was collected as controls from 11 healthy, reproductive age women undergoing elective gynecological surgery, without evidence of uterine diseases or endometriosis. Among 11 normal healthy women, 6 were in proliferative phase while 5 were in secretory phase. Women who received exogenous hormonal therapy in the 3 months before tissue collection or had endometrial pathology were excluded. Collected tissue samples were processed immediately; an aliquot of tissue stored at −80°C in RNAlater for RNA isolation and the remaining tissue was stored in 4% formaldehyde overnight and processed for histological studies.

Animals

C57BL/6J wild-type mice were purchased from Charles River Laboratories (Wilmington, Massachusetts) and The Jackson Laboratory (Bar Harbor, Maine), respectively. Mice were housed and maintained (4-5 per cage) in a room (21°C ± 1°C) with a 12-hour light/dark cycle (7:00 AM to 7:00 PM) with ad libitum access to food (Purina Chow; Purina Mills, Richmond, Indiana) and water, in the Yale Animal Resources Center (YARC) at Yale School of Medicine. All animal experiments were conducted in accordance with an approval protocol from the Yale University Institutional Animal Care and Use Committee (IACUC).

Mouse Model of Endometriosis

Endometriosis in mice was surgically induced under aseptic conditions and anesthesia using a modified method previously described.[34] Uterine horns were removed from wild-type female donor mice at diestrus, opened longitudinally, cut into fragments of 3 mm, and transplanted onto the peritoneal wall of recipient mice and affixed with absorbable suture. Three lesions of equal size were systematically transplanted in each mouse. Ectopic tissues were collected 2 weeks and 4 weeks after grafting.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from endometriotic lesions by TRIzol reagent (Life Technologies, Carlsbad, California) followed by purification using Quiagen cleaning kit (Valencia, California) to prepare cDNA with 50 ng RNA in a 20-μL reaction mixture by iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify gene expression using specific primers and SYBR Green (Bio-Rad Laboratories) and optimized in the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories). The primers used are CXCL12: forward 5′-AACACTCCAAACTGTGCCCT-3′, reverse 5′-CTCTCACATCTTGAACCTCTTGTT-3′; CXCR7: forward 5′-GCAGAGCTCACAGTTGTTGC-3′, reverse 5′-GCTGATGTCCGAGAAGTTCC-3′.

The specificity of the amplified transcript and the absence of primer dimers were confirmed by a melting curve analysis. Gene expression was normalized to the expression of β-actin for each sample. Relative mRNA expression for each gene was calculated using the comparative cycle threshold (Ct) method, also known as the $2^{\Delta\Delta C(T)}$ method.[35,36] All experiments were carried out in triplicate, and nuclease-free water was used as a negative control replacing the cDNA template.

Immunohistochemistry

Tissue samples from patients with endometriosis and endometriotic lesions from mice were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer tissue sections were mounted on slides followed by 10 minutes boiling in sodium citrate (pH 6) for antigen retrieval and blocking using 10% serum (Vector Laboratories, Burlingame, California). Slides were incubated at 4°C overnight with anti-CXCR7 primary antibody clone 11G8 (1:1000; R & D Systems, Minneapolis, Minnesota) followed by 30 minutes at room temperature with appropriate biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, California), and detected using ABC Vectastain Elite reagents with DAB plus H_2O_2 (Vector Laboratories, Burlingame, California). Tissue sections were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, Missouri). Images of stained sections were captured using Olympus BX-51 microscope (Olympus, Waltham, MA, USA). Stained cells were quantified by monitoring the average numbers of positively stained cells relative to the total number of cells from 6 randomly chosen fields.

Statistical Analysis

Data were analyzed using Prism (GraphPad Software Inc, La Jolla, California). An unpaired Student *t* test or one-way analysis of variance (ANOVA) was used to determine the statistical significance between mRNA expression levels. Data are expressed as means ± SEM. The Mann-Whitney rank followed by Dunn's test was used to compare percentage of labeled cells (PLC). P value <.05 was considered significant.

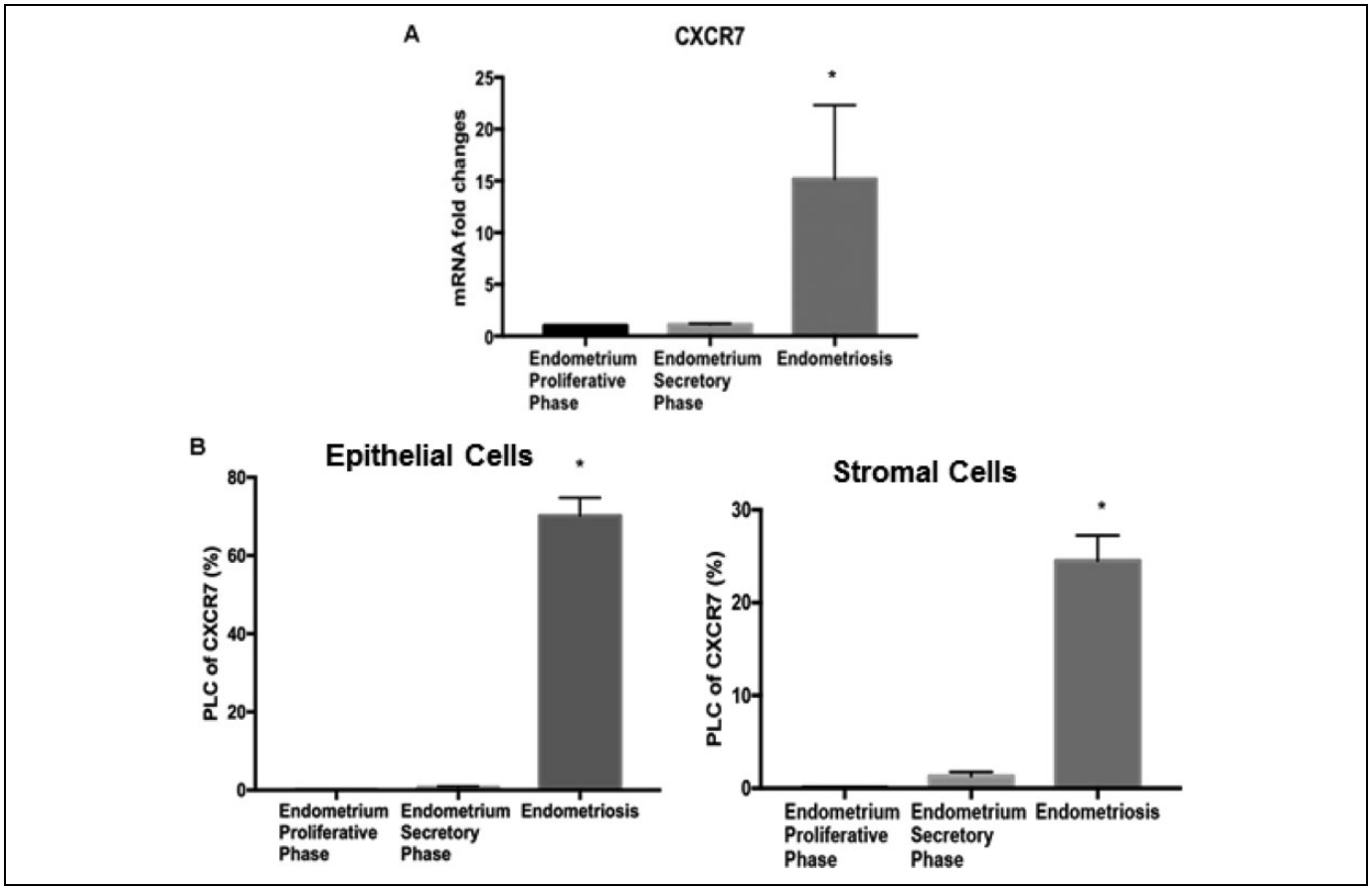

Figure 1. *CXCR7* expression. A, Expression of *CXCR7* mRNA levels in eutopic normal endometrium (proliferative phase set as baseline and equal to 1.0) and ectopic endometriosis. *CXCR7* mRNA levels were significantly increased in ectopic endometriosis. B, Quantitative analyses of the levels of *CXCR7* in human normal endometrium during proliferative, secretory phase of the menstrual cycle, and endometriosis. PLC indicates percentage of labeled cells.

Results

CXCR7 *Is Overexpressed in Human Endometriosis*

CXCR7 mRNA expression was analyzed in normal human eutopic endometrium in both the proliferative and secretory phase and compared to human endometriotic lesions. As shown in Figure 1A, *CXCR7* mRNA levels were significantly higher (14-fold) in endometriotic lesions than in normal endometrium. Immunohistochemistry (IHC) analysis revealed that *CXCR7* protein levels were undetectable or present at very low levels with a slight, although not significant, predominance in the secretory phase of the menstrual cycles (day 21 of normal ovulatory women) both in the epithelial and in stromal compartments (Figures 1B and 2) in normal endometrium tissue obtained from control patients without endometriosis. In contrast, expression of *CXCR7* was clearly detected on >70% of ectopic glands with a predominance of the epithelial layer over the stromal compartment (Figures 1 and 2). *CXCR7* is also expressed at low level in endothelial cells (mostly venule endothelium) in normal endometrium; however, a marked upregulation of *CXCR7* occurred in endometriosis vasculature. Specifically, in addition to endothelial cells, vascular smooth muscle cells into the endometriosis implants were markedly stained. Interestingly, fibromuscular cells associated with deep endometriosis were also markedly stained.[37]

CXCR7 *Is Overexpressed in Murine Model of Endometriosis*

The analysis of endometriosis implants in mice was performed after 2 and 4 weeks after the engraftment of donor uterine fragments in peritoneal wall of immune and reproductive competent recipient mice. Results were compared with uteri of sham-treated mice. In endometriosis mice, *CXCL12* mRNA levels were increased gradually from the day of endometriosis induction, reached a peak at 15th day, and regressed after 30 days. Consistently with human findings, *CXCR7* expression was markedly higher in endometriosis in comparison with the normal uterus, without a significant difference between 2 and 4 weeks postengraftment (Figure 3A). IHC revealed higher staining compared to normal uteri, mostly in epithelial cells (Figure 3).

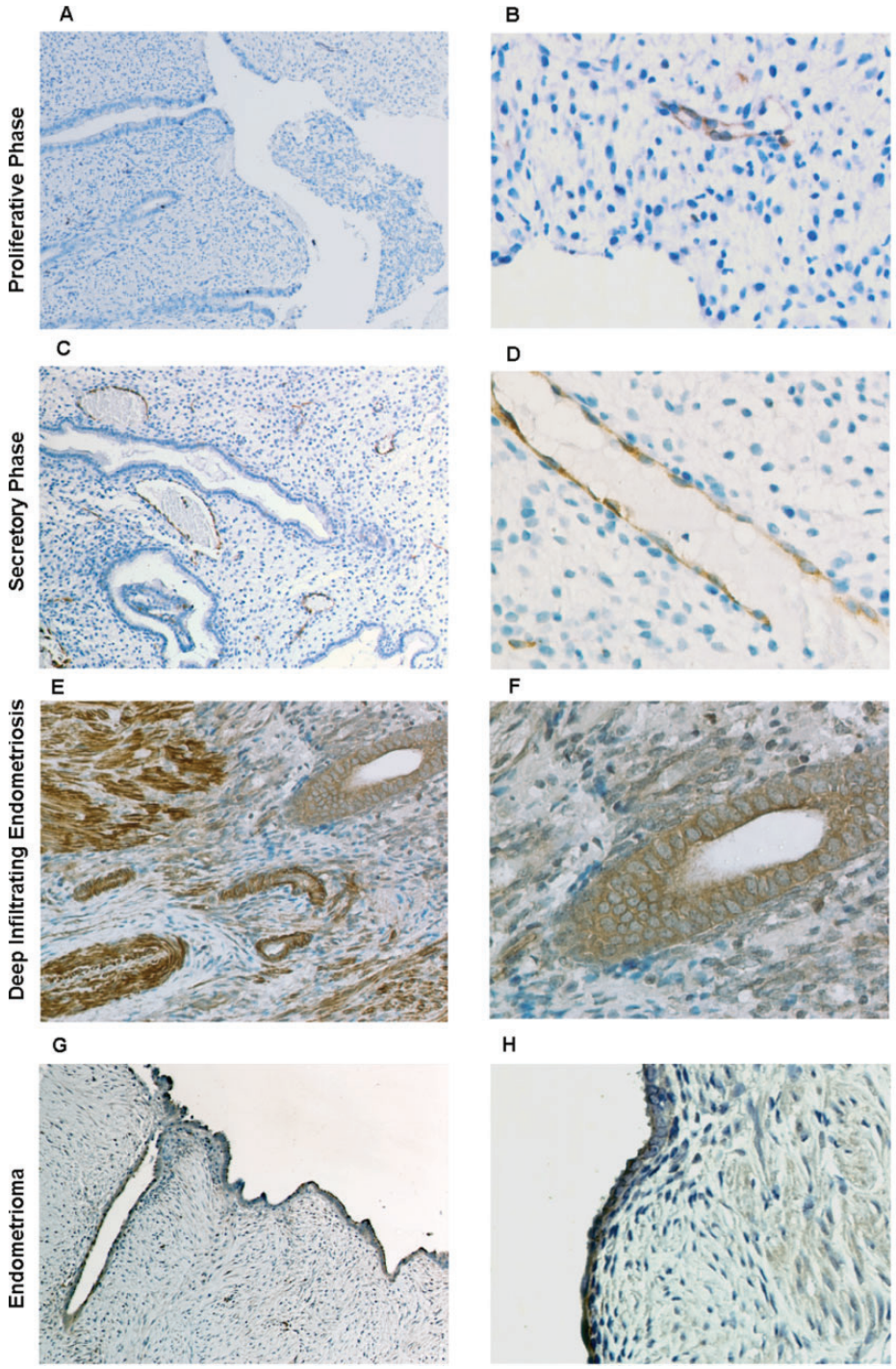

Figure 2. Representative images of *CXCR7* protein expression by IHC staining in eutopic endometrium and ectopic endometriosis. A, B, Normal endometrium proliferative phase (original magnification ×100, ×400, respectively). C, D, Normal endometrium secretory phase (×100, ×400, respectively). E, Deep infiltrating endometriosis (×200). F, Deep infiltrating endometriosis, ectopic gland (×400). G, H, Endometrioma (×100, ×400, respectively). *CXCR7* protein is highly expressed in deep endometriosis and endometrioma compared to phases in normal eutopic endometrium.

Discussion

In this study, we determined that *CXCR7* expression is increased in endometriosis in human as well as in a murine model when compared to the respective controls. Normal endometrial epithelium and stroma showed undetectable or very low expression of *CXCR7,* without any significant changes across menstrual cycle phases, while endometriosis showed a dramatic *CXCR7* upregulation of both mRNA and

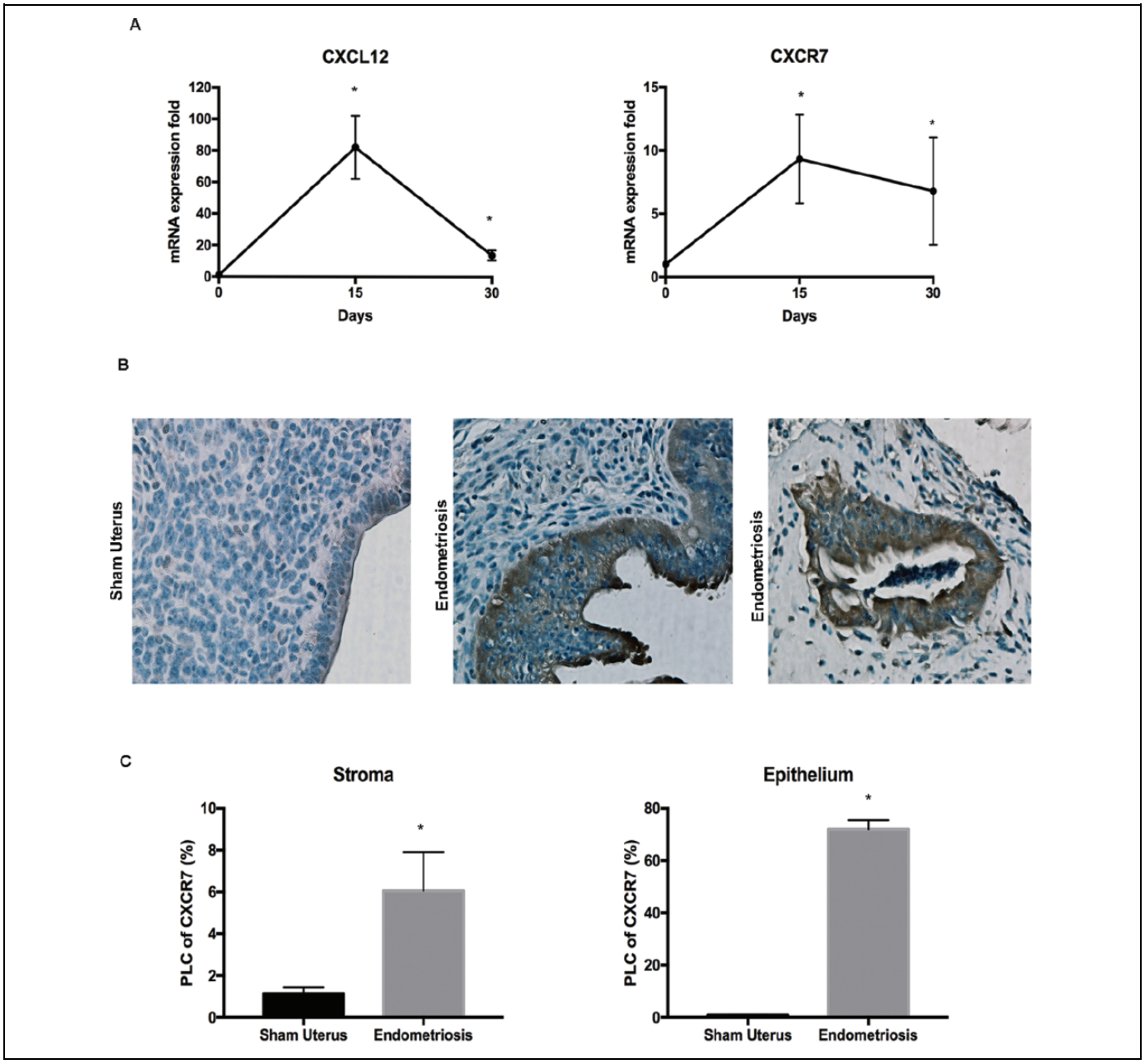

Figure 3. Expression of *CXCR7* and *CXCL12* in endometriosis of murine model. A, Expression of *CXCR7* and *CXCL12* in mouse ectopic lesion over 2 and 4 weeks was analyzed by qRT-PCR. *CXCL12* levels peaks at 15th day and regressed after 30 days, after induction of endometriosis while *CXCR7* levels stays at high levels even after 30 days. mRNA levels are expressed relative to transcript level in eutopic uterine donor tissue, set at 1.0. B, Representative images of IHC showing high levels of *CXCR7* protein expression in stroma and epithelial cells of ectopic uteri of endometriosis compared to sham control. C, Shows the percentage of labeled cells (PLC) based on IHC staining shown in B. Results are shown in mean $\pm$ SEM. *$P < .05$. IHC indicates Immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction.

protein. This finding confirmed previous immunostaining data showing that normal endometrium has very low or no *CXCR7* expression.[38] We further characterized the absence of significant effects exerted by ovarian steroids on *CXCR7* expression in normal endometrial tissue since no significant changes between the proliferative and secretory phase were detected. In normal endometrium, the *CXCR7* is also expressed in endothelial cells of some endometrial vessels. Smooth muscle and pericytes are reduced in the superficial layer of the endometrium, and the most superficial vessels consist only of endothelial cells.[39] In the secretory phase, the growth of the subepithelial capillary plexus takes place and endothelial cells maintain a marked *CXCR7* staining. Endometriosis implants are typically characterized by glandular epithelium and stroma surrounded by abundant vascularization (as a result of neoangiogenesis) and myofibroblasts.[28] We showed that *CXCR7* is

upregulated and widely expressed in the vascular smooth muscle cells and in endothelial cell of endometriosis-associated vessels. This finding is in agreement with previous data showing that *CXCR7* exhibited robust staining in a large percentage of tumor vasculature from human malignancies.[40,41]

CXCR7 has a crucial role in vascular remodeling in physiological as well as pathological conditions. *CXCR7* activation by *CXCL12* stimulates angiogenesis *in vivo* and promotes tube formation in human umbilical vein endothelial cells (HUVEC).[42] *CXCR7* plays a key role in maintaining endothelial integrity, through which it regulates vascular response to endothelial denudation injury.[42,43] Moreover, *CXCR7* activation induces directional migration and polarization of endothelial cells in response to *CXCL12*.[42]

Interestingly, unlike normal endometrium, ectopic glands in endometriosis implants exhibit a positive staining with predominance in epithelial cells. This novel feature of ectopic glands confirms profound cellular dysregulation in endometriosis compared to normal endometrium.[44] Lastly, cells surrounding vessels thought to be myofibroblasts exhibit strong positive *CXCR7* staining in agreement with other pathological conditions characterized by fibroblast recruitment and fibrosis.[45]

The mouse model of endometriosis recapitulated human features. Uterine grafting in the abdomen is associated with a tremendous increase in intralesion *CXCL12* expression after 2 and 4 weeks after grafting. This finding is associated with an increased expression of *CXCR7* demonstrating the existence of an upregulated *CXCL12-CXCR7* axis in endometriosis. This observation confirms the crucial role of chemokines in the endometriosis microenvironment. This hypothesis is also supported by adhesion studies demonstrating, in vitro, that *CXCR7* expression is regulated by inflammatory cytokines (Interleukin-1β [IL-1β], IL-8, and *CXCL12*) on endothelial cells and promotes maximal interactions when expressed concomitantly on both tumor cells and activated endothelium.[29,40] *CXCR7* is also induced in human endothelial cells and in bone marrow-derived mesenchymal stem cells under hypoxic and acidic conditions, consistent with the endometriosis microenvironment.[46]

Based on the current knowledge of the *CXCL12-CXCR4/CXCR7* axis, we hypothesize that it has a dual role in the endometriosis microenvironment: (a) enhancing local proliferative and anti-apoptotic cellular pathways in different cellular populations harboring the receptors and (b) promoting bone marrow–derived stem cell trafficking into endometriosis favoring the development of ectopic lesions. Future studies will address the functional implications of these findings. In conclusion, specific upregulation of *CXCR7* in different cellular populations of endometriosis microenvironment may represent a novel target for new nonhormonal treatments of this chronic inflammatory disease.

Declaration of Conflicting Interests

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