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Bone Marrow Stem Cell Chemotactic Activity Is Induced by Elevated *CXCl12* in Endometriosis

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Abstract

Endometriosis is an inflammatory gynecological disorder caused by the growth of endometrial tissue outside the uterus. Endometriosis produces chemokines, including *CXCL12*, that attract bone marrow cells to the lesions. In this study, we describe the expression, localization, and chemotactic activity of *CXCL12* in endometriotic lesions. Biopsies were collected both from women with endometriosis undergoing laparoscopy and control endometrium from women without endometriosis. Expression of *CXCl12* and *CXCR4* messenger RNA was increased approximately 4- and 6-fold, respectively, in endometriosis compared to eutopic endometrium. Immunohistochemistry of lesions revealed that *CXCR4* was expressed in the stroma and epithelium in both endometriosis and control eutopic endometrium. The level of *CXCR4* protein expression was significantly higher in all cellular compartments of the endometriotic lesions compared to control endometrium. *CXCL12* protein expression was also higher in endometriotic lesions and was greatest in the epithelial compartment. *CXCL12* was increased more in the condition media of cultured endometriosis than in controls as measured by enzyme-linked immunosorbent assay. Transwell chamber migration was used to demonstrate 2-fold increased chemoattraction of mouse bone marrow stem cells toward *CXCL12* in the endometriotic-conditioned medium compared with eutopic endometrium. Our results indicate that a preferential recruitment of stem cells to endometriosis can explain how endometriosis outcompetes eutopic endometrium in recruiting the limited supply of circulating stem cells. The *CXCL12*/*CXCR4* signaling axis is a potential target for the treatment of endometriosis and its associated disorders.

Keywords

CXCL12, CXCR4, chemotactic activity, endometriosis, stem cells, bone marrow

Introduction

Endometriosis is a benign, estrogen (E2)-dependent, and invasive disease that is defined as the ectopic growth of endometrial glands and stroma outside the uterus. The prevalence of this disease is approximately 5% to 10% of reproductive-age women.[1] The ectopic growth of this tissue causes inflammation, fibrosis, ovarian cysts, and adhesions, which result in dysmenorrheal, chronic pelvic pain, dyspareunia, and infertility. Although the etiology is not fully characterized, endometriosis is a multifactorial disorder, resulting from retrograde menstruation, ectopic differentiation of stem cells, dysregulated immune/inflammatory responses, aberrant expression of multiple genes, and exposure to environmental factors.[2-8] Various cellular mechanisms are activated, which allow survival, adhesion, proliferation, and invasion of endometrial cells that reach the peritoneal cavity.[9,10] The development of endometriosis involves altered activation of immune factors that induce a permissive environment leading to the survival of endometrial cells ectopically.[11] In fact, there is a strong evidence to support the role of immune and inflammatory mechanisms in endometriosis.[12,13]

Chemokines are small (8-12 kDa) polypeptides, which are characterized by their proinflammatory mediators, and play a role in cancer as regulators of angiogenesis, invasion, and metastases, cell growth, proliferation, and tissue remodeling.[14,15] The best-known function of the chemokines is the regulation of migration of various cells in the body; more than 40 chemokines and approximately 20 receptors have been implicated in the migration of cells endogenously. Chemokine receptors are 7-transmembrane G protein-coupled

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receptors that are usually linked to G proteins.[16,17] The chemokine receptor 4 (*CXCR4*) and its specific ligand, *CXCL12* (also known as stromal-derived factor 1), are reported to act in a paracrine fashion in cancer, promoting tumor growth and development, angiogenesis, and metastasis to tissues where *CXCL12* is expressed.[18] *CXCL12* and *CXCR4* also have a role in placental function.[19,20] Both the receptor and its ligand are also expressed by trophoblasts in the first trimester that penetrate maternal decidual blood vessels.[21] *CXCL12* may also be produced by decidualized stromal cells.[22] *CXCL12*/*CXCR4* signaling in trophoblast cell cultures has been shown to stimulate antiapoptotic pathways and promote cell survival.[21,23,24]

The interaction of *CXCR4* with *CXCL12* plays a key role in the mobilization and homing of stem cells.[25] We have previously reported that toxicant exposure, medications, and ischemia–reperfusion can alter the migration of BM-derived stem cells (BMDSCs) to the uterus; however, the molecular mechanism responsible for the recruitment and engraftment of these cells is unknown.[26-28] Recent work from our laboratory demonstrated that *CXCL12* and its receptor have an essential role in the migration of bone marrow cells (BMCs) toward endometrial cells.[29] Based on these data, we hypothesized that the *CXCR4–CXCL12* axis is involved in endometriosis by promoting the invasion and engraftment of stem cells at ectopic sites. In this study, we report the increased expression of *CXCR4* and *CXCL12* in endometriosis and the chemoattractant function of *CXCl12* in these lesions.

Materials and Methods

Sample Collection

Biopsies of endometriosis were obtained from 11 patients undergoing laparoscopy. Control endometrium was obtained from 11 patients without endometriosis. Endometriosis was confirmed histologically. All of the women were between 20 and 40 years of age and had regular menstrual cycles lasting between 25 and 35 days. No patients received hormonal treatments, including gonadotropin-releasing hormone agonist or sex steroids, nor did they use intrauterine contraception for at least 6 months prior to surgery. Approval for the collection of specimens was obtained from Yale University Human Investigations Committee.

RNA Isolation

Tissue samples collected from endometriosis and controls were processed immediately or frozen at −80°C in RNALater solution. The tissue was homogenized in TRIzol (100 mg/1 mL) reagent (Invitrogen, Carlsbad, California), and homogenates were kept on ice for 5 minutes, then 0.2 mL of chloroform (per 1 mL TRIzol) was added to each tube separately, and then homogenates were vortexed for 15 seconds. Samples were then incubated at room temperature (RT) for 3 minutes and centrifuged at 12 000 rpm at 4°C for 15 minutes. Next, the aqueous layer from each sample was transferred to a fresh tube, and the RNA was precipitated by adding 0.5 mL of isopropyl alcohol (per 1 mL TRIzol) to each sample and incubated at RT for 10 minutes. All tubes were centrifuged at 10 000 rpm to form the RNA pellets, which were then collected, washed with 75% ethanol, and dissolved in RNase-free water. The total RNA was purified using the Qiagen RNeasy Cleanup Kit (Qiagen, Valencia, California), according to the manufacturer's protocol and quantified by a NanoDrop spectrophotometer (ThermoFicher Scientific, MA, USA). Purified RNA was immediately used for cDNA synthesis or stored at −80°C until use.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Purified RNA (25 ng) was reverse transcribed in 10 μL reaction mixture using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green (Bio-Rad) and optimized in the MyQ Single Color Real-Time PCR Detection System (Bio-Rad). The specificity of the amplified transcript and the absence of primer dimers were confirmed by a melting curve analysis. All products yielded the predicted melting temperature. Gene expression was normalized to the expression of β-actin. Relative messenger RNA (mRNA) expression of each gene was calculated using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).[30,31] The primers used are *CXCL12*: forward-AACACTCCAAAC TGTGCCCT, reverse-CTCTCACATCTTGAACCTCTTGTT; *CXCR4*: forward-GCAGAGGAGTTAGCCAAGATGT, reverse-CATTGGGGTAGAAGCGGTCA; and β-actin: forward-ATCAAGGAGAAGCTCTGCTACATC, reverse, TCAGACTCGGCTGGAAGAGA. All experiments were carried out twice in triplicate. Negative controls were run at the same time without reverse transcriptase in the reaction.

Immunohistochemistry

The deidentified formalin-fixed, paraffin-embedded endometrium and endometriosis tissues were used to make tissue sections. Tissue sections from biopsies (each category containing at least 3 samples in the normal proliferative phase and 3 samples in the secretory phase) were deparaffinized followed by dehydration with xylene and ethanol, stained with hematoxylin and eosin. The presence of endometriosis and the menstrual cycle phase were confirmed by histology. For immunohistochemistry (IHC) analysis, sections were rinsed for 5 minutes in water, and an antigen retrieval was carried out in 0.01 mol/L sodium citrate (pH 6) buffer for 12 minutes, followed by 20-minute cooling and rinsing in phosphate-buffered saline with 0.1% Tween (PBST) for 5 minutes. Later, sections were incubated in 3% hydrogen peroxide for 10 minutes, followed by three 5-minute PBST washes for 3 times and blocked with 2% normal goat serum. Sections were incubated with primary antibodies anti-*CXCL12* (Millipore, Temecula, California) or anti-*CXCR4*

(Thermo Fisher Scientific, MA, USA) in a humidified chamber for overnight at 4°C followed by washings and then incubated with biotinylated secondary antibody for 1 hour at RT. Sections were washed and then incubated with avidin-biotinylated enzyme complex for 30 minutes at RT and washed twice before staining for 2 minutes for *CXCL12* and 1 minute for *CXCR4* with diaminobenzidine tetrachloride. Sections were washed in water for 5 minutes and counterstained for 20 seconds with hematoxylin (20% in tap water) and dehydrated through alcohol, cleared in xylene, and mounted in DePeX. Negative control sections were processed in the same way but substituting primary antibodies with normal goat immunoglobulin G. Analysis of *CXCL12* and *CXCR4* expression was evaluated in each endometrial compartment (epithelium, glands, and stroma). The number of stained cells and the intensity of staining were evaluated in 5 high-power fields on each slide by 3 evaluators without knowledge of the specimen source. Staining was quantified using the H-score method.[32,33] The Mann-Whitney rank test was used for statistical analysis.

Cell Culture

Primary cell cultures were prepared by chopping the tissue collected from 5 normal endometrium and 5 endometriosis lesions. The finely minced tissue was incubated in Hanks balanced salt solution containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 25 mm), 1% penicillin/streptomycin, collagenase (1 mg/mL, 15 U/mg), and DNase (0.1 mg/mL, 1500 U/mg) for 45 minutes at 37°C with agitation. During and at the end of the incubation, the tissue was pipetted gently to disperse the cells every 15 minutes. Endometrial cells were pelleted, washed, and suspended in Ham's dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) (1:1) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin B. A mixture of endometrial cells (epithelial and stromal) was passed through a 40-μm sieve, which allowed stromal cells to pass through while epithelial cells are retained on the sieve (Millipore, Billerica, Massachusetts). The stromal cells were cultured for an additional 48 hours before carrying out the experiments.

CXCL12 Protein Assay of Conditioned Medium by Enzyme-Linked Immunosorbent Assay

The *CXCL12* protein concentration in the conditioned medium from primary cell cultures was measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minnesota), according to the manufacturer's instructions. Endometrial cells from endometriotic lesions and normal endometrium were cultured in DMEM/F12 supplemented with 10% FBS and 1% penicillin and streptomycin in a 6-well plate (2×10^5 cells/well). The supernatants or conditioned media were collected from 48-hour old cell cultures and were used immediately or frozen at −80°C until use.

Cell Migration Assay

The migration of mouse bone marrow cells (mBMCs) was carried out by transwell migration assay using 8-μm pore size polycarbonate membrane (Millipore, Billerica, Massachusetts). The conditioned medium (600 μL) collected from cultures of both endometriosis and normal endometrial cells after 48 hours was added into the lower chamber, and 200 μL of cells (1×10^6 cells) was placed into the upper insert. The cells in the upper insert were serum starved for 24 hours. After 16 hours, in a humidified CO_2 incubator at 37°C, the nonmigrating cells were scraped with a cotton swab from the top of the membrane. The cells migrating across the membrane were fixed, stained, and lysed according to the manufacturer's instructions. Optical densities were read in triplicate at 560 nm using a Bio-Rad Laboratories (Hercules, California) plate reader. To determine the relative percentage of migration, results were compared to the control with 100% migration. Each experiment was performed 3 times in triplicate using specimens from 5 subjects without endometriosis and 5 subjects with endometriosis. Results are reported as a chemotactic index, defined as cells migrating in response to the conditioned supernatants divided by cells response to the serum-free DMEM/F12 medium. The Mann-Whitney *U* test was used to assess the significance of the difference in the acquired data.

Statistical Analysis

Results from qRT-PCR and ELISA were analyzed by 1-way analysis of variance with SPSS 11.0. The Mann-Whitney rank test was used to compare migration assay data and H-scores. A *P* value (<.05) was considered significant.

Results

Increased Expression of CXCL12 and CXCR4 in Women With Endometriosis

The qRT-PCR results showed increased mRNA expression of *CXCL12* and *CXCR4* in women with endometriosis compared to women without endometriosis. As shown in Figure 1A and B, CXCR4 expression was increased nearly 4-fold, whereas *CXCL12* was increased 6.5-fold in endometriosis compared to normal endometrium. In order to confirm that the increased mRNA expression of *CXCL12* resulted in an increase in secretion of the cytokine, its protein levels were determined in the conditioned media from primary cell cultures obtained from endometriotic lesions and normal endometrium using the ELISA. As shown in Figure 1C, *CXCL12* protein levels were increased by 2.3-fold in the conditioned media from primary stromal cell cultures of endometriotic lesions (3144 ± 324 ng/mL) compared with the conditioned media of normal endometrium stromal cells (1348 ± 251 ng/mL).

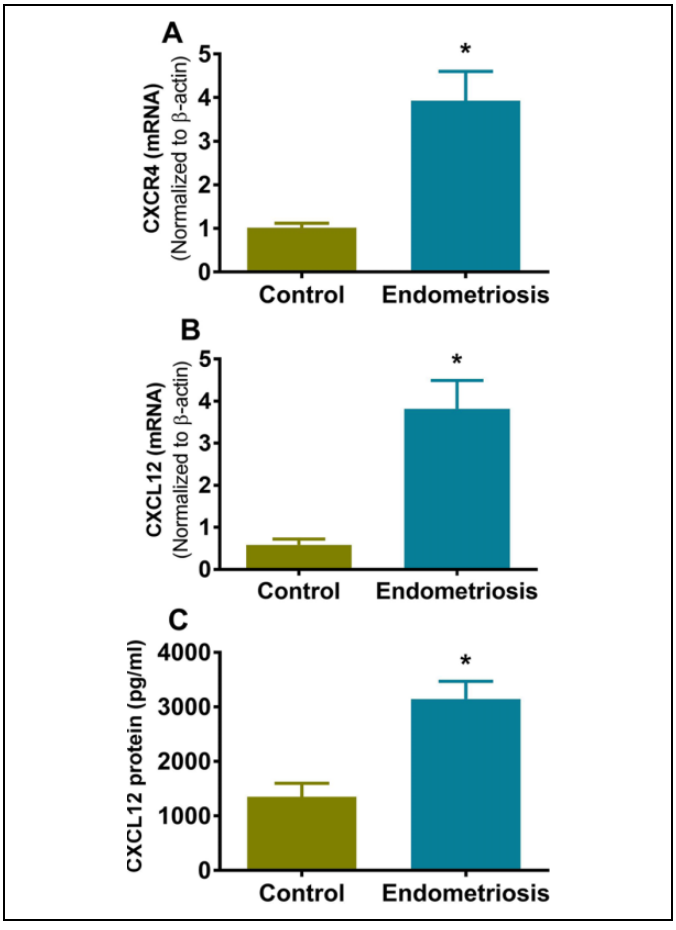

Figure 1. Increased expression of *CXCR4* and *CXCL12* in women with endometriosis. A and B, Quantitative real-time polymerase chain reaction (qRT-PCR) of CXCR4 and CXCL12, respectively. The messenger RNA (mRNA) levels of both genes were increased significantly (*CXCR4*, $P < .02$; CXCL12, $P < .0001$) in women with endometriosis compared to women without endometriosis. Bars represent the mean $\pm$ standard error of the mean (SEM) of 10 controls and 9 endometriosis samples from distinct subjects. C, Quantification of secreted *CXCL12* protein by enzyme-linked immunosorbent assay (ELISA) tested in conditioned media of primary cell culture. The protein content was significantly higher in the conditioned medium of cells from endometriosis compared to normal endometrium ($P < .001$). Each bar represents the mean $\pm$ SEM of 4 independent samples from unique subjects. Each experiment was performed in triplicate. *Statistically significant difference ($P < .05$) versus normal endometrium.

Increased CXCR4 *and* CXCL12 *Protein in Endometriotic Epithelium and Stroma*

The localization and protein expression of *CXCR4* and *CXCL12* were studied in both endometriotic lesions and normal endometrium using IHC. The *CXCR4* was expressed in the stroma, glands, and epithelium in both endometriosis and normal endometrial tissue; however, the intensity was significantly higher in all cellular compartments of the endometriotic lesions when compared to normal endometrium (Figure 2). *CXCL12* expression was also higher in endometriotic lesions and was greatest in the epithelial compartment as shown in Figure 2. The increased protein levels of *CXCR4* and *CXCL12* in endometriotic lesions compared to normal endometrium were confirmed by H-score on the stained sections. Table 1 shows the H-score for *CXCR4* was 243 for endometriosis versus 142 for normal endometrium ($P < .01$), whereas the H-score for *CXCL12* was 260 versus 160 ($P < .02$) in endometriosis and control endometrium, respectively. In glandular epithelium, *CXCR4* immunostaining was predominantly localized in the cytoplasm, toward the lumen. The staining for *CXCL12* was positive in the epithelial compartment and appeared to be stronger in luminal epithelium compared with the glands. The intensity was higher in endometriosis compared to normal endometrium. The *CXCL12* and *CXCR4* staining showed no significant difference between the proliferative and secretory phases.

Effect of CXCl12 *on Migration of BMSCs*

A migration assay was carried out to determine the chemoattractant activity of *CXCL12* on mBMCs, using the conditioned media from primary cultures of both endometriotic lesions and normal endometrium. Previously, we have shown that *CXCL12* was necessary and sufficient to attract BMSCs to conditioned media from normal endometrial stromal cells.[29] In this study, we show that conditioned medium from endometriotic cells also significantly induced the migration of mBMCs. The migration of BMSCs was greater toward conditioned medium from endometriotic cells than toward the conditioned medium from normal endometrium cells as shown in Figure 3 (2.37-fold; $P < .0001$).

Discussion

In previous studies, we have shown that the chemoattractant activity of the chemokine receptor *CXCR4* is due to its specific ligand *CXCL12* in the conditioned media of primary cultures of normal endometrium from women without endometriosis.[29] Here, we demonstrate that the expression and secretion of *CXCL12* is elevated in endometriosis compared to normal endometrium. We demonstrated that the expression of both *CXCR4* and *CXCL12* is upregulated in endometriotic lesions relative to normal endometrium. These results are in agreement with the previous reports,[11,24,34,35] which described the upregulation of *CXCR4* in both ectopic and eutopic endometrium of patients with endometriosis, as well as in endometriotic lesions in a rat model of endometriosis[34] and the peritoneal fluid from endometriosis patients.[36-38] More recently, it is been reported that the activation of the *CXCR4–CXCL12* signaling axis is involved in the pathophysiology of endometriosis by promoting invasion and growth of endometrial cells ectopically[39,40] and is responsible for the survival of human secretory phase endometrial stromal cells by the inhibition of autophagy.[41] We demonstrated previously that E2 treatment at physiological levels induced the expression of both *CXCR4* and *CXCL12* in mBMCs and in human endometrial stromal cells (hESCs), respectively, and enhanced the chemoattraction activity of

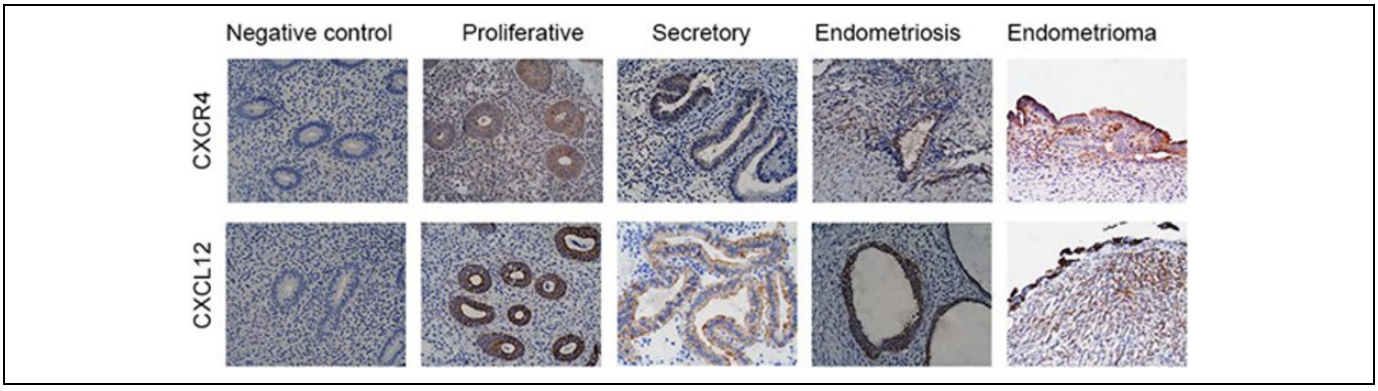

Figure 2. Detection of *CXCR4* and *CXCL12* by immunohistochemical staining. A, The immunostaining of *CXCR4* and *CXCL12* of negative control, proliferative phase, secretory phase, endometriosis, and endometrioma tissue sections, using anti-CXCR4 and anti-CXCL12 antibodies. The intensity of staining of *CXCR4* and *CXCL12* was greater in endometriosis and endometrioma compared to normal endometrium.

Table 1. Quantification of *CXCR4* and *CXCL12* of immunostained sections by H-score.

H-Score				
	Negative Control	Normal Endometrium	Endometriosis	*P*-value
CXCR4	100	142	243	$P < 0.01$
CXCL12	100	160	260	$P < 0.02$
	Proliferative	Secretory	*P*-value	
CXCR4	152	130	NS	
CXCL12	160	163	NS	

The average H-score is significantly higher in endometriosis than normal endometrium. Statistical analysis was performed using Mann-Whitney rank sum test, and $P < .05$ was considered statistically significant.

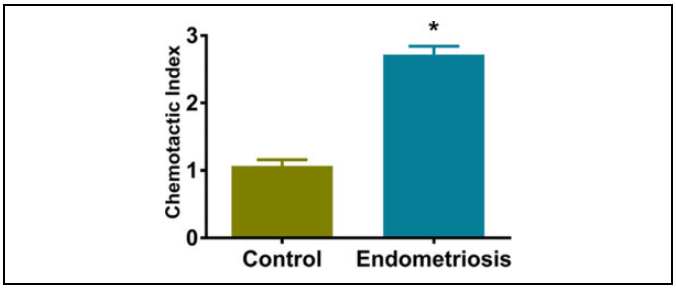

Figure 3. The chemotactic activity of *CXCL12* in conditioned medium by migration assay. The chemoattractant activity of mouse bone marrow cells (mBMCs) was determined by migration assay. The migration of mBMCs toward hESC-conditioned medium obtained from primary cell culture of endometriotic lesions or from normal endometrium was determined by seeding cells (5×10^4/well) on inserts and measuring the migration toward serum-free medium alone (negative control) or 48-hour hESCs serum-free supernatant. Data are shown as a chemotactic index (CI): cells migrating in response to the conditioned supernatants divided by cells responding to the serum-free medium. Bars represent the mean $\pm$ standard error of the mean (SEM) of 4 samples from controls and endometriosis, each performed in triplicate. The CI is significantly greater for endometriosis than normal endometrium. *Statistically significant difference ($P < .0001$) versus normal endometrium.

CXCR4.[29] Similarly, Ruiz et al reported that progesterone treatment results in the downregulation of *CXCR4* and *CXCL12* in endometrial cell lines and estradiol treatment results in an increase in epithelial *CXCL12* expression (although nonsignificant in their study).[40] Microarray studies that show the increased mRNA expression of both *CXCR4* and *CXCL12* in endometriotic lesions further support our results.[1,35]

Bone marrow (BM) contains multiple cell types including mesenchymal stem cells, which have the capacity to differentiate into a number of numerous types of cells. Bone marrow–derived stem cells are involved in the regeneration of the endometrium and contribute to the remarkable regenerative capacity of the endometrium.[28,42,43] Bone marrow–derived stem cells have been shown to engraft to normal endometrium as well as endometriosis.[4] Previously, our laboratory described the engraftment of BMDSCs into the uterus of women who received single-antigen human leukocyte antigen–mismatched BM transplants[42] and the engraftment of stem cells into the endometrium of mice.[4] We have also demonstrated the increased recruitment of BMDSCs to the site of uterine injury in female mice when the BM of male mice was transplanted into female mice.[4,44] The functional importance of stem cell flux to the uterus was demonstrated by experiments showing that BM cell delivery to mice after uterine injury improved the reproductive performance.[45] We have recently demonstrated that endometriosis can arise from the endometrium or from an exogenous sources including BM as a result of ectopic differentiation of stem cells.[4] In fact, endometriosis is more effective than endometrium in recruiting circulating BMDSCs from the circulation.[26] Endometriosis competes with normal endometrium for a limited supply of stem cells, depriving the eutopic endometrium of stem cells needed for optimal regeneration. One of the mechanisms that explains this engraftment is chemokine signaling. Chemokines are small cytokine molecules that attract various cell types by their chemotactic function and regulate the mobilization and homing of stem cells.[28,45,46] The chemokine receptor *CXCR4* is commonly expressed on the surface of stem cells that are attracted toward its ligand *CXCL12*. *CXCL12* is produced by many cell types, including

the stroma and epithelium of the endometrium and is generally expressed at sites of inflammation and injury.[47-49] The interaction between *CXCR4* and *CXCL12* allows the *CXCR4–CXCL12* signaling axis to be involved in angiogenesis, tissue repair, migration, and invasion.[50] Bone marrow–derived stem cells that express the *CXCR4* receptor migrate toward hESCs that express and secrete the *CXCL12* ligand; this migration was further increased by estradiol treatment, which induced *CXCL12* expression in endometrial cells.[29] Our present data demonstrate that BMDSCs were attracted to endometrial stromal cell–conditioned medium, and the migration was increased toward endometriosis-conditioned media that contain high concentrations of *CXCL12*. Variations in *CXCL12* concentration create a chemical gradient that directs the migration of stem cells.[47] Our study is in agreement with other studies that show higher levels of *CXCR4* and *CXCL12* in epithelium of ovarian endometriosis and ovarian cancer tissue when compared to normal ovary.[51,52] Moreover, endometriotic lesions have been shown to contain populations of mesenchymal stem cells that contribute in the development and progression of endometriosis.[53-58] *CXCL12* has been previously identified as an E2-regulated gene in E2 receptor-positive ovarian and breast cancer cells.[59] Previously, we have shown that in the endometrium, E2 significantly increased *CXCL12* expression, suggesting a mechanism by which stem cells are recruited to the uterus in reproductive-age women.[59] It is likely that this recruitment decreases after menopause when the uterus is no longer needed for reproduction. Premenopausal E2s provide a favorable environment for inducing *CXCL12* and enhancing BM-derived stem cell migration to both normal and ectopic endometrium. The inflammatory environment of endometriosis may program higher *CXCL12* expression that favors preferential stem cell recruitment to endometriosis.

In conclusion, the activation of the *CXCR4–CXCL12* signaling axis plays a critical role in stem cell migration that results in both the formation of new endometriotic lesions, as well as the inappropriate incorporation of stem cells into the eutopic endometrium. The high *CXCL12* production in endometriosis causes increased incorporation of stem cells into endometriosis. Furthermore, the elevated *CXCL12* production by endometriosis relative to normal endometrium provides a mechanism by which endometriosis preferentially recruits the limited supply of circulating stem cells. Future treatments for endometriosis may alter the inappropriate migration of stem cells, specifically due to the activation of the *CXCR4–CXCL12* signaling pathway. Inhibition of this signaling axis may have potential therapeutic value in the treatment of endometriosis and its associated disorders.

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Declaration of Conflicting Interests

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