Biology of Reproduction, 2018, 99(6), 1137–1148
doi:10.1093/biolre/ioy147
Research Article
Advance Access Publication Date: 25 June 2018

OXFORD

Research Article

Cysteine-X-cysteine motif chemokine ligand 12 and its receptor CXCR4: expression, regulation, and possible function at the maternal–conceptus interface during early pregnancy in pigs†

Jisoo Han[1], Wooyoung Jeong[2], Min Jeong Gu[3], Inkyu Yoo[1], Cheol-Heui Yun[3], Jinyoung Kim[2] and Hakhyun Ka[1,*]

[1]Department of Biological Science and Technology, Yonsei University, Wonju, Republic of Korea; [2]Department of Animal Resources Science, Dankook University, Cheonan, Republic of Korea and [3]Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea

*__Correspondence:__ Department of Biological Science and Technology, Yonsei University, Wonju 26493, Republic of Korea. Tel: +82-33-760-2369; Fax: +82-33-760-2183; E-mail: hka@yonsei.ac.kr

†__Grant Support__: This study was supported by the Next Generation BioGreen 21 Program (#PJ01110301), Rural Development Administration, Republic of Korea.
Edited by Dr. Haibin Wang, PhD, Xiamen University.

Received 15 March 2018; Revised 29 May 2018; Accepted 23 June 2018

Abstract

Cysteine-X-cysteine (CXC) motif chemokine ligand 12 (CXCL12) and its receptor, CXC chemokine receptor type 4 (CXCR4), are involved in regulating the proliferation, migration, and survival of trophoblast cells and the maternal immune response in humans and mice. The present study examined the expression, regulation, and function of *CXCL12* and *CXCR4* at the maternal–conceptus interface during pregnancy in pigs. The endometrium expressed *CXCL12* and *CXCR4* mRNAs with the greatest *CXCL12* abundance on Day 15 of pregnancy. CXCL12 protein was localized mainly in endometrial epithelial cells, while CXCR4 protein was localized in subepithelial stromal cells, vascular endothelial cells, and immune cells in blood vessels in the endometrium during the estrous cycle and pregnancy. CXCL12 protein was detected in uterine flushing on Day 15 of pregnancy. The conceptus during early pregnancy and chorioallantoic tissues during mid-to-late pregnancy expressed *CXCL12* and *CXCR4*. Interferon-γ increased the abundance of *CXCL12*, but not *CXCR4* mRNA in endometrial explants. Recombinant CXCL12 (rCXCL12) protein dose-dependently increased migration of cultured porcine trophectoderm cells and peripheral blood mononuclear cells (PBMCs). Furthermore, rCXCL12 caused migration of T cells, but not natural killer cells, in PBMCs. This study revealed that interferon-γ-induced *CXCL12* and its receptor, *CXCR4,* were expressed at the maternal–conceptus interface and increased the migration of trophectoderm cells and T cells at the time of implantation in pigs. These results suggest that CXCL12 may be critical for the establishment of pregnancy by regulating trophoblast migration and T cell recruitment into the endometrium during the implantation period in pigs.

Summary Sentence

A chemokine CXCL12 induced by interferon-γ of conceptus origin in the endometrium is involved in activation of trophectoderm cell migration and recruitment of T cells at the maternal–conceptus interface in pigs.

Key words: pig, pregnancy, uterus, endometrium, CXCL12.

Introduction

Well-coordinated interactions between the developing conceptus (embryo/fetus and associated extraembryonic membranes) and the maternal endometrium during the implantation period lead to appropriate placentation and organogenesis of the conceptus and endometrial remodeling of the uterus, resulting in successful pregnancy [1]. Various molecules including steroid hormones and cytokines derived from the conceptus, endometrium, and/or ovary are involved in the interactions between the conceptus and the endometrium [2, 3]. During the implantation period in pigs, the conceptus migrates within the uterine lumen to obtain an area sufficient for attachment to the endometrium. The conceptus undergoes a morphological transformation from spherical to ovoid, tubular, and then filamentous forms [1]. In pigs, on approximately Day 12 of pregnancy, the rapidly elongating conceptus synthesizes and releases estrogen, which acts as a signal for maternal recognition of pregnancy, and interleukin-1β2 [1, 4]. Subsequently, the conceptus secretes two types of interferons (IFNs) between Days 12 and 20 of pregnancy, with the highest amounts on Days 15 and 16 [5–7]. These IFNs are IFN-γ (IFNG), a type II IFN with major antiviral activity in the uterine lumen, and IFN-δ (IFND), a type I IFND with minor IFN activity [5, 7]. Although some evidence indicates that IFNs activate immune-related molecules in the endometrium [8–11], the functions of IFNs at the maternal–conceptus interface during early pregnancy are not fully understood in pigs.

Chemokines are small chemoattractant cytokines that are classified based on their structural motifs into four main subfamilies: cysteine (C), cysteine-cysteine (CC), cysteine-X-cysteine (CXC), and cysteine-X-3 cysteines (CX3C). More than 50 chemokines have been identified to date [12, 13]. Chemokines bind to G-protein coupled receptors that are differentially expressed by hematopoietic and nonhematopoietic cells, including endothelial, epithelial, and neural cells [14]. Chemokines mediate cell functions such as leukocyte infiltration, cellular proliferation, differentiation, migration, apoptosis, angiogenesis, and cancer metastasis [15–18]. At the maternal–conceptus interface, chemokines produced by the endometrium and the conceptus are critical for the establishment and maintenance of pregnancy in several mammalian species [19–24].

Among chemokines, the CXC-motif chemokine ligand 12 (CXCL12) is a 12-kDa protein that induces diverse intracellular signaling cascades by binding to its receptor, CXC receptor 4 (CXCR4) [25]. The receptor is expressed in many tissues and cell types including B cells, T cells, neutrophils, and monocytes, as well as endothelial and epithelial cells [26]. CXCL12 mediates a variety of physiological events including adhesion and transendothelial migration of hematopoietic cells and induction of a number of chemokines such as CC-motif ligand 2 (CCL2), interleukin 8, and CXCL10, during inflammatory reactions in several organ systems [27]. The importance of CXCL12 and CXCR4 interaction at the maternal–conceptus interface has been demonstrated in humans [28, 29], mice [30], and sheep [31]. In humans, CXCL12 expressed by invasive trophoblasts induces migration of natural killer (NK) cells expressing CXCR4 into decidua. CXCL12 also increases invasiveness of trophoblasts by promoting metalloproteinases 2 and 9 production in an autocrine manner during the first trimester [28, 29, 32]. In mice, CXCL12 and CXCR4 are expressed at the maternal–fetal interface, and CXCL12 increases migration of regulatory T cells into the uterus during pregnancy [33]. *CXCL12* and *CXCR4* expressed in endometrial epithelial cells and the conceptus trophectoderm during early pregnancy are involved in placental vascularization in sheep [31, 34].

Although the presence of several chemokines including CCL2, CCL4, CCL23, CCL28, CXCL9, CXCL10, and CXCL11 at the maternal–conceptus interface has been reported in pigs [21, 22, 24, 35, 36], the expression, regulation, and function of CXCL12 and its receptor CXCR4 at the maternal–conceptus interface have not been determined. To understand the function of CXCL12 in the uterus during pregnancy in pigs, we determined the following: (1) the expression of *CXCL12* and *CXCR4* in the uterine endometrium during the estrous cycle and pregnancy and in conceptuses during early pregnancy and chorioallantoic tissues during mid-to-late pregnancy; (2) localization of *CXCL12* and *CXCR4* at the maternal–conceptus interface; (3) effect of IFNG on *CXCL12* and *CXCR4* expression in endometrial tissues; (4) the presence of CXCL12 in uterine luminal flushings on Day 15 of pregnancy; (5) effect of CXCL12 on proliferation and migration of conceptus trophectoderm cells; and (6) effect of CXCL12 on migration of peripheral blood mononuclear cells (PBMCs).

Materials and methods

Animals and tissue preparation

All experimental procedures involving animals were conducted in accordance with the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University (Approval No. YWC-P120). Sexually mature, crossbred female pigs were assigned randomly to either cyclic or pregnant status. The reproductive tracts of gilts were obtained immediately after slaughter on either Days 12 or 15 of the estrous cycle or Days 12, 15, 30, 60, 90, or 114 of pregnancy (n = 4–6 gilts/day/status). Pregnancy was confirmed by the presence of apparently normal filamentous conceptuses in uterine flushings on Days 12 and 15 and the presence of embryos and placenta in the later days of pregnancy. Uterine flushings were obtained with 50 ml phosphate-buffered saline (PBS) (pH 7.4) (25 ml/uterine horn). Conceptus tissues on Days 12 and 15 of pregnancy were obtained from uterine flushings, and chorioallantoic tissues were obtained from days 30, 60, 90, and 114 of pregnancy (n = 3–4/day). Flushings were clarified by centrifugation (3000× *g* for 10 min at 4°C), aliquoted, and frozen at −80°C until analyzed.

Endometrium, dissected free of myometrium, was collected from the middle portion of each uterine horn, snap-frozen in liquid nitrogen, and stored at −80°C for RNA extraction. For immunohistochemistry, cross-sections of endometrium and conceptus tissues

Table 1. Summary of PCR primer sequences and expected product sizes.

Primer	Sequence of forward (F) and reverse (R) primers (5′ → 3′)	Annealing Temperature (°C)	Product size (bp)	No. of cycles	GenBank accession no.
CXCL12	F: GTC AGC CTG AGC TAC AGA TGC R: TGT TTA AAG CTT TCT CCA GGT ATT C	60	196	40	NM_001009580.1
CXCR4	F: ATC ATC TTC TTA ACT GGC ATA GTG G R: TGA CAG TAT AGA TGA CAT GGA CTG C	60	217	40	NM_213773.1
RPL7	F: AAG CCA AGC ACT ATC ACA AGG AAT ACA R: TGC AAC ACC TTT CTG ACC TTT GG	60	172	40	NM_001113217

were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and embedded in paraffin as previously described [37].

Total RNA extraction and reverse transcription-polymerase chain reaction for *CXCL12* and *CXCR4* cDNA

Total RNA was extracted from endometrial, conceptus, chorioallantoic tissues, and PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and integrity was validated following electrophoresis using 1% agarose gels.

Four micrograms of total RNA from endometrial, conceptus, and chorioallantoic tissues were treated with DNase I (Promega, Madison, WI, USA) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNAs. The cDNA templates were diluted 1:4 with nuclease-free water and amplified by PCR using Taq polymerase (Takara Bio, Shiga, Japan). PCR conditions and sequences of the primer pairs are listed in Table 1. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of amplified PCR products was verified by sequence analysis after cloning into the pCRII vector (Invitrogen).

Quantitative real-time reverse transcription-polymerase chain reaction

To analyze levels of *CXCL12* and *CXCR4* mRNAs in endometrial and chorioallantoic tissues, real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA, USA) and Power SYBR Green PCR Master Mix (Applied Biosystems). Final reaction volumes were 20 μl with 2 μl cDNA, 10 μl 2X Master mix, 2 μl each primer (100 nM), and 4 μl dH_2O. PCR conditions and primer pair sequences are listed in Table 1. Results are reported as expression relative to level detected on Day 12 of the estrous cycle, Day 30 of pregnancy, or control explant tissues after normalization of transcript amount to the endogenous porcine ribosomal protein L7 (*RPL7*) control by the $2^{-\Delta\Delta CT}$ method [38].

Immunohistochemical analysis

Sections of the porcine endometrium during the estrous cycle and pregnancy and the conceptus on Days 12 and 15 of pregnancy were immunostained to determine the type(s) of cells that expressed CXCL12 and CXCR4 proteins. Sections (5 μm) were deparaffinized and rehydrated in alcohol gradients. For antigen retrieval, tissue sections were boiled in citrate buffer (pH 6.0) for 10 min. Tissue sections were washed with PBST (PBS with 0.1% Tween-20) three times, and a peroxidase block was performed with 0.5% (v/v) H_2O_2 in methanol for 30 min. Tissue sections were blocked with 10% normal goat serum for 30 min at room temperature. Mouse monoclonal anti-CXCL12 (3 μg/ml; R&D Systems, Minneapolis, MN, USA) or mouse monoclonal anti-CXCR4 (5 mg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 5% normal goat serum was added for incubation overnight at 4°C in a humidified chamber. For each tissue tested, purified isotype-matched normal mouse IgG_1 (Vector Laboratories, Burlingame, CA, USA) was substituted for the primary antibody as a negative control. Tissue sections were washed with PBST three times. Biotinylated goat anti-mouse secondary antibody (1 μg/ml; Vector Laboratories) was added and incubated for 1 h at room temperature. Following washes with PBST, streptavidin-peroxidase conjugate (Invitrogen) was added for 10 min at room temperature. Sections were washed with PBST, and aminoethyl carbazole substrate (Invitrogen) was added for 10 min at room temperature. Tissue sections were washed in water, counterstained with Mayer's hematoxylin, and coverslipped.

Explant cultures

Endometrium on Day 12 of the estrous cycle was dissected from the myometrium and placed in warm phenol red-free Dulbecco modified Eagle medium/F-12 (DMEM/F-12) (Sigma, St. Louis, MO, USA) containing penicillin G (100 IU/ml) and streptomycin (0.1 mg/ml) as described previously [37], with some modification. Endometrium was minced with a scalpel blade into 2- to 3-mm^3 pieces, and 500 mg was placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/ml insulin, 10 ng/ml transferrin, and 10 ng/ml hydrocortisone (Sigma). To determine the effects of IFNG on *CXCL12* and *CXCR4* expression, endometrial explant tissues were cultured immediately after mincing with 0, 1, 10, or 100 ng/ml recombinant porcine IFNG (Sigma) with both estradiol-17β (E_2; 10 ng/ml) and progesterone (P_4; 30 ng/ml) at 37°C for 24 h. Explant tissues were harvested, and total RNA was extracted for real-time RT-PCR to determine the expression of *CXCL12* and *CXCR4*. Experiments were conducted using endometria from three gilts on Day 12 of the estrous cycle. Treatments were performed in triplicate using endometrial tissues obtained from each of the three gilts.

Immunoblot analysis

To detect CXCL12 protein in the uterine lumen on Day 15 of the estrous cycle and pregnancy, immunoblots were generated as described previously [21]. Uterine flushings were concentrated using centrifugal filters with pore size of molecular weight of 10,000 according to the manufacturer's recommendations (Millipore, Billerica, MA, USA). Forty micrograms of protein from concentrated

uterine flushings were separated on 12% SDS-PAGE gels, followed by electrotransfer onto nitrocellulose membranes. Nonspecific binding was blocked with 5% (w/v) fat-free milk in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST) buffer for 1 h at room temperature. Blots were incubated overnight at 4°C with 1 μg/ml mouse monoclonal anti-CXCL12 antibody (R&D Systems), 1 μg/ml mouse monoclonal anti-CXCL12 antibody neutralized with 100 ng recombinant human CXCL12 protein (rCXCL12; R&D Systems), or 1 μg/ml isotype-matched normal mouse IgG_1 (Vector Laboratories) diluted in 2% (w/v) fat-free milk in TBST. Blots were washed in TBST at room temperature three times for 10 min each, incubated with peroxidase-conjugated goat anti-mouse secondary antibody (1:5000; Abfrontier, Seoul, Republic of Korea) for 1 h at room temperature, and rinsed with TBST for 30 min at room temperature. To show the specificity of immunostaining, blots incubated with anti-CXCL12 antibody alone or secondary antibody alone were also included in immunoblotting. Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico, Pierce Chemical Co, Rockford, IL, USA) according to the manufacturer's recommendations using an ImageQuant LAS4000 Imager (GE Lifesciences, Piscataway, NJ, USA).

Porcine trophectoderm cell culture

A mononuclear porcine trophectoderm (pTr) cell line established from Day 12 conceptuses for in vitro studies was described previously [39–41]. pTr cells were cultured in phenol red-free DMEM/F-12 with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 1% Zell Shield (Minerva Biolabs, Berlin, Germany), 0.1 mM each nonessential amino acid, 1 mM sodium pyruvate, 2 mM glutamine, and 0.7 mM insulin in 5% CO_2 in air at 37°C. When the density of cells was approximately 80% confluency, cells were passaged at 1:3, and frozen stocks were prepared at each passage. For experiments, monolayer cultures of pTr cells were grown in culture medium to 80% confluency in 100-mm tissue culture plates.

pTr cell proliferation assays

pTr cells were subcultured in 24-well plates at 50% confluency in DMEM/F-12, serum starved for 24 h, and treated with 0, 0.1, 1, 10, 25, 50, or 100 ng/ml rCXCL12 (R&D Systems) at 37°C for 48 h. Cell numbers were determined as described previously [40, 41]. Medium was removed from cells by vacuum aspiration, and cells were fixed in 50% ethanol for 30 min, followed by vacuum aspiration of fixative. Fixed cells were stained with Janus Green B in PBS (0.2% w/v) for 3 min at room temperature. Stain was removed using a vacuum aspirator, and plates were dipped into water and destained by gentle shaking. Remaining water was removed by shaking, and stained cells were lysed in 0.5 N HCl. Absorbance at 595 nm was determined using a microplate reader. As described previously [41, 42], cell numbers were calculated from absorbance using the formula: cell number = (absorbance – 0.00462)/0.00006926. Experiments were independently replicated three times with different batches of pTr cells.

pTr cell migration assays

Migration assays were conducted with pTr cells as described previously [43]. pTr cells (50,000 cells per 100 μl serum-free medium) were seeded on 8-μm pore Transwell inserts (Corning-Costar, Corning, NY, USA). Wells (n = 3 replicates/treatment) were treated with 0, 0.1, 1, 10, 25, 50, or 100 ng/ml rCXCL12. After 12 h, the cells on the upper side of inserts were removed with a cotton swab. To evaluate cells that migrated onto the lower surface, inserts were fixed in methanol for 10 min. The Transwell membranes were then air-dried and stained using hematoxylin (Sigma, St. Louis, MO) for 30 min. The inserted membranes were washed gently several times with tap water to remove excess stain. The cells on the upper side of the inserts were removed with a cotton swab. The Transwell membranes were removed and placed on a glass slide with the side containing cells facing up, covered it using Permount solution. Migrated cells were systematically counted in five nonoverlapping locations covering approximately 70% of the insert membrane growth area using using a microscope DM3000 (Leica). Experiments were repeated three times.

Porcine PBMC migration assays

Whole blood from approximately 6-month-old pigs was diluted with PBS at 1:1, and PBMCs were isolated by density gradient centrifugation (400× *g* for 25 min without brake) using Ficoll-Paque Plus (Amersham Bioscience, Buckinghamshire, UK). PBMCs suspended in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (Invitrogen) were used for in vitro migration assays to measure response to rCXCL12 using 0.33-cm^2 polycarbonate membrane inserts with 5.0-μm pores in 24-well tissue culture plates (Corning-Costar). Inserts were preincubated in RPMI 1640 containing 5% FBS for 1 h. Media was removed, and inserts were placed in wells containing 500 μl media with 0, 2, 5, 10, 30, or 90 ng/ml rCXCL12. Single-cell suspensions of 1×10^5 PBMCs in 200 μl media were loaded onto inserts. Plates were incubated at 37°C and in 5% CO_2 for 90 min, and migrated cells were collected and analyzed. The cells that migrated to the lower chamber but remained attached to inserts were recovered by removing cells in the upper chamber with remaining suspension. Attached cells were recovered by placing the bottom of inserts into 500 μl ice-cold PBS containing 0.05% (w/v) trypsin-EDTA and tapping lightly. Migration index was calculated as follows: migration index = [(cell number in lower chamber with chemokine) – (cell number in lower chamber without chemokine)]/(number of cells initially added).

To investigate phenotypes of migrated cells from PBMCs, cells that migrated after treatment with 10 ng/ml rCXCL12 were analyzed using mouse anti-porcine CD3e biotin (clone PPT3; Southern Biotech, Birmingham, AL, USA), CD4 FITC (clone 74-12-4; BD Biosciences, San Jose, CA, USA), CD8a PE (clone 76-2-11; BD Biosciences), and CD16 biotin (clone FCG7; BD Biosciences). Biotin-conjugated antibody was detected using allophycocyanin-linked streptavidin (BioLegend, San Diego, CA, USA) for $CD3^+$ T cells and NK cells. Cells were incubated for 20 min at 4°C in the dark, washed, and marker expression was measured using flow cytometry (FACSCantoII, BD Biosciences). Flow cytometric data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Experiments were independently replicated at least three times in triplicate with different batches of PBMCs from three different pigs.

Statistical analysis

Data from real-time RT-PCR for *CXCL12* and *CXCR4* expression during the estrous cycle and pregnancy were analyzed by ANOVA using the general linear models procedures of SAS (Cary, NC). As sources of variation, models included day, pregnancy status (cyclic or pregnant, Days 12 and 15 post-estrus), and interactions to evaluate steady-state levels of *CXCL12* and *CXCR4* mRNAs. Data from

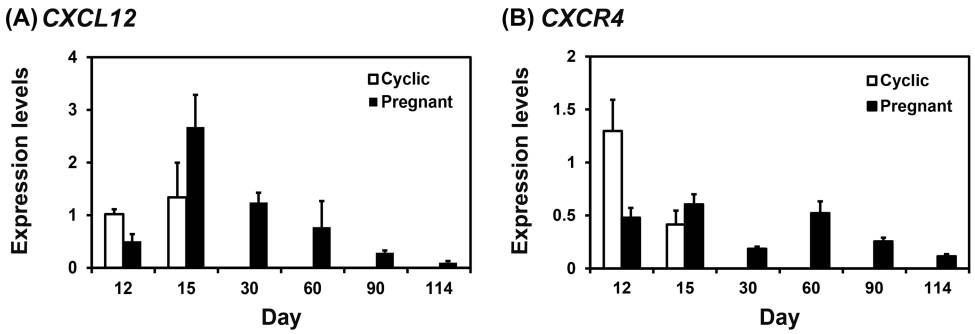

Figure 1. Expression of *CXCL12* (A) and *CXCR4* (B) mRNA in the endometrium during the estrous cycle and pregnancy in pigs. Endometrial tissue samples from cyclic and pregnant gilts were analyzed by real-time RT-PCR. Data are reported as expression relative to that detected on Day 12 of the estrous cycle after normalization of the transcript amount to the endogenous *RPL7* control. Data are presented as mean with standard error.

real-time RT-PCR to assess effects of pregnancy day in the endometrium (Days 12, 15, 30, 60, 90, and 114) and in chorioallantoic tissue (Days 30, 60, 90, and 114) on *CXCL12* and *CXCR4* expression were analyzed by least squares regression analysis. Data from IFNG dose-response studies and cell proliferation and migration assays were analyzed by one-way ANOVA followed by Tukey post-test. Data are presented as mean with SEM. Differences were considered significant if $P < 0.05$.

Results

Expression of *CXCL12* and *CXCR4* in the endometrium during the estrous cycle and pregnancy

To determine if *CXCL12* and *CXCR4* were expressed in the endometrium during the estrous cycle and pregnancy in pigs, we determined the relative abundance of *CXCL12* and *CXCR4* mRNAs in the endometrium using real-time RT-PCR (Figure 1). On Days 12 and 15 postestrus, the abundance of *CXCL12* mRNA was affected by day ($P < 0.01$) and day × status ($P < 0.05$), but not by pregnancy status, and was greater on Day 15 of pregnancy than on Day 15 of the estrous cycle. The abundance of *CXCR4* mRNA was affected by day × status ($P < 0.05$), but not by day or pregnancy status, and was greater on Day 12 of the estrous cycle than on Day 12 of pregnancy. During pregnancy, the abundance of *CXCL12* and *CXCR4* mRNAs changed in the endometrium with the greatest abundance on Day 15 of pregnancy (linear effect of day for *CXCL12* and *CXCR4*, $P < 0.05$).

Localization of CXCL12 and CXCR4 in the endometrium during the estrous cycle and early pregnancy and of the CXCL12 in the pig uterine lumen

Having determined that *CXCL12* and *CXCR4* are differentially expressed in the endometrium during pregnancy, we determined the cell types expressing CXCL12 and CXCR4 proteins in the endometrium using immunohistochemistry. Immunoreactive CXCL12 was localized predominantly to luminal and glandular epithelial cells in the endometrium (Figure 2A). CXCL12 was also detected in chorionic epithelial cells and the allantoic membrane during pregnancy. Immunoreactive CXCR4 protein in the endometrium was localized to subepithelial stromal cells, vascular endothelial cells, and some immune cells in blood vessels during the estrous cycle and pregnancy (Figure 2B).

Because *CXCL12* was expressed in endometrial epithelial cells, we determined if CXCL12 protein was secreted into the uterine lumen. We obtained uterine flushings from Day 15 of the estrous cycle and pregnancy and analyzed CXCL12 protein using immunoblotting. CXCL12 protein of approximately 35 kDa was detected in uterine flushings on Day 15 of pregnancy, but not on Day 15 of the estrous cycle (Figure 3A). Specific signals for CXCL12 protein in uterine flushings on Day 15 of pregnancy were decreased by pretreating the primary anti-CXCL12 antibody with recombinant CXCL12 protein (Figure 3B), and not detected when mouse control IgG_1 in substitute for anti-CXCL12 antibody, anti-CXCL12 alone, or secondary antibody alone were treated in immunoblotting (Figure 3C–E), although nonspecific signals were detected due to secondary antibody (Figure 3E).

Expression and localization of *CXCL12* and *CXCR4* mRNA and protein in conceptuses during early pregnancy and mRNAs in chorioallantoic tissues in later pregnancy

Using RT-PCR on cDNA from conceptuses on Days 12 and 15 of pregnancy and porcine PBMCs, we found that *CXCL12* mRNA was detectable in conceptuses on both pregnancy days. *CXCR4* mRNA was detectable in conceptuses from Day 15 of pregnancy and in PBMCs (Figure 4A). We further determined the localization of CXCL12 and CXCR4 proteins in conceptus tissues on Days 12 and 15 of pregnancy. Immunohistochemistry revealed that CXCL12 and CXCR4 were localized predominantly to trophectoderm cells (Figure 4B).

We used real-time RT-PCR to determine if the abundance of *CXCL12* and *CXCR4* mRNAs changed in chorioallantoic tissues during pregnancy. Analysis of *CXCL12* and *CXCR4* mRNA in chorioallantoic tissues on Days 30, 60, 90, and 114 of pregnancy showed that *CXCL12* mRNA decreased from Day 30 to term pregnancy (linear effect, $P < 0.05$), while *CXCR4* mRNA increased from Day 30 to term pregnancy (linear effect, $P < 0.05$) (Figure 4C).

Effects of IFNG on *CXCL12* and *CXCR4* expression in uterine endometrial tissues

Because the abundance of *CXCL12* and *CXCR4* mRNAs in the endometrium was greatest on Day 15 of pregnancy, we investigated factors that induce *CXCL12* and *CXCR4* expression in the endometrium. Because the conceptus secretes IFNs, IFNG, and IFND,

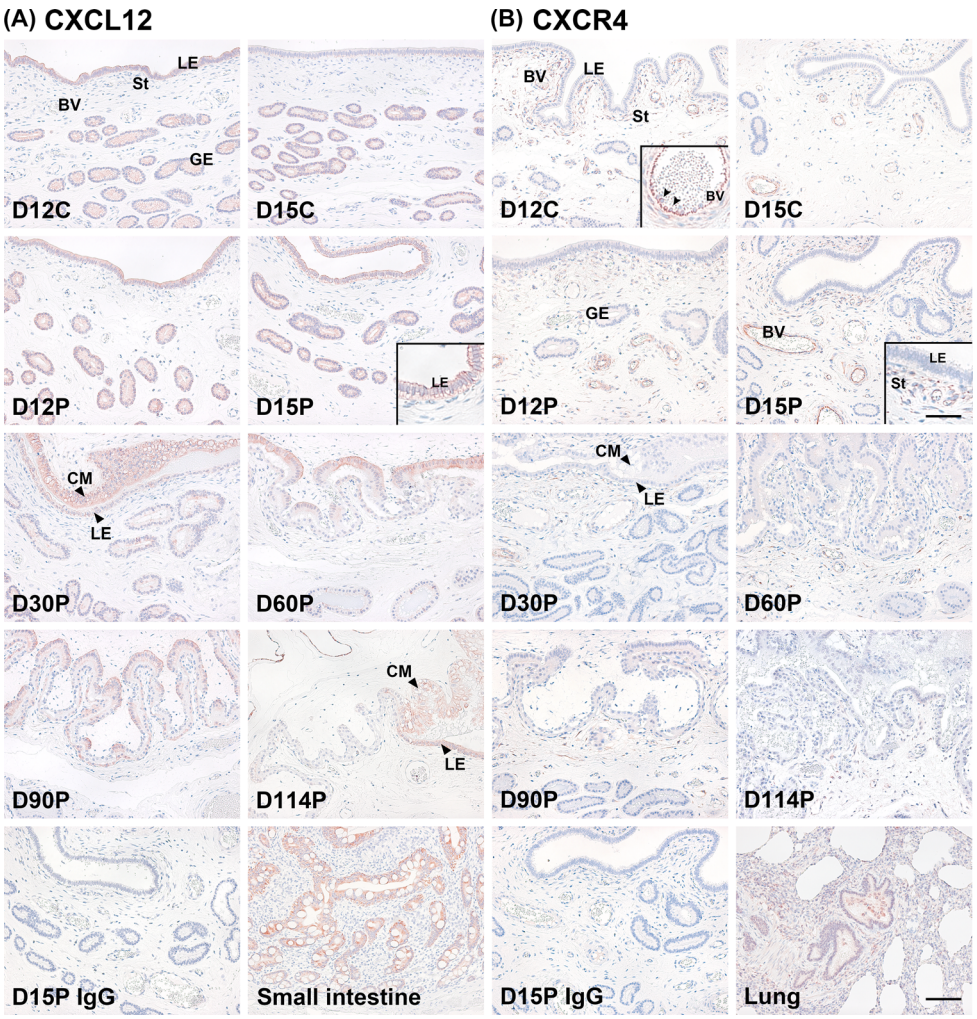

Figure 2. Localization of CXCL12 (A) and CXCR4 (B) proteins by immunohistochemistry in the endometrium during the estrous cycle and pregnancy in pigs. Immunohistochemistry for CXCL12 and CXCR4 proteins in uterine endometrium during the estrous cycle and early pregnancy in pigs. Representative uterine sections from Day 15 of pregnancy immunostained with normal mouse IgG_1 (IgG) as a negative control and tissue sections from small intestine and lung as positive controls for CXCL12 and CXCR4 immunostaining, respectively, are shown. D, Day; C, estrous cycle; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; St, stroma; BV, blood vessels; CM, chorioallantoic membrane. Bars = 100 μm and 50 μm in inset.

into the uterine lumen, with the greatest amount on Day 15 of pregnancy, and IFNG induces many endometrial genes [1, 7, 44], we hypothesized that IFNG from conceptuses would affect endometrial expression of *CXCL12* and *CXCR4*. We treated endometrial explant tissues from Day 12 of the estrous cycle with different doses of IFNG. The abundance of *CXCL12* mRNA significantly increased with IFNG at 100 ng/ml ($P < 0.01$) compared to the control (0 ng/ml), but the abundance of *CXCR4* mRNA was not affected by IFNG (Figure 5).

Effect of CXCL12 on proliferation and migration of pTr cells

Because endometrial expression of *CXCL12* was highest on pregnancy Day 15, CXCL12 proteins were secreted into the uterine lumen, and *CXCR4* was expressed in the conceptus trophectoderm during early pregnancy, we hypothesized that CXCL12 would affect conceptus development during early pregnancy. We analyzed the effect of CXCL12 protein on proliferation and migration of pTr cells, a primary porcine trophectoderm cell line [39]. After determining that pTr cells expressed *CXCR4* (Figure 6A), we treated pTr cells with increasing doses of rCXCL12. Proliferation of pTr cells was not affected by rCXCL12 (Figure 6B). However, pTr cell migration significantly increased with rCXCL12 at 1, 10 ($P < 0.05$), 25, 50, and 100 ng/ml ($P < 0.01$) compared to the control (0 ng/ml) (Figure 6C and D).

Effects of CXCL12 on PBMC migration

As *CXCL12* expression in endometrial epithelial cells during pregnancy was greatest on Day 15 (Figures 1 and 2), CXCL12 is critical for lymphocyte recruitment [20, 26], and *CXCR4* was expressed in blood vessel immune cells and PBMCs (Figures 2 and 4), we hypothesized that CXCL12 would affect immune cell recruitment into the endometrium during early pregnancy. We analyzed the chemotactic effect of CXCL12 protein on PBMC migration. Treatment of PBMCs with 10 ng/ml or higher rCXCL12 significantly increased migration of PBMCs compared to the control ($P < 0.05$) (Figure 7A). To determine if recruitment of T cells and NK cells into the endometrium during early pregnancy was affected by CXCL12, we an-

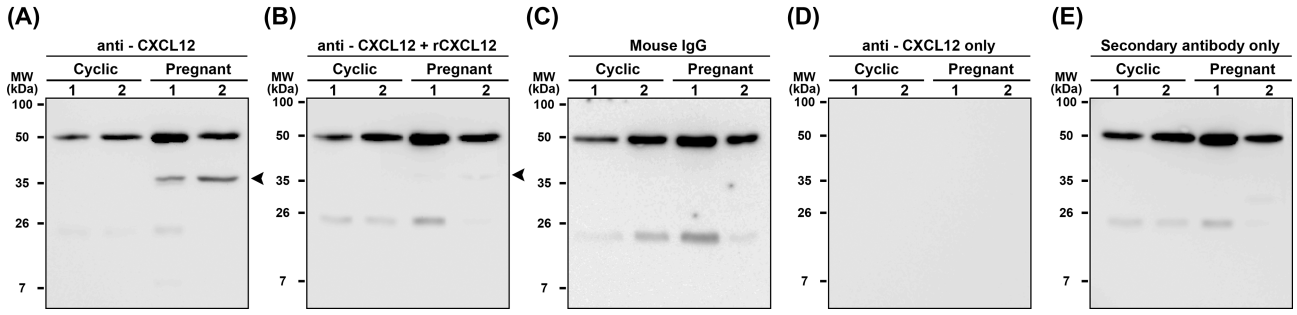

Figure 3. Immunoblot analysis of CXCL12 proteins in uterine flushings on Day 15 of the estrous cycle and pregnancy in pigs. **(A)** Uterine flushings were obtained from Day (D) 15 of the estrous cycle (Cyclic) and pregnancy (Pregnant) and determined for the presence of CXCL12 protein by immunoblotting. To show the specificity of immunoblotting, blots were treated with anti-CXCL12 antibody neutralized with 100 ng recombinant human CXCL12 protein (B), isotype-matched normal mouse IgG_1 (IgG) in substitute for anti-CXCL12 antibody (C), anti-CXCL12 antibody (D), or secondary antibody alone (E) in immunoblotting. Each lane represents analysis of uterine flushings from different gilts. Arrow head indicates CXCL12 immunostaining signal. MW, molecular weight.

alyzed the effects of rCXCL12 on the migration of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells and NK cells among PBMCs. Treatment with rCXCL12 significantly increased the number of migrated $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells ($P < 0.01$), but not NK cells, compared to the control (Figure 7B). Representative images of dot blots for $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+CD4^+CD8^+$ T cells (Figure 7C) and NK cells (Figure 7D) analyzed by flow cytometry are presented.

Discussion

The significant findings of this study in pigs are as follows: (1) *CXCL12* and its specific receptor *CXCR4* were expressed in the endometrium in a pregnancy status- and stage-dependent manner; (2) endometrial expression of CXCL12 and CXCR4 proteins was cell-type specific; (3) CXCL12 protein was detectable in uterine flushings on Day 15 of pregnancy; (4) *CXCL12* and *CXCR4* were expressed in early stage conceptuses and chorioallantoic tissues from mid-to-term pregnancy; (5) IFNG increased expression of *CXCL12* but not *CXCR4* in endometrial tissues; (6) rCXCL12 increased migration but not proliferation of conceptus trophectoderm cells; and (7) rCXCL12 induced migration of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ T cells, but not NK cells. To the best of our knowledge, this is the first report to characterize the expression of the CXCL12 chemokine and its receptor CXCR4 at the maternal–conceptus interface and describe the potential role of CXCL12 on trophectoderm cell function and immune cell recruitment during pregnancy in pigs.

CXCL12 and its receptor *CXCR4* are expressed at the maternal–conceptus interface during early pregnancy in humans, mice, and sheep [28, 31, 45, 46]. In this study of pigs, our results showed that *CXCL12* and *CXCR4* were expressed in the endometrium during the estrous cycle and pregnancy and in the conceptus during pregnancy. CXCL12 is known to be expressed mainly by trophoblast cells during early pregnancy in humans and mice [28, 30] and by endometrial epithelial cells, stroma, and conceptus trophectoderm in sheep [31, 47]. In pigs, CXCL12 expression was detected in endometrial epithelial cells and trophectoderm, but not in stroma. These data indicate that expression of CXCL12 in trophoblast cells is common, whereas the cell types expressing CXCL12 in the endometrium are species-specific. CXCR4 is expressed in monocytes, B cells, and naive T cells in peripheral blood and in some other cell types such as endothelial cells and smooth muscle cell progenitors in various tissues [48, 49]. CXCR4 is expressed in decidual stromal cells and immune cells such as decidual NK cells and T cells in the human endometrium [28, 29, 32] and decidual NK cells in mice [30]. It is detected in endometrial epithelial cells and trophoblast cells in sheep [34]. In this study, CXCR4 expression was detected in stromal and vascular endothelial cells and leukocytes in blood vesssels, but not in epithelial cells, in the endometrium and in trophectoderm cells in conceptuses in pigs. These data indicate that localization of CXCR4 expression in the endometrium depends on the species, and CXCL12 may act on endometrial cells and conceptus trophectoderm cells in an autocrine and/or paracrine manner.

In pigs, conceptuses produce a large amount of IFNs, IFND, and IFNG, between Days 14 and 16 of pregnancy to regulate endometrial functions for successful implantation and conceptus development [1]. Intrauterine infusion of conceptus secretory proteins containing IFNG and IFND into cyclic pigs increases endometrial expression of swine leukocyte antigens (SLAs), *SLA-1*, *SLA-2*, *SLA-3*, *SLA-6*, *SLA-7*, and *SLA-8*, β2-microglobulin (*B2M*), signal transducer and activator of transcription 1 (*STAT1*), and interferon-regulatory factor 1 (*IRF1*) [8–10]. Treatment of endometrial explants with IFNG induces an increase in *SLA-DQA* and *SLA-DQB*, which are expressed in stromal and endothelial cells in the endometrium in pigs [11]. Recently, we have shown that IFNG increases endometrial expression of chemokines, *CXCL9*, *CXCL10*, and *CXCL11*, which are expressed in the endometrium with the greatest abundance on Day 15 during pregnancy in pigs [22]. Because the abundance of *CXCL12* and *CXCR4* mRNAs during pregnancy was greatest on Day 15 and decreased thereafter, we hypothesized that *CXCL12* and *CXCR4* expression may be regulated by conceptus-derived IFNs in the endometrium in pigs. The results of the current study showed that the expression of *CXCL12* but not *CXCR4* increased with IFNG in endometrial explant tissues, indicating that IFNG originating from the conceptus may induce expression of *CXCL12* in the endometrium during the implantation period in pigs. The effect of IFND on CXCL12 and CXCR4 expression and the regulatory mechanism of endometrial *CXCR4* expression during the implantation periods still need to be determined.

Since *CXCL12* was expressed by endometrial epithelial cells and the conceptus trophectoderm during early pregnancy in pigs, we postulated that CXCL12 might be secreted into the uterine lumen. Indeed, CXCL12 protein of approximately 35 kDa was detected in uterine flushings on Day 15 of pregnancy. These findings suggest that CXCL12 from the endometrium and conceptus acts on conceptus

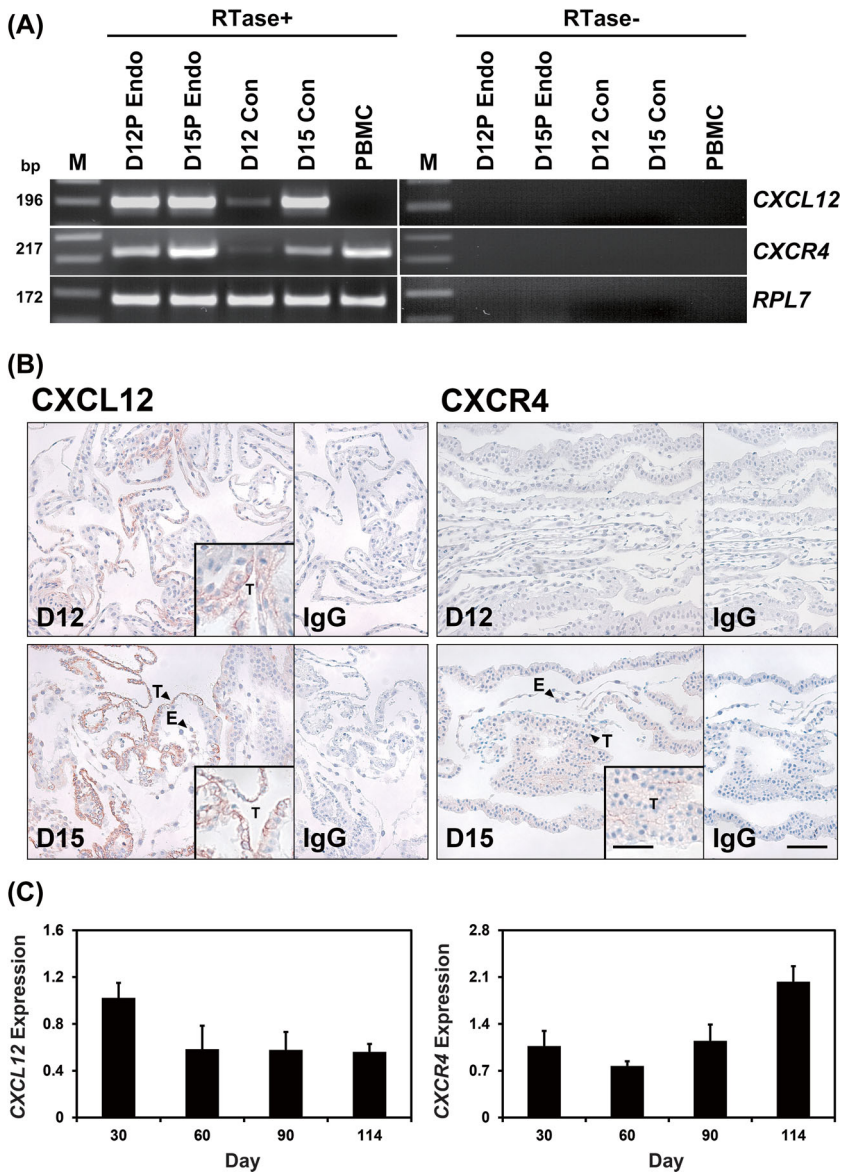

Figure 4. Expression of *CXCL12* and *CXCR4* in conceptuses from Days 12 and 15 of pregnancy and chorioallantoic tissues during later pregnancy. (A) RT-PCR of *CXCL12* and *CXCR4* mRNA from pregnancy Day 12 and 15 conceptuses using total RNA. *RPL7* was used as a positive control. RTase +/–, with (+) or without (–) reverse transcriptase; M, molecular marker; D12P Endo, endometrium on Day 12 of pregnancy; D15P Endo, endometrium on Day 15 of pregnancy; D12 Con, Day 12 conceptus; D15 Con, Day 15 conceptus; PBMC, peripheral blood mononuclear cells. (B) Immunohistochemistry for CXCL12 and CXCR4 in conceptuses from Days (D) 12 and 15 of pregnancy. T, trophectoderm; E, endoderm. Scale bar = 50 μm. (C) Real-time RT-PCR analysis of the expression of *CXCL12* and *CXCR4* mRNA in chorioallantoic tissues on Days 30, 60, 90, and 114 of pregnancy. Data are reported as expression relative to that detected on Day 30 of pregnancy after normalization of the transcript amount to the endogenous *RPL7* control, and data are presented as mean with standard error.

development in an autocrine and/or paracrine manner. Interestingly, the size of CXCL12 proteins detected in uterine flushings was greater than the exprected size of approximately 10–12 kDa [50]. It is well known that CXCL12 dimerizes and forms a complex with heparin oligosaccharides in the extracellular space in physiological conditions [50, 51]. In addition, CXCL12 protein aggregates in buffers above pH 7.0 in the presence of salts such as phosphate and sulfate [52]. Thus, it seems that the greater size of CXCL12 protein in uterine flushings may be cuased by the immunoblotting conditions in this study. Nevertheless, the biogenesis and structural characteristics of porcine CXCL12 protein found in uterine flushings need further analysis.

During the implantation period in pigs, attachment of trophectoderm to the maternal endometrial epithelial cells and proliferation and migration in the uterus are key features of the implanting conceptus [1, 53]. Also, elongated porcine conceptuses undergo cell proliferation and migration during this period [1, 39]. Thus, based on the localization of CXCL12 and CXCR4 at the maternal–conceptus interface and the effect of CXCL12 on activation of cell proliferation and migration of cell types including hematopoietic cells and epithelial cells [54], we hypothesized that CXCL12 may activate proliferation and migration of the conceptus trophectoderm during early pregnancy in pigs. Our data showed that rCXCL12 increased migration but not proliferation of porcine primary

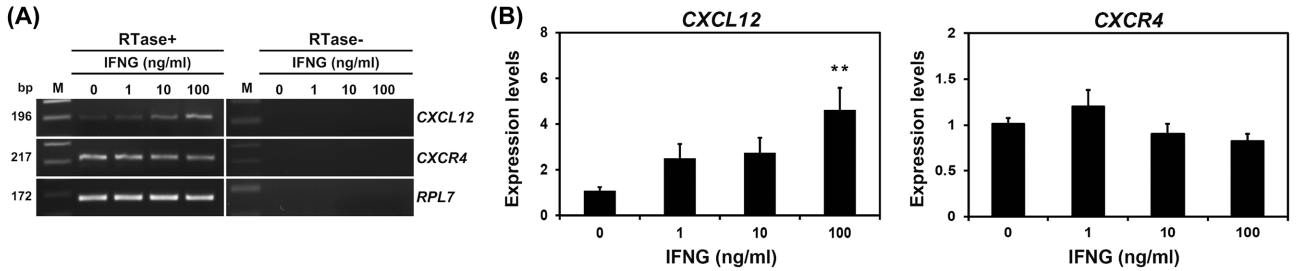

Figure 5. Effects of IFNG on *CXCL12* and *CXCR4* mRNA in endometrial explant cultures. (A) RT-PCR analysis of *CXCL12* and CXCR4 mRNAs in endometrial explant tissues treated with increasing doses of IFNG. RPL7 was used as a positive control for RT-PCR. RTase +/−, with (+) or without (−) reverse transcriptase; M, molecular marker. (B) Real-time RT-PCR analysis of effects of IFNG on *CXCL12* and *CXCR4* mRNA in endometrial explant cultures. Endometrial explants from gilts on Day 12 of the estrous cycle were cultured with 0, 1, 10, or 100 ng/ml IFNG. Abundance of mRNA expression determined by real-time RT-PCR analyses was relative to that for *CXCL12* and *CXCR4* mRNAs in the control group (0 ng/ml IFNG) of endometrial explants after normalization of transcript amounts to *RPL7* mRNA. Data are presented as mean with standard error. These treatments were performed in triplicate using tissues obtained from each of the three gilts. The asterisks denote statistically significant differences: $^{**}P < 0.01$.

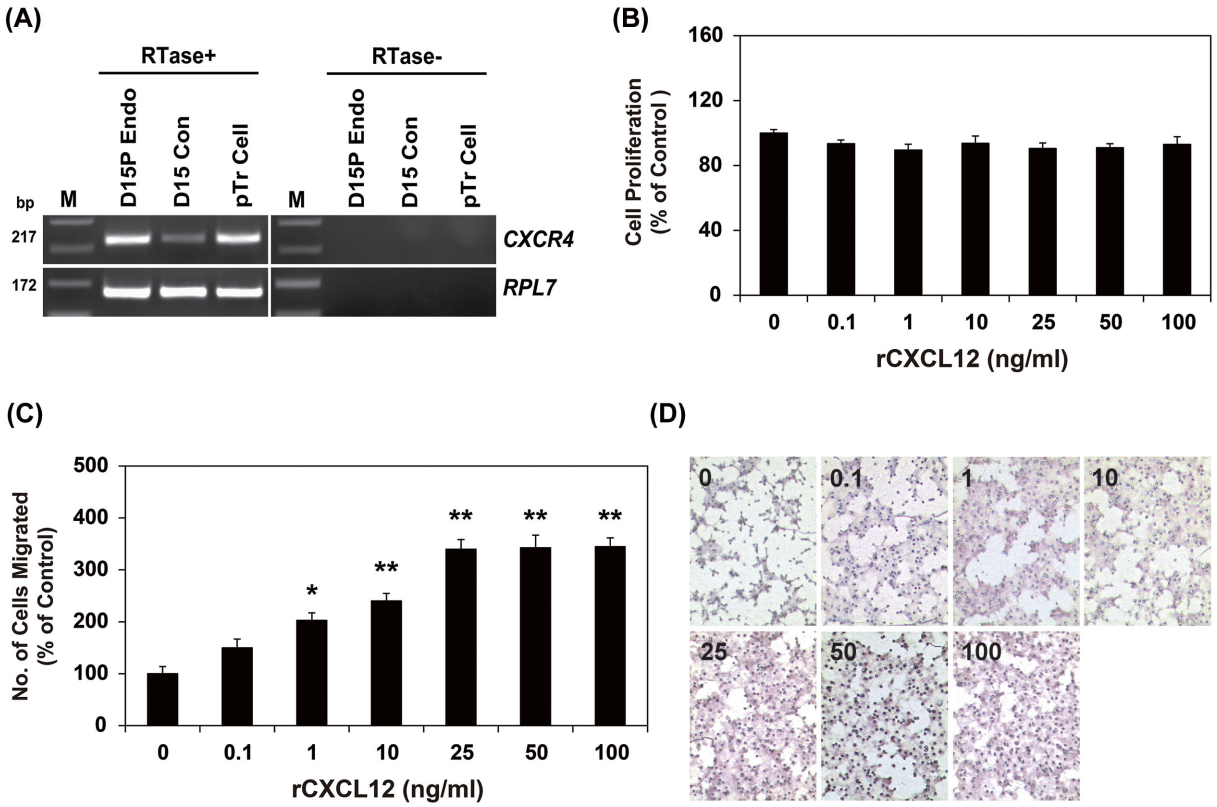

Figure 6. Effect of CXCL12 on pTr cell proliferation and migration. (A) RT-PCR analysis of *CX*CR4 mRNA in the pTr cell line. RPL7 was used as a positive control for RT-PCR. RTase +/− , with (+) or without (−) reverse transcriptase; M, molecular marker; D15 Endo, endometrium from Day 15 of pregnancy. D15 Con, Day 15 conceptus. (B) Effect of rCXCL12 on pTr cell proliferation. After serum starvation for 24 h, pTr cells were treated with 0, 0.1, 1, 10, 25, 50, or 100 ng/ml recombinant human CXCL12 (rCXCL12) at 37°C for 48 h in triplicate. (C) Effect of rCXCL12 on pTr cell migration. Serum-starved pTr cells were seeded on 8-μm pore Transwell inserts and treated with 0, 0.1, 1, 10, 25, 50, or 100 ng/ml rCXCL12 in triplicate for 12 h. Unmigrated cells on the upper side of inserts were removed. Cells that migrated were counted systematically in five nonoverlapping locations. Independent experiments for cell proliferation and migration were replicated three times. Data are presented as mean with standard error. The asterisks denote statistically significant differences: $^{*}P < 0.05$; $^{**}P < 0.01$. (D) A representative image of pTr cells in migration assay using Trans-well membrane. Migrated cells were fixed in methanol and stained with hematoxylin. The rCXCL12 doses treated are indicated. Original magnification, ×20.

trophectoderm cells, suggesting that CXCL12 produced by the endometrium and conceptus may be involved in migration of the conceptus trophectoderm and act as a histotroph at the maternal–conceptus interface. In addition, we found that chorioallantoic tissues during mid-to-late pregnancy decreased *CXCL12* and increased *CXCR4* mRNAs toward the end of pregnancy. CXCL12 may also be involved in migration of trophoblast cells for placental development during late pregnancy in pigs, but the details of CXCL12 signaling in chorioallantoic tissues during mid-to-late pregnancy in pigs need to be further investigated.

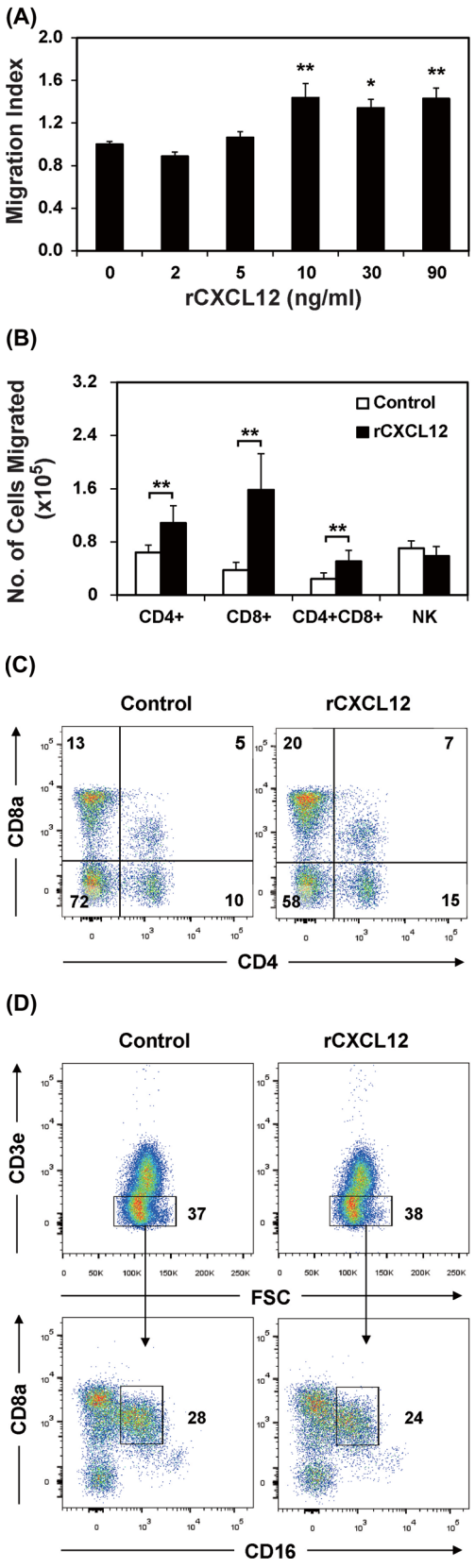

Figure 7. Effects of CXCL12 on migration of PBMCs and $CD4^+$, $CD8^+$, $CD4^+CD8^+$ T cells, and NK cells in PBMCs. (A) Effects of CXCL12 on peripheral blood mononuclear cell (PBMC) migration. PBMCs were loaded onto inserts in wells of 5.0-μm-pore transwell plates containing complete media with 0, 2, 5, 10, 30, or 90 ng/ml rCXCL12. Total numbers of migrated cells were counted. Experiments were replicated at least three times in triplicate, with different batches of PBMCs from three gilts. Data are presented as mean with standard error. (B) Effects of CXCL12 on migration of $CD4^+$, $CD8^+$, $CD4^+CD8^+$ T cells, and NK cells. PBMCs were loaded onto inserts in wells containing 500 ml of complete media alone (Control) or with 10 ng/ml rCXCL12 (rCXCL12). Migrated cells were examined by staining with mouse anti-porcine CD3, CD4, CD8a, or CD16 antibodies. $CD3^+$ T cells and NK cells ($CD3^-CD8a^+CD16^+$) were analyzed by flow cytometry. Experiments were replicated at least three times in triplicate with different batches of PBMCs from three gilts. Data are presented as mean with standard error. The asterisks denote statistically significant differences: $*P < 0.05$; $**P < 0.01$. (C) A representative image of dot blots for $CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+CD4^+CD8^+$ T cells analyzed by flow cytometry in (B). (D) A representative image of dot blots for $CD3^-CD8a^+CD16^+$ NK cells analyzed by flow cytometry in (B). Numbers in dot blots indicate the percentages of cells migrated in flow cytometric analysis.

Because the major function of CXCL12 is chemotaxis [26], and CXCR4 is expressed in endometrial stroma and PBMCs, we hypothesized that CXCL12 may be involved in migration of immune cells for recruitment into the endometrium during early pregnancy in pigs. Analysis of the chemotactic effect of CXCL12 protein on PBMC migration showed that rCXCL12 increased migration of PBMCs, suggesting that this chemokine may act on recruitment of immune cells into the endometrium. Among immune cells, T and NK cells are the major types that express CXCR4 [20, 55]. These cells have critical functions at the maternal–conceptus interface during pregnancy in pigs [56, 57]. In addition, expression of T cell receptor A (*TCRA*) and *TCRB* and an NK cell marker, *FCGR3A* (Fc fragment of IgG, low affinity IIIa, receptor), is highest in the endometrium on Day 15 of pregnancy in pigs [22]. Thus, we analyzed which types of PBMCs migrated and found that migration of $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD8^+$ T cells, but not NK cells, increased with rCXCL12. Our previous study showed that chemokines CXCL9, CXCL10, and CXCL11, which show maximal expression in the endometrium on Day 15 of pregnancy in pigs, induced migration of both T and NK cells [22]. Thus, it is likely that recruitment of immune cells into the endometrium during the implantation period is differentially regulated depending on the chemokine type in pigs. In addition, the redundant actions of several chemokines regulate recruitment of T cells into the endometrium during the implantation period in pigs. In humans, CXCL12 expressed by trophoblast cells induces migration of CXCR4-expressing NK cells into decidua [28, 29]. However, CXCL12 did not affect NK cell migration in pigs. Because other chemokines including CCL3 and CCL4 also affect recruitment of NK cells into the endometrium in humans [58, 59], endometrial NK cell recruitment is likely to be regulated by other chemokines including CXCL9, CXCL10, and CXCL11, rather than CXCL12 in pigs. Overall, these results indicate that IFNG from the conceptus induces endometrial expression of the chemokine CXCL12, which activates infiltration of T cells into the endometrium during early pregnancy in pigs. However, further studies are needed to understand the specific roles of these infiltrated immune cells in the endometrium for the establishment of pregnancy in pigs.

Results of this study showed that *CXCR4* was expressed by vascular endothelial cells in the endometrium. Although no data are available on CXCR4 expression in vascular endothelial cells in the endometrium in other species, the importance of CXCR4 expression

during angiogenesis has been well documented [60–62]. In addition, blocking CXCR4 signaling by treating pregnant sheep with a CXCR4 antagonist decreases expression of vascular endothelial growth factor in the caruncle of the endometrium and fetal membrane tissues [47]. This result suggests that CXCL12-CXCR4 signaling is involved in angiogenesis at the maternal–conceptus interface in sheep. Thus, CXCL12 may be associated with vascular development in the endometrium to support increased blood flow for the developing placenta and fetus during pregnancy in pigs. The effect of CXCL12 on endothelial cell function, however, needs further investigation.

In conclusion, our results indicate that the chemokine CXCL12 and its receptor CXCR4 are expressed in the endometrium in a pregnancy stage- and cell type-specific manner and conceptus tissues during pregnancy. Conceptus-derived IFNG induces endometrial expression of CXCL12, which may act on migration of trophectoderm cells and recruitment of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells into the endometrium. These findings provide important insights into the regulation of conceptus development and maternal immune responses to IFNG of conceptus origin at the maternal-fetal interface in pigs, which form a true epitheliochorial placenta.

References

1. Bazer FW, Johnson GA. Pig blastocyst-uterine interactions. *Differentiation* 2014; **87**(1–2):52–65.
2. Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, Bayless K. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol Hum Reprod* 2010; **16**(3):135–152.
3. Spencer TE, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy. *Reprod Biol Endocrinol* 2004; **2**(1):49.
4. Mathew DJ, Newsom EM, Guyton JM, Tuggle CK, Geisert RD, Lucy MC. Activation of the transcription factor nuclear factor-kappa B in uterine luminal epithelial cells by interleukin 1 Beta 2: a novel interleukin 1 expressed by the elongating pig conceptus. *Biol Reprod* 2015; **92**(4):107.
5. La Bonnardiere C, Martinat-Botte F, Terqui M, Lefevre F, Zouari K, Martal J, Bazer FW. Production of two species of interferon by Large White and Meishan pig conceptuses during the peri-attachment period. *Reproduction* 1991; **91**(2):469–478.
6. Mege D, Lefevre F, Labonnardiere C. The porcine family of interferon-omega: cloning, structural analysis, and functional studies of five related genes. *J Interferon Res* 1991; **11**(6):341–350.
7. Cencic A, Guillomot M, Koren S, La Bonnardiere C. Trophoblastic interferons: do they modulate uterine cellular markers at the time of conceptus attachment in the pig? *Placenta* 2003; **24**(8–9):862–869.
8. Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Jaeger LA, Spencer TE, Bazer FW, Johnson GA. Pig conceptuses increase uterine interferon-regulatory factor 1 (IRF1), but restrict expression to stroma through estrogen-induced IRF2 in luminal epithelium. *Biol Reprod* 2007; **77**(2):292–302.
9. Joyce MM, Burghardt RC, Geisert RD, Burghardt JR, Hooper RN, Ross JW, Ashworth MD, Johnson GA. Pig conceptuses secrete estrogen and interferons to differentially regulate uterine STAT1 in a temporal and cell type-specific manner. *Endocrinology* 2007; **148**(9):4420–4431.
10. Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Bazer FW, Johnson GA. Uterine MHC class I molecules and beta 2-microglobulin are regulated by progesterone and conceptus interferons during pig pregnancy. *J Immunol* 2008; **181**(4):2494–2505.
11. Kim M, Seo H, Choi Y, Shim J, Bazer FW, Ka H. Swine leukocyte antigen-DQ expression and its regulation by interferon-gamma at the maternal-fetal interface in pigs. *Biol Reprod* 2012; **86**(2):43.
12. Mantovani A. Chemokines. Introduction and overview. *Chem Immunol* 1999; **72**:1–6.
13. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; **354**(6):610–621.
14. Mackay CR. Chemokines: immunology's high impact factors. *Nat Immunol* 2001; **2**(2):95–101.
15. Luster AD. Chemokines–chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998; **338**(7):436–445.
16. Borroni EM, Bonecchi R, Buracchi C, Savino B, Mantovani A, Locati M. Chemokine decoy receptors: new players in reproductive immunology. *Immunol Invest* 2008; **37**(5–6):483–497.
17. Zlotnik A, Yoshie O, Nomiyama H. The chemokine and chemokine receptor superfamilies and their molecular evolution. *Genome Biol* 2006; **7**(12):243.
18. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, Gimotty PA, Gilks CB, Lal P, Zhang L, Coukos G. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells. *Nature* 2011; **475**(7355):226–230.
19. Salamonsen LA, Hannan NJ, Dimitriadis E. Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. *Semin Reprod Med* 2007; **25**(6):437–444.
20. Du MR, Wang SC, Li DJ. The integrative roles of chemokines at the maternal-fetal interface in early pregnancy. *Cell Mol Immunol* 2014; **11**(5):438–448.
21. Choi Y, Seo H, Han J, Yoo I, Kim J, Ka H. Chemokine (C-C motif) ligand 28 and its receptor CCR10: expression and function at the maternal-conceptus interface in pigs. *Biol Reprod* 2016; **95**(4):84–84.
22. Han J, Gu MJ, Yoo I, Choi Y, Jang H, Kim M, Yun CH, Ka H. Analysis of cysteine-X-cysteine motif chemokine ligands 9, 10, and 11, their receptor CXCR3, and their possible role on the recruitment of immune cells at the maternal-conceptus interface in pigs. *Biol Reprod* 2017; **97**(1):69–80.
23. Nagaoka K, Sakai A, Nojima H, Suda Y, Yokomizo Y, Imakawa K, Sakai S, Christenson RK. A chemokine, interferon (IFN)-gamma-inducible protein 10 kDa, is stimulated by IFN-tau and recruits immune cells in the ovine endometrium. *Biol Reprod* 2003; **68**(4):1413–1421.
24. Croy BA, Wessels J, Linton N, Tayade C. Comparison of immune cell recruitment and function in endometrium during development of epitheliochorial (pig) and hemochorial (mouse and human) placentas. *Placenta* 2009; **30**(Suppl A):26–31.
25. Janssens R, Mortier A, Boff D, Ruytinx P, Gouwy M, Vantilt B, Larsen O, Daugvilaite V, Rosenkilde MM, Parmentier M, Noppen S, Liekens S et al. Truncation of CXCL12 by CD26 reduces its CXC chemokine receptor 4- and atypical chemokine receptor 3-dependent activity on endothelial cells and lymphocytes. *Biochem Pharmacol* 2017; **132**:92–101.
26. Murdoch C. CXCR4: chemokine receptor extraordinaire. *Immunol Rev* 2000; **177**:175–184.
27. Oh JW, Drabik K, Kutsch O, Choi C, Tousson A, Benveniste EN. CXC chemokine receptor 4 expression and function in human astroglioma cells. *J Immunol* 2001; **166**:2695–2704.
28. Hanna J, Wald O, Goldman-Wohl D, Prus D, Markel G, Gazit R, Katz G, Haimov-Kochman R, Fujii N, Yagel S, Peled A, Mandelboim O. CXCL12 expression by invasive trophoblasts induces the specific migration of CD16- human natural killer cells. *Blood* 2003; **102**:1569–1577.
29. Wu X, Jin LP, Yuan MM, Zhu Y, Wang MY, Li DJ. Human first-trimester trophoblast cells recruit CD56brightCD16- NK cells into decidua by way of expressing and secreting of CXCL12/stromal cell-derived factor 1. *J Immunol* 2005; **175**:61–68.
30. Takashima A, Ishikawa F, Kuwabara T, Tanaka Y, Kinoshita T, Ito M, Kakiuchi T. Uterine natural killer cells severely decrease in number at gestation day 6 in mice. *Biol Reprod* 2013; **89**:101.
31. Ashley RL, Antoniazzi AQ, Anthony RV, Hansen TR. The chemokine receptor CXCR4 and its ligand CXCL12 are activated during implantation and placentation in sheep. *Reprod Biol Endocrinol* 2011; **9**:148.
32. Zhou WH, Du MR, Dong L, Yu J, Li DJ. Chemokine CXCL12 promotes the cross-talk between trophoblasts and decidual stromal cells in human first-trimester pregnancy. *Hum Reprod* 2008; **23**:2669–2679.
33. Lin Y, Xu L, Jin H, Zhong Y, Di J, Lin QD. CXCL12 enhances exogenous CD4+CD25+ T cell migration and prevents embryo loss in non-obese diabetic mice. *Fertil Steril* 2009; **91**:2687–2696.

34. Quinn KE, Ashley AK, Reynolds LP, Grazul-Bilska AT, Ashley RL. Activation of the CXCL12/CXCR4 signaling axis may drive vascularization of the ovine placenta. *Domest Anim Endocrinol* 2014; **47**:11–21.
35. Jeong W, Bae H, Lim W, Bazer FW, Song G. Differential expression and functional roles of chemokine (C-C motif) ligand 23 and its receptor chemokine (C-C motif) receptor type 1 in the uterine endometrium during early pregnancy in pigs. *Dev Comp Immunol* 2017; **76**:316–325.
36. Lim W, Bae H, Bazer FW, Song G. Cell-specific expression and signal transduction of C-C motif chemokine ligand 2 and atypical chemokine receptors in the porcine endometrium during early pregnancy. *Dev Comp Immunol* 2018; **81**:312–323.
37. Seo H, Kim M, Choi Y, Lee CK, Ka H. Analysis of lysophosphatidic acid (LPA) receptor and LPA-induced endometrial prostaglandin-endoperoxide synthase 2 expression in the porcine uterus. *Endocrinology* 2008; **149**:6166–6175.
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402–408.
39. Ka H, Jaeger LA, Johnson GA, Spencer TE, Bazer FW. Keratinocyte growth factor is up-regulated by estrogen in the porcine uterine endometrium and functions in trophectoderm cell proliferation and differentiation. *Endocrinology* 2001; **142**:2303–2310.
40. Kim J, Song G, Wu G, Bazer FW. Functional roles of fructose. *Proc Natl Acad Sci* 2012; **109**:E1619E1628.
41. Kim J, Song G, Wu G, Gao H, Johnson GA, Bazer FW. Arginine, leucine, and glutamine stimulate proliferation of porcine trophectoderm cells through the MTOR-RPS6K-RPS6-EIF4EBP1 signal transduction pathway. *Biol Reprod* 2013; **88**:113.
42. Raspotnig G, Fauler G, Jantscher A, Windischhofer W, Schachl K, Leis HJ. Colorimetric determination of cell numbers by Janus green staining. *Anal Biochem* 1999; **275**:74–83.
43. Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, Johnson GA, Bazer FW. Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. *Matrix Biol* 2010; **29**:369–382.
44. Johnson GA, Bazer FW, Burghardt RC, Spencer TE, Wu G, Bayless KJ. Conceptus-uterus interactions in pigs: endometrial gene expression in response to estrogens and interferons from conceptuses. *Soc Reprod Fertil Suppl* 2009; **66**:321–332.
45. Dominguez F, Galan A, Martin JJ, Remohi J, Pellicer A, Simon C. Hormonal and embryonic regulation of chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B in the human endometrium and the human blastocyst. *Mol Hum Reprod* 2003; **9**:189–198.
46. Barrientos G, Tirado-Gonzalez I, Freitag N, Kobelt P, Moschansky P, Klapp BF, Thijssen VL, Blois SM. CXCR4+ Dendritic cells promote angiogenesis during embryo implantation in mice. *Angiogenesis* 2013; **16**:417–427.
47. Quinn KE, Reynolds LP, Grazul-Bilska AT, Borowicz PP, Ashley RL. Placental development during early pregnancy: Effects of embryo origin on expression of chemokine ligand twelve (CXCL12). *Placenta* 2016; **43**:77–80.
48. Aiuti A, Tavian M, Cipponi A, Ficara F, Zappone E, Hoxie J, Peault B, Bordignon C. Expression of CXCR4, the receptor for stromal cell-derived factor-1 on fetal and adult human lymphohematopoietic progenitors. *Eur J Immunol* 1999; **29**:1823–1831.
49. Petit I, Jin D, Rafii S. The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. *Trends Immunol* 2007; **28**:299–307.
50. Holmes WD, Consler TG, Dallas WS, Rocque WJ, Willard DH. Solution studies of recombinant human stromal-cell-derived factor-1. *Protein Expr Purif* 2001; **21**:367–377.
51. Ray P, Lewin SA, Mihalko LA, Lesher-Perez SC, Takayama S, Luker KE, Luker GD. Secreted CXCL12 (SDF-1) forms dimers under physiological conditions. *Biochem J* 2012; **442**:433–442.
52. Veldkamp CT, Peterson FC, Pelzek AJ, Volkman BF. The monomer-dimer equilibrium of stromal cell-derived factor-1 (CXCL 12) is altered by pH, phosphate, sulfate, and heparin. *Protein Sci* 2005; **14**:1071–1081.
53. Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues Organs* 2002; **172**:202–217.
54. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res* 2010; **16**:2927–2931.
55. Piao HL, Tao Y, Zhu R, Wang SC, Tang CL, Fu Q, Du MR, Li DJ. The CXCL12/CXCR4 axis is involved in the maintenance of Th2 bias at the maternal/fetal interface in early human pregnancy. *Cell Mol Immunol* 2012; **9**:423–430.
56. Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol Rev* 2006; **214**:161–185.
57. Engelhardt H, Croy BA, King GJ. Evaluation of natural killer cell recruitment to embryonic attachment sites during early porcine pregnancy. *Biol Reprod* 2002; **66**:1185–1192.
58. Red-Horse K, Drake PM, Gunn MD, Fisher SJ. Chemokine ligand and receptor expression in the pregnant uterus: reciprocal patterns in complementary cell subsets suggest functional roles. *Am J Pathol* 2001; **159**:2199–2213.
59. Drake PM, Gunn MD, Charo IF, Tsou CL, Zhou Y, Huang L, Fisher SJ. Human placental cytotrophoblasts attract monocytes and CD56(bright) natural killer cells via the actions of monocyte inflammatory protein 1alpha. *J Exp Med* 2001; **193**:1199–1212.
60. Hasan SS, Tsaryk R, Lange M. Endothelial Notch signalling limits angiogenesis via control of artery formation. Nat Cell Biol 2017; **19**:928–940.
61. Xu J, Liang J, Meng YM, Yan J, Yu XJ, Liu CQ, Xu L, Zhuang SM, Zheng L. Vascular CXCR4 expression promotes vessel sprouting and sensitivity to sorafenib treatment in hepatocellular carcinoma. *Clin Cancer Res* 2017; **23**:4482–4492.
62. Zhang Q, Xu F, Shi Y, Chen YW, Wang HP, Yu X, Li Y. C-X-C motif chemokine receptor 4 promotes tumor angiogenesis in gastric cancer via activation of JAK2/STAT3. *Cell Biol Int* 2017; **41**:854–862.