Differential Regulation of CXCL5 by FGF2 in Osteoblastic and Endothelial Niche Cells Supports Hematopoietic Stem Cell Migration

Kyung-Ae Yoon,¹ Hye-Sim Cho,² Hong-In Shin,¹ and Je-Yoel Cho³

Stem cell maintenance requires a specific microenvironment. Hematopoietic stem cells (HSCs) are mainly maintained by the endosteal osteoblast (OB) niche, which provides a quiescent HSC microenvironment, and the vascular niche, which regulates the proliferation, differentiation, and mobilization of HSCs. The systemic administration of FGF2 failed to induce normal hematopoiesis in bone marrow (BM) by reducing SDF-1, an important factor for hematopoiesis. Interestingly, SDF-1 levels were decreased in the OBs, but increased in vascular endothelial C166 cells when FGF2 was administered. We hypothesized that FGF2 induces changes in HSC migration from BM; therefore, we investigated FGF2-induced factors of HSC migration by a microarray chip. We searched the genes that were decreased in primary OBs, but increased in C166 cells upon FGF2 treatment. We confirmed selected genes that function in the extracellular region and identified the CXCR2-related chemokine candidate LIX/*Cxcl5*. A chemotaxis assay showed that CXCL5 induced the migration of HSCs (CD34⁻/low LSK). Our data suggest that the differential regulation of the chemokine CXCL5 between OBs and endothelial cells upon FGF2 treatment is involved in HSC mobilization from the OB niche or BM to peripheral blood.

Introduction

THE STEM CELL NICHE CAN REGULATE the balance between stem cell quiescence and proliferation. The niche of adult stem cells is critical for tissue homeostasis and is formed by supporting cells that provide a microenvironment. Hematopoietic stem cell (HSC) self-renewal and differentiation are regulated by interactions with a variety of cells, including osteoblasts (OBs) and sinusoidal endothelial cells, in bone marrow (BM) [1–3]. Alterations of HSC niche function might induce pathological conditions, such as acute myeloid leukemia and myelodysplasia [4,5].

FGF2 plays important roles in the growth, differentiation, migration, and survival of a variety of cells; it also plays roles in hematopoiesis, angiogenesis, and bone formation [6,7]. Clinically, elevated levels of FGF2 have been seen in abnormal conditions, such as leukemia and some solid tumors [8]. It is also known that the chemokine SDF-1/CXCL12 directs the movement of various hematopoietic cells, including hematopoietic stem cells and progenitor cells (HSPCs), and regulates many hematopoietic responses including survival, proliferation, and migration, and homing [9,10]. Thus, it is

assumed that elevated FGF2 might also affect OB and endothelial cell function as stem cell niches for HSPCs.

In FGF2-administrated mice that show a phenotype similar to clonal myeloid disorders, the expression of SDF-1, a chemoattractant for HSC mobilization and homing, in BM stromal cells is significantly decreased along with other chemokines. However, several pieces of evidence support the theory that the SDF-1/CXCR4 axis may not be entirely required for mobilization and homing by HSCs [11,12]. Thus, angiocrine factors, including FGF2 in endothelial cells, might regulate HSCs expansion and differentiation. This evidence suggests that an altered HSC niche may induce an alternative HSC niche and eventually affect HSPC expansion, differentiation, and mobilization [13]. Alterations of SDF-1 by FGF2 might be partially responsible for this mobilization; however, SDF-1 may not be the sole factor responsible for the early mobilization of HSPCs induced by FGF2.

We studied the effect of FGF2 on *Sdf-1* mRNA expression and SDF-1 secretion between endosteal OBs (osteoblastic niche cells) and C166 endothelial cells (vascular niche cells). Furthermore, novel genes involved in the differential regulation of OB and the endothelial niche cells upon FGF2

¹Department of Oral Pathology, School of Dentistry, IHBR, Kyungpook National University, Daegu, Korea.

²ProtAnBio, Co., Daegu, Korea.

³Department of Veterinary Biochemistry, BK21 and Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul, Korea.

treatment were investigated by microarray analysis. We found that the CXCL5 chemokine is differentially regulated between OBs and endothelial cells and can initiate the migration of $CD34^{-/low}$ LSK. Furthermore, treating $CD34^{-/low}$ LSK (hematopoietic stem cell) with CXCL5 resulted in cellular migration. Upon FGF2 elevation, our data suggested that CXCL5 might act as a chemokine for $CD34^{-/low}$ LSK migration from the BM to the vascular niche.

Materials and Methods

Primary OB and BM cell isolation

Primary OBs were isolated according to a previously described protocol with a few modifications [14,15]. Briefly, femurs and tibiae from 8- to 12-week-old mice were removed and treated with 0.1% collagenase (GIBCO, Grand Island, NY) and 0.13% dispase (GIBCO) for 1 h to remove connective tissue and muscle from the periosteum. Epiphyses from the cleaned femurs and tibiae were removed and flushed. Marrow-depleted bones were chopped into fine bone fragments (1- to 2-mm fragments) and crushed with a mortar and pestle. The OBs from the femurs and tibiae were isolated by enzymatic digestion in 0.1% collagenase and 0.5% dispase for 90 min. Bone cells derived by enzymatic digestion were harvested by centrifugation and cultured in the minimum essential medium (MEM) Alpha Modification (Hyclone, South Logan, UT) containing 15% fetal bovine serum (FBS) (Hyclone) and 1% antibiotic-antimycotic (Anti-Anti, GIBCO). Cells isolated by the enzymatic treatment were termed enzymatic long bone-derived osteoblasts (ELBOSs). BM cells were isolated from femurs and tibiae by flushing with phosphate-buffered saline (PBS) containing 2% FBS. Red blood cells (RBCs) were removed using an RBC lysis buffer (Sigma-Aldrich, St. Louis, MO).

Cell culture

Mouse endothelial C166 cells were from the ATCC (American Type Culture Collection). C166 cells were cultured in the Dulbecco's modified Eagle's medium (DMEM/high glucose) (Hyclone) with 10% FBS and Anti-Anti.

RNA isolation and real-time reverse transcriptase-polymerase chain reaction

Total RNA was isolated from ELBOSs and C166 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (1.5 μ g) was reverse-transcribed using the Omniscript Reverse Transcription kit (QIAGEN, Valencia, CA). To measure mRNA levels, fluorescence-based real-time polymerase chain reaction (PCR) was performed with the DNA Engine OPTICON[®]2 system (Bio-Rad, Hercules, CA). SYBR green (Molecular Probe[®]; Invitrogen) and Go Taq[®] Flexi DNA polymerase (Promega, Madison, WI) were used for PCR reactions.

Alizarin red staining (bone nodule assays)

ELBOSs were plated at 7×10^4 cells per well in a 12-well plate and cultured with α -MEM containing 15% FBS and 1% Anti-Anti. Confluent ELBOSs were treated with ascorbic acid (Sigma-Aldrich; 100 μ g/mL) and 5 mM β -glycophosphate

(Sigma-Aldrich). After 21 days, mineralization of bone nodules was detected in cultured cells by alizarin red staining. The cells were washed twice with PBS, and fixed with 4% paraformaldehyde (PFA) for 1 h, and then stained with 0.4 M alizarin red S (AR-S; Sigma-Aldrich), pH 4.2, for 10 min at room temperature.

Western blot analysis

ELBOSs were lysed in a lysis buffer (RIPA buffer, 50 mM Tris-Cl[pH 8.0], 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF; ELPis, Daejeon, Korea) containing a protease cocktail (Roche, Mannheim, Germany). Equal amounts of cell lysate protein (μ g/lane) were subjected to SDS-PAGE and transferred to membranes. The membranes were incubated with primary antibodies (Runx2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Osteopontin, and N-cadherin (Iowa University)), overnight at 4°C, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were developed using a chemiluminescence detection system (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to an x-ray film.

Immunocytochemistry

Cells (7×10^4) were plated on glass slides before experiments. The cells were treated with 100% EtOH, followed by fixation in 4% PFA for 20 min (4°C). The fixed cells were permeabilized with 0.1% Triton-X in PBS and blocked with 5% goat serum in 0.1% Triton-X/PBS for 1 h at room temperature. Cells were treated with an Osteopontin antibody (0.023 μ g/ μ L; Iowa University) and incubated overnight in a moist chamber (4°C). The washed cells were incubated with an Alexa 594-conjugated anti-mouse secondary antibody (1:200 in 0.05% goat serum; Invitrogen) in a moist chamber for 1 h (RT). Slides were counterstained and mounted in a medium containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to stain the nuclei.

Enzyme-linked immunosorbent assay

ELBOS and C166 cells were seeded on 35-mm dishes. Semiconfluent cells were treated with FGF2 at various concentrations and for various amounts of times. The culture supernatant samples were collected after removing particulates by centrifugation. The enzyme-linked immunosorbent assay (ELISA) for the detection of murine SDF-1 (CXCL12) and LIX (CXCL5) in culture supernatants was performed using a mouse CXCL12/SDF-1 alpha Quantikine ELISA kit and CXCL5/LIX Duo set (R&D Systems, Minneapolis, MN).

Fluorescence-activated cell-sorting analysis

Whole BM cells were flushed from the femurs and tibiae of C57BL/6 mice using PBS supplemented with 2% FBS. The cells were filtered through a 45- μ m cell strainer to obtain individual cells. The cells were stained using Alexa Fluor[®] 488-conjugated lineage marker antibodies (mouse lineage mixture; CD3, CD11b, CD45R, Ly-6C/G, and Ter-119; Invitrogen, Camarillo, CA), PE-conjugated CD117 (cKit) (eBioscience, San Diego, CA), PE-Cy7 Sca-1 (BD Bioscience, San Jose, CA), and a brilliant violet-conjugated CD34

antibody (BD Bioscience) for 30 min on ice to sort HSCs. Flow cytometric experiments were performed using a FACS Aria II™ (BD Bioscience).

Migration assay

Sorted CD34⁻/low LSK cells were suspended in the RPMI medium (Hyclone) with 10% FBS. Cells were placed on the top well of a transwell for migration assays (BD Falcon, Franklin Lakes, NJ). A medium containing murine CXCL5 or SDF-1/CXCL12 (PEPROTECH, Rocky Hill, NJ) was added to the bottom well and the cells were allowed to migrate for 3 h at 37°C. The percentage of migration was calculated by dividing the number of cells in the bottom well by the total cell input multiplied by 100.

Microarray

Cultured ELBOS and C166 cells were treated with FGF2 (20 ng/mL) for 48 h. Total RNA was isolated from FGF2-treated cells using an RNeasy RNA isolation kit (QIAGEN) according to the manufacturer's protocol. RNA 6000 Nano-chips were used to determine the concentration and purity/integrity of RNA samples using a Nanodrop ND-1000 and Agilent 2100 Bioanalyzer. Gene expression profiling was performed using a Mouse Gene 1.0 ST array system (Affymetrix, Santa Clara, CA).

Statistics

Data were expressed as mean ± standard deviation (SD). For statistical analysis, a *P*-value calculator for the Stu-

dent's *t*-test was used. The group results were subjected to statistical analysis with the Student's *t*-test to assess differences compared to the control group. A probability (*P* value) less than 0.05 was considered to be statistically significant.

Results

ELBOSs have the characteristics of osteoblastic cells

To study the role of OBs on the migration of HSCs, osteoblastic cells were obtained through the enzymatic digestion of mice long bones (femurs and tibiae) after removing the BM. These cells were termed ELBOSs. Long bone-derived OBs acquired from long bone showed characteristics of osteoblastic niche cell that support or maintain hematopoietic progenitor cells [14,15]. We confirmed that the isolated ELBOSs expressed various osteogenic makers, including Osteopontin, Osterix, *Runx2*, Collagen type I, and Alkaline phosphatase (*Alp*) at high levels compared with the whole BM (WBM) (Fig. 1A–E). We also examined these gene expressions and compared to MC3T3E1 cells, in which the levels of these gene expressions were not different from ELBOS cells (data not shown). The ELBOSs were able to mineralize bone under an osteogenic differentiation condition for 21 days, as demonstrated by Alizarin Red S staining (Fig. 1F). Taken together, these data indicate that the ELBOSs demonstrate both osteogenic maker gene expression and the functional properties of osteoblastic cells.

FIG. 1. The osteoblast phenotype of enzymatic long bone-derived osteoblasts (ELBOSs). (A–E) To confirm the expression of osteoblast maker genes, quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analyses of mRNAs isolated from ELBOSs were compared with those from whole bone marrow (BM) cells. The relative expression levels of Osteopontin (A), Osterix (B), *Runx2* (C), type I collagen (D), and alkaline phosphatase (E) to *Gapdh* are presented. (F) ELBOS were cultured in the osteogenic medium (OM) (ascorbic acid: 50 ng/mL; 5 mM β -glycophosphate) for 21 days to induce calcium deposition. After osteogenic induction, Alizarin red staining was performed to show nodule formation in ELBOSs. Control cells were cultured in the growth medium (GM) for 21 days. **P* < 0.05 compared with control.

Expression of HSC niche-related genes and proteins in the ELBOs

OBs are also important in the regulation of hematopoiesis and HSPC proliferation. Therefore, we next tested whether these ELBOs also expressed niche-related genes and proteins. Expression levels of various niche-related genes, including stromal cell-derived factor 1 (*Sdf-1*)/*Cxcl12*, stem cell factor (*Scf*), N-cadherin (*N-cad*), and Jagged-1 (*Jag-1*) were significantly high in ELBOs compared to BM (Fig. 2A). We also checked niche genes in the established preosteoblast cell line, MC3T3-E1 cells, and compared the levels of the genes to ELBOs (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/scd). These genes, which are expressed in OB and/or sinusoidal endothelial cells,

are known to affect HSC mobilization. These ELBOs also expressed the osteoblastic niche-related proteins Runx2, N-cadherin, and Opn (Fig. 2B). High levels of *Runx2* in OBs are known to promote HSPC properties [16]. Conditional *Bmpr1a*-deficient mice that can inducibly delete *Bmpr1a* in bone-marrow stroma display an increased number of N-cadherin-positive OBs and HSCs [1]. Osteopontin (Opn), an acidic glycoprotein produced by OBs, is a known useful marker for HSC-interacting OB cells [17,18]. These OPN-positive cells play a role in HSC proliferation and mobilization in the cyclophosphamide/granulocyte colony-stimulating factor (G-CSF)-induced state. Opn proteins were expressed in most ELBOs, as confirmed by immunocytochemistry (Fig. 2C). These results demonstrated that ELBOs have the properties of osteoblastic niche cells.

FIG. 2. Expression of hematopoietic stem cell (HSC) niche-related genes and proteins in ELBOs. **(A)** Total RNA was isolated from ELBOs. The mRNA expression levels of *Sdf-1* (*Cxcl12*), *Scf*, *Jag-1*, and *N-cad* relative to *Gapdh* were analyzed by qRT-PCR and presented as ratios to the BM's relative values. **(B)** The expression levels of HSC niche-related proteins, including Runx2, N-cad, and Opn were confirmed by western blot in the total lysates of ELBOs (1, 2, and 3 indicate 3 different ELBOs lysates from 3 different mice). **(C)** ELBOs were stained using an Opn antibody. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole. * $p < 0.05$ compared with control (BM).

Effects of FGF2 on the expression of HSC niche-related genes and the secretion of SDF-1/CXCL12 in ELBOS and endothelial C166 cells

The systemic administration of FGF2 fails to induce normal hematopoiesis in BM through the reduced expression of SDF-1 *in vivo* [19]. We investigated whether FGF2 could regulate the expression of the HSC niche-related genes *Sdf-1*, *N-cad*, *Scf*, and *Jag-1* in ELBOSs and C166 in endothelial niche cells. The expression levels of *Sdf-1* and *Jag-1* were decreased in ELBOSs upon FGF2 treatment (Fig. 3A). However, *Scf* mRNA expression was increased in ELBOSs in a dose-dependent manner, and *N-cad* was slightly increased, at an FGF2 concentration of 80 ng/mL (Fig. 3A). The expression patterns of *Sdf-1*, *Scf*, *N-cad*, and *Jag-1* were similar in FGF2-treated MC3T3-E1 cells, when compared to ELBOSs (Supplementary Fig. S1B). The expression levels of *Sdf-1* and *Jag-1* mRNAs were significantly decreased in a time-dependent manner until after 24 h of FGF2 treatment (Fig. 3B). However, the expression

levels of the *Opn* mRNAs were significantly increased in the ELBOSs compared with the non-FGF2-treated control cells (Fig. 3C). When measured up to 72 h after the treatment using an SDF-1-specific ELISA, the secretion of SDF-1 protein in the culture medium of ELBOS cells was significantly decreased even at a dosage of 20 ng/mL FGF2 (Fig. 3D, E).

Interestingly, the expression levels of *Sdf-1* and *Jag-1* were increased in endothelial C166 cells upon FGF2 treatment (Fig. 4A). The secretion of SDF-1 protein was also increased in the culture medium of C166 cells during FGF2 treatment (Fig. 4B, C). The responses of stem cell niche-related SDF-1 protein and *Jag-1* gene expression levels to FGF2 treatment in endothelial C166 cells were increased opposite to those of the ELBOSs. We speculated that the FGF2 treatment led to an altered HSC niche migration microenvironment in BM through an opposite pattern that decreased in ELBOS, but increased in C166 cells of HSC niche-related gene expression and SDF-1 secretion between the ELBOS niche and the C166 endothelial niche cells.

FIG. 3. Altered expression of HSC niche-related genes and SDF-1 secretion by FGF2 in ELBOSs. **(A)** Confluent ELBOSs were cultured in 2% fetal bovine serum (FBS) medium (starvation conditions). The starved ELBOSs were treated with FGF2 (20 or 80 ng/mL) for 48 h. The expression levels of the HSC niche-related genes *Sdf-1* (*Cxcl12*), *N-cad*, *Scf*, and *Jag-1* relative to *Gapdh* were analyzed by qRT-PCR. **(B and C)** The starved ELBOSs were treated with FGF2 (20 ng/mL). Total RNA was isolated at the indicated times. Levels of *Sdf-1* (**B**), *Jag-1* (**B**), and *Opn* (**C**) expression were analyzed by qRT-PCR. The expression levels of each mRNA relative to *Gapdh* were compared with the untreated controls at each time point. **(D)** ELBOSs were cultured in a medium containing FGF2 at various concentrations (0, 20, 40, 80 ng/mL) for 48 h. SDF-1 protein in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). **(E)** SDF-1 protein levels were also measured in ELBOSs treated with 20 ng/mL of FGF2 for the given time periods. * $P < 0.05$ compared with control (**A**, **B**, **C**, **E**) or no treatment (**D**).

FIG. 4. Altered expression of HSC niche-related genes and SDF-1 secretion by FGF2 in C166 cells. **(A)** Confluent C166 cells were cultured in 2% FBS medium for starvation. The starved C166 cells were treated with FGF2 (20 ng or 80 ng/mL) for 48 h. The expression levels of the HSC niche-related genes *Sdf-1* (*Cxcl12*) and *Jag-1* relative to *Gapdh* were analyzed by qRT-PCR. **(B)** C166 cells were cultured in a medium with FGF2 at various concentrations (0, 20, 40, 80 ng/mL) for 48 h. SDF-1 levels in culture supernatants were measured by ELISA. **(C)** Confluent C166 cells were cultured in a medium with FGF2 (20 ng/mL). The culture supernatants were harvested at the indicated times. SDF-1 secretion was measured by ELISA. * $P < 0.05$ compared with control (**A**, **C**) or no treatment (**B**).

Effects of FGF2 on the gene expression profiles of the ELBOS and C166 cells

The recruitment or migration of CXCR4-expressing HSC from the osteoblastic niche to the vascular niche might depend on the SDF-1 chemokine. However, recently reported data suggest that SDF-1 alone cannot support the relationship between the OB/vascular niche and HSC migration. To

identify other candidate chemokine factors that are differentially influenced by FGF2 between ELBOSs, (osteoblastic niche cells) and C166 cells (endothelial niche cells), microarray analyses were performed using total RNA from ELBOSs or C166 cells grown in the presence or absence of FGF2 for 48 h. Table 1 displays a subset of selected genes that show the differential regulation by FGF2. We focused on genes upregulated by FGF2 in C166 cells, but downregulated in ELBOSs, comparing the expression levels with those of untreated cells. We then selected genes whose coding proteins are localized to the extracellular or membrane region and in the BM. Some candidate genes, including R-spondin 2 homolog (*Rspo2*), Chemokine (CXC motif) ligand 5 (*Cxcl5*), Matrix metalloproteinase 13 (*Mmp13*), Haptoglobin (*Hp*), and Clusterin (*Clu*), were confirmed based on their differential expression levels when using real-time reverse transcriptase-PCR. The expression of these genes was significantly increased in the FGF2-treated C166 cells, whereas they were decreased in the FGF2-treated ELBOSs (Fig. 5A–E). Among the listed genes, we focused on the chemokine candidate *Cxcl5* (also known as LIX, lipopolysaccharide-induced CXC chemokine).

Chemokine CXCL5 is differentially regulated by FGF2 between ELBOSs and endothelial cells

Many chemokines, including SDF-1 and G-CSF, have been reported to mobilize HSPCs or differentiated blood cells [20,21]. G-CSF is reported to induce HSC and progenitor cell mobilization through a decrease of SDF-1 in OBs. FGF2 also

TABLE 1. SELECTED GENES SHOWING AN INCREASE IN C166 CELLS, BUT A DECREASE IN ENZYMIC LONG BONE-DERIVED OSTEOBLASTS DUE TO FGF2 TREATMENT

Localization	Expression ratio (Fold increase in C166/Fold decrease in ELBOS)	Gene symbol	Gene description
Extracellular region	28.191	<i>Rspo2</i>	R-spondin 2 homolog (<i>Xenopus laevis</i>)
	27.233	<i>Penk</i>	Preproenkephalin
	14.638	<i>Cxcl5</i>	Chemokine (C-X-C motif) ligand 5
	10.924	<i>Igsf10</i>	Immunoglobulin superfamily, member 10
	9.185	<i>Mmp13</i>	Matrix metalloproteinase 13
	9.172	<i>Cpxm1</i>	Carboxypeptidase × 1 (M14 family)
	7.658	<i>Hp</i>	Haptoglobin
	5.934	<i>Isg15</i>	ISG15 ubiquitin-like modifier
	5.457	<i>Clu</i>	Clusterin
	4.633	<i>Rspo3</i>	R-spondin 3 homolog (<i>Xenopus laevis</i>)
	2.647	<i>Crisp2</i>	Cysteine-rich secretory protein 2

The lists and ratios were calculated from a microarray study. ELBOS, enzymatic long bone-derived osteoblasts.

FIG. 5. Gene expression levels altered by FGF2 were confirmed by qRT-PCR in ELBOSs and C166 cells. Confluent ELBOSs and C166 cells were treated with or without FGF2 (20 ng/mL). Total RNAs were isolated from FGF2-treated and untreated cells at 48 h. The mRNA expression levels of *Rspo2* (A), *Cxcl5* (B), *Mmp13* (C), *Hp* (D), and *Clu* (E) relative to *Gapdh* were analyzed by qRT-PCR in FGF2-treated ELBOSs and C166 cells. * $P < 0.05$ compared with control.

decreases SDF-1 expression in BM stromal cells [22,23]. Therefore, we next investigated *Cxcl5* expression changes induced by FGF2 in ELBOS and C166 cells. When FGF2 was used at various concentrations (0–80 ng/mL) for 48 h, *Cxcl5* was dramatically decreased in ELBOSs, but increased in C166 cells (Fig. 6A, B). The effects of FGF2 concentration were somewhat different on the gene expression levels between ELBOSs and C166 cells. Low concentration of FGF2 (20 ng/mL) had an effect on the ELBOSs. However, C166 cells required a higher concentration to induce high levels of gene expression changes (Fig. 6A). The expression level of *Cxcl5* in ELBOSs (20 ng/mL FGF2) was slightly increased at 3 h, and then significantly decreased from 6–48 h. In the case of C166 cells (80 ng/mL FGF2), *Cxcl5* expression was gradually increased from 6–48 h (Fig. 6B). We also investigated the secreted levels of CXCL5 protein in the culture medium of C166 cells and ELBOSs during FGF2 treatment. In the ELBOS culture medium, secreted CXCL5 protein was significantly decreased at various dosages of FGF2 in a time-dependent manner (Fig. 7A, B). However, FGF2 treatment incrementally induced CXCL5 protein expression in the culture medium of C166 cells in a dose- and time-dependent manner (Fig. 7C, D). Taken together, these data showed that the FGF treatment exerted opposing effects on ELBOSs and C166 endothelial cells with regard to the expression patterns of CXCL5. These data also showed that FGF2-induced HSC niche cells, OBs, and endothelial cells could alter CXCL5 expression. These changes might facilitate the migration of HSCs from the osteoblastic niche to the vascular niche.

The migration of $CD34^{-/low}$ LSK in response to CXCL5

CXC chemokines mediate their effects by interacting with 7-transmembrane G-protein-coupled receptors [24,25]. ECR⁺ CXC chemokine, CXCL1, CXCL2, CXCL5, and CXCL5 all interact with CXCR2. To verify the expression of CXCR2 in

FIG. 6. *Cxcl5* mRNA levels were decreased by FGF2 in ELBOSs, but increased in C166 cells. (A) ELBOSs and C166 cells were treated with FGF2 at the given concentrations. *Cxcl5* levels relative to *Gapdh* were analyzed by qRT-PCR. (B) FGF2-treated ELBOSs (20 ng/mL) and C166 cells (80 ng/mL) were harvested to isolate RNAs at the indicated times. *Cxcl5* levels relative to *Gapdh* were compared with the control at each time point by qRT-PCR. * $P < 0.05$ compared with no treatment (A) or control (B).

FIG. 7. CXCL5 secretion was decreased in ELBOs, but not in C166 cells by FGF2 treatment. (A and C) ELBOs and C166 cells were cultured in a medium containing FGF2 at various concentrations (0, 20, 40, 80 ng/mL) for 48 h. CXCL5 protein in culture supernatants was measured by ELISA. (B and D) CXCL5 protein levels were also measured in ELBOs (20 ng/mL) and C166 cells (80 ng/mL) treated with FGF2 for the given time periods. * $P < 0.05$ compared with no treatment (A, C) or control (B, D).

HSPCs, we sorted to enrich HSPCs (CD45⁺Lin⁻ cells) from BM cells [1–3]. PCR analysis showed an expression of *Cxcr2* in sorted HSPCs and WBM, which served as a positive control (Fig. 8A, B). Various chemokine receptors, including CCR3, CCR9, and CXCR4, are expressed in HSCs [26]. We next investigated the migration of CD34⁻LSK cells to CXCL5 using transwell culturing. Sorted CD34⁻LSK cells were added to the top chamber of a transwell, and the CXCL5-containing medium (0–1000 ng/mL) was placed in the bottom well. High concentration (1 μ g/mL) of CXCL5 was treated for the maintenance of HSC [27]. CD34⁻LSK cells, which migrated in response to CXCL5, were collected and counted from the bottom well (Fig. 8D). CXCL5 induced an increase of CD34⁻LSK cells migration in a dose-dependent manner, whereas the percentage of cell migration was not significantly increased at the highest concentration (1000 ng/mL) used. These data indicated that CXCL5, as well as SDF-1, was elevated in vascular endothelial cells by FGF2, which might lead to an induction of CD34⁻LSK migration from the ELBOs niche cells, where CXCL5 is decreased by FGF2.

Discussion

The stem cell niche, which is composed of a specialized population of cells, provides external cues to regulate adult stem cell self-renewal and differentiation [28,29]. The HSC niche consists of complex cellular cells, including OBs (osteoblastic niche), CXCL12-abundant reticular cells, nestin-positive mesenchymal stem cells (MSCs), and sinusoidal endothelial cells (vascular niche) in BM [30]. In this study, we demonstrated that SDF-1 is differentially regulated by FGF2 in osteoblastic ELBOs niche cells and endothelial niche cells. Furthermore, by microarray approaches, we also found that CXCL5 is downregulated in ELBOs, but upregulated in endothelial niche cells by FGF2.

In a steady state/untreated niche, OBs control the HSC number and negatively regulate HSC expansion or maintain quiescence. However, OBs are induced to a more active state in an activated niche or a cytokine-induced state by irradiation, bleeding, etc. In this study, we used endosteal OB, ELBOs cells from mouse long bones. We showed that these cells have osteoblastic characteristics in terms of the marker

expression and bone nodule formation by osteogenic induction. Primary OBs derived from both long bone and neonatal calvariae could support and enhance hematopoietic properties [15,31]. Recently, it was reported that *Runx2*, a master transcription factor for OB differentiation, is important to enhance or maintain for HSC function and properties, and its expression level is high at the early stage of OB differentiation or in freshly isolated OB [16,32]. *Runx2* protein was abundant in ELBOs without any stimulation, and the primary OBs derived from long bone tissues. Stage-specific markers for OBs may be required or utilized for detailed stage-wise sorting. However, the characteristics of the osteolineage cell population that support HSC have been controversial. Recent data showed MSCs and more mature osteoblastic cells also provide HSC support in HSC regulation within the BM microenvironment [1,33]. It was also reported that multiple osteoblastic lineage cells could also support HSCs at least in vitro [31,34].

Endothelial cells play essential roles as niche cells in HSC proliferation, differentiation, and mobilization [35]. We used C166 mouse endothelial cells as vascular niche cells, which are known to have endothelial characteristics, cobblestone morphology. The C166 cells rearrange into tube-like structures on Matrigel and express Vcam-1 (vascular cell adhesion molecular 1), Meca-99. The cells thus are known to support proliferation of primitive HSCs or HSPCs [36,37]. It was difficult to acquire and culture pure primary endothelial cells from mouse tissues. However, there are several mouse endothelial cell lines established from various tissues, including axillary lymph node [38–40], embryonic yolk sacs [37], and aorta, brain, and heart capillaries [41]. These cells were demonstrated to be used as tools for in vitro studies regarding endothelial cell function.

We selected C166, mouse endothelial cells. As stated above, C166 cells could support hematopoietic progenitor and stem cells. We could not use human endothelial cells, such as the human umbilical vein endothelial cell, because we also performed microarray comparison between mouse endothelial cells and ELBOs to discover new candidate genes for HSC migration-affecting gene identification. FGF2 has an effect on hematopoiesis through SDF-1 reduction in bone marrow stromal cells in vitro or in vivo [19,22]. FGF2

FIG. 8. CXCL5 induces CD34^{-/low} LSK migration. **(A)** Representative FACS dot graph of CD45-positive and lineage-negative cells (Q1) isolated from BM. **(B)** The expression of *Cxcr2*, the chemokine receptor of CXCL5 in HSPCs (sorted CD45⁺Lin⁻ cells from BM), mRNA compared with whole BM (WBM) as a control. **(C)** FACS analysis for CD34^{-/low} LSK cells (bottom panel: P7) on bone marrow mononuclear cells. **(D)** Sorted CD34^{-/low} LSK cells from BM were added to the top well, which is on the bottom well containing medium alone (0.1% bovine serum albumin), or CXCL5, at the given concentrations (0-1000 ng/mL). CD34^{-/low} LSK cells migrating to the bottom well were counted and are presented as the percentage of cells input to the top wells. **P* < 0.05 compared with no treatment.

strongly inhibited SDF-1 secretion, and the expression of other FGFs, FGF4, 7, 9, 16, 19 also decreased SDF-1 in bone marrow stromal cells [22]. Mice that received systemic administration of FGF2 showed characteristics of clonal myeloid disorders, which are characterized by a reduced BM cellularity, an induced immature myeloid cell mobilization, extramedullary hematopoiesis, thickened bone trabeculae, and increased BM vascularization with dilation of sinusoids. Increased SDF-1 expression induced by FGF2-regulated signaling in endothelial cells might also regulate angiogenesis [42].

Soluble chemokines or cytokine molecules involved in cell migration include SDF-1 [43,44] and G-CSF [45]. The chemokine and angiocrine factors in endothelial cells are also important in balancing the self-renewal and differentiation of HSCs and migration [13]. Our data showed that the expression levels of *Sdf-1* and *Jag-1* mRNAs were significantly decreased in ELBOs, but increased in C166 cells by FGF2. Our data are also supported by a report that SDF-1 levels are gradually decreased during OB differentiation [46]. Recent reports also showed that FGF-2 mediates in vivo expansion of murine HSPCs via proliferation of their supportive

stromal cells by reducing CXCL12 [47]. FGF2 also slightly increased the mRNA levels of other genes expressed in OBs, including *N-cad* and *Scf*, in ELBOs. These results were similar to those from a previous report, where the loss of *Sdf-1* in the BM stroma of adult mice induced a decrease of *Jag-1* and an increase of *N-cad* [48]. The secretion level of FGF2-induced SDF-1 in the ELBOS culture medium was decreased; this was not the case in the C166 culture medium. Thus, we could mimic the effect of FGF2 on OB and endothelial cells in BM in vitro.

SDF-1 is not solely responsible for HSC mobilization upon FGF2 elevation. Thus, in this study, we searched for other chemokines, especially those differentially regulated in ELBOS and C166 cells upon FGF2 treatment, using a microarray assay. We determined that genes that were decreased in ELBOs, but increased in endothelial cells by FGF2 treatment included *Rspo 2* and *3*, *Cxcl5*, *Mmp13*, *Hp* and *Clu*.

Rspo2 and *Rspo3* are secreted glycoproteins containing cysteine-rich domains and a thrombospondin type I domain that is related to the regulation of the Wnt signaling pathway [49]. *Rspo2* knockout mice exhibit limb loss and lung hypoplasia, and *Rspo3* knockout mice have embryonic vascular deficiencies. *Rspo3* signaling is required to support endothelial cell growth and remodeling [50,51]. Thus, combined with our microarray data, it is feasible that FGF2 induction and the increased niche function of endothelial cells by FGF2 induction might also, in part, be due to the supportive role of *Rspo3* and *2* in cell growth and remodeling.

CXCL5 belongs to the ELR⁺ (glutamic acid-leucine-arginine)⁺ CXC chemokines. CXCL5 is associated with neutrophil influx into inflamed tissues [25,52] and with neutrophil homeostasis. This chemokine also has a proliferative effect on HSC maintenance [27]. CXCL5 binds to the receptor CXCR2 on the cell membrane. In mice, in addition to CXCL5 (also known as LIX, lipopolysaccharide-induced CXC chemokine), three other ELR⁺ members, including CXCL1 (also known as KC, keratinocyte-derived chemokines), CXCL2 (also known as MIP, macrophage inflammatory protein-2), and CXCL15 (also known as lungkine) also interact with the CXCR2 receptor. Other ELR⁺CXC chemokines, CXCL1 and CXCL2, were expressed in mature OBs (Col2.3 OB) and endothelial cells from BM; their increased expression in endothelial cells was not decreased in ELBOs upon FGF2 treatment. G-CSF, which is widely administered as a clinical mobilizer, is induced upon HSC mobilization in mice and induced an increase in *Cxcl2* mRNA and a decrease of *Sdf-1* in bone marrow endothelial cells [53]. Thus, CXCL2 might also enhance HSC mobilization when combined with G-CSF. Our data showed that CXCL5 expression and secretion was significantly increased by FGF2 in C166 cells in a dose- and time-dependent manner, whereas it was decreased in ELBOs (Figs. 6 and 7). More importantly, sorted HSPCs (CD45⁺Lin⁻ cells) were expressed CXCR2 and sorted CD34⁻/low LSK migrated to the chamber containing CXCL5 (Fig. 8). Together, our data strongly suggest that the CXCL5/CXCR2 axis might also be involved in HSC homing and/or mobilization. In the future, we plan to develop an OB stage-specific CXCL5 knockout and to investigate HSC mobilization upon FGF2 treatment in vivo. However, before the mouse model is developed, our current microarray discovery and in vitro validation shows the possibility of a role for CXCL5 as a HSC and HSPC chemokine responding to FGF2.

It is known that FGF2 levels can be abnormally increased in pathological situations, including leukemia, myeloid metaplasia, and other clonal myeloid disorders [8,54]. In the BM of patients with clonal myeloproliferation, several characteristics, including an increase in the number of stromal cells and increases in osteosclerosis and angiogenesis, have been demonstrated [55,56]. BMSCs (bone marrow stromal cells) and MC4 OBs have an induced osteocyte-like phenotype due to long-term FGF2 treatments [57]. Because it is generally accepted that FGF2 increases OB populations and promotes early OB differentiation [58,59], it is likely that elevated FGF2 levels might suppress stem cell niche function because FGF2 decreases SDF-1 and CXCL5 secretion in ELBOs, and thus releases HSCs to the vascular niche environment.

FGFs bind and activate four tyrosine kinase FGF receptors (FGFR1-4), which are alternatively spliced forms [60]. FGF2 binds some alternative splicing FGFR1-3 and FGFR4 except FGFR2IIIb and FGFR3IIIb [61,62]. We confirmed FGFRs expression patterns in ELBOs and C166 cells to explain different responses of SDF-1 and CXCL5 to FGF2 treatment. All FGFR types (Fgfr1-3) were expressed except Fgfr4 in ELBOs and C166 cells (Data not shown). In summary, the FGF2-induced OB and endothelial niche environment (activated niche/cytokine induced) were similar to the abnormal microenvironment that gives rise to the pathological conditions, such as clonal myeloid disorders. These conditions supported HSCs or HSPCs migration through downregulating in ELBOs and upregulating in C166 cells the chemokines SDF-1 and CXCL5 in both the osteoblastic niche and the vascular endothelial niche. The FGF2-activated niche/cytokine-induced niche could induce a change in chemokine expression in OBs and endothelial cells, and then support HSCs or HSPCs migration more than a steady state/untreated niche.

Acknowledgments

This work was supported by grants from the National Research Foundation of Korea (NRF), which was funded by the Ministry of Education, Science and Technology (no. 20110019355 & 2009-0077615).

Author Disclosure Statement

No competing financial interests exist.

References

1. Zhang J, C Niu, L Ye, H Huang, X He, WG Tong, J Ross, J Haug, T Johnson, et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425:836–841.
2. Calvi LM, GB Adams, KW Weibrecht, JM Weber, DP Olson, MC Knight, RP Martin, E Schipani, P Divieti, et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425:841–846.
3. Kiel MJ and SJ Morrison. (2006). Maintaining hematopoietic stem cells in the vascular niche. *Immunity* 25:862–864.
4. Lane SW, DT Scadden and DG Gilliland. (2009). The leukemic stem cell niche: current concepts and therapeutic opportunities. *Blood* 114:1150–1157.
5. Raaijmakers MH, S Mukherjee, S Guo, S Zhang, T Kobayashi, JA Schoonmaker, BL Ebert, F Al-Shahrour, RP Hasser-

- jian, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 464:852–857.
6. Bikfalvi A, S Klein, G Pintucci and DB Rifkin. (1997). Biological roles of fibroblast growth factor-2. *Endocr Rev* 18:26–45.
7. Thisse B and C Thisse. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev Biol* 287:390–402.
8. Nguyen M, H Watanabe, AE Budson, JP Richie, DF Hayes and J Folkman. (1994). Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. *J Natl Cancer Inst* 86:356–361.
9. Aiuti A, IJ Webb, C Bleul, T Springer and JC Gutierrez-Ramos. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 185: 111–120.
10. Lataillade JJ, D Clay, C Dupuy, S Rigal, C Jasmin, P Bourin and MC Le Bousse-Kerdiles. (2000). Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood* 95:756–768.
11. Pelus LM and S Fukuda. (2006). Peripheral blood stem cell mobilization: the CXCR2 ligand GROβ rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Exp Hematol* 34:1010–1020.
12. Christopherson KW, 2nd, G Hangoc, CR Mantel and HE Broxmeyer. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science* 305:1000–1003.
13. Kobayashi H, JM Butler, R O'Donnell, M Kobayashi, BS Ding, B Bonner, VK Chiu, DJ Nolan, K Shido, L Benjamin and S Rafii. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* 12:1046–1056.
14. Mayack SR and AJ Wagers. (2008). Osteolineage niche cells initiate hematopoietic stem cell mobilization. *Blood* 112: 519–531.
15. Balduino A, SP Hurtado, P Frazao, CM Takiya, LM Alves, LE Nasciutti, MC El-Cheikh and R Borojevic. (2005). Bone marrow subendosteal microenvironment harbours functionally distinct haemosupportive stromal cell populations. *Cell Tissue Res* 319:255–266.
16. Chitteti BR, YH Cheng, DA Streicher, S Rodriguez-Rodriguez, N Carlesso, EF Srouf and MA Kacena. Osteoblast lineage cells expressing high levels of Runx2 enhance hematopoietic progenitor cell proliferation and function. *J Cell Biochem* 111:284–294.
17. Nilsson SK, HM Johnston, GA Whitty, B Williams, RJ Webb, DT Denhardt, I Bertoncello, LJ Bendall, PJ Simmons and DN Haylock. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* 106:1232–1239.
18. Stier S, Y Ko, R Forkert, C Lutz, T Neuhaus, E Grunewald, T Cheng, D Dombkowski, LM Calvi, SR Rittling and DT Scadden. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J Exp Med* 201:1781–1791.
19. Nakayama T, N Mutsuga and G Tosato. (2007). Effect of fibroblast growth factor 2 on stromal cell-derived factor 1 production by bone marrow stromal cells and hematopoiesis. *J Natl Cancer Inst* 99:223–235.
20. Levesque JP, J Hendy, Y Takamatsu, PJ Simmons and LJ Bendall. (2003). Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by G-CSF or cyclophosphamide. *J Clin Invest* 111:187–196.
21. Pelus LM, D Horowitz, SC Cooper and AG King. (2002). Peripheral blood stem cell mobilization. A role for CXC chemokines. *Crit Rev Oncol Hematol* 43:257–275.
22. Nakayama T, N Mutsuga and G Tosato. (2007). FGF2 post-transcriptionally down-regulates expression of SDF1 in bone marrow stromal cells through FGFR1 IIIc. *Blood* 109: 1363–1372.
23. Yoon KA, YM Chae and JY Cho. (2009). FGF2 stimulates SDF-1 expression through the Erm transcription factor in Sertoli cells. *J Cell Physiol* 220:245–256.
24. Murphy PM. (1996). Chemokine receptors: structure, function and role in microbial pathogenesis. *Cytokine Growth Factor Rev* 7:47–64.
25. Kobayashi Y. (2006). Neutrophil infiltration and chemokines. *Crit Rev Immunol* 26:307–316.
26. Wright DE, EP Bowman, AJ Wagers, EC Butcher and IL Weissman. (2002). Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J Exp Med* 195:1145–1154.
27. Choong ML, YP Yong, AC Tan, B Luo and HF Lodish. (2004). LIX: a chemokine with a role in hematopoietic stem cells maintenance. *Cytokine* 25:239–245.
28. Yin T and L Li. (2006). The stem cell niches in bone. *J Clin Invest* 116:1195–1201.
29. ter Huurne M, CG Figdor and R Torensma. Hematopoietic stem cells are coordinated by the molecular cues of the endosteal niche. *Stem Cells Dev* 19:1131–1141.
30. Kunisaki Y and PS Frenette. (2012). The secrets of the bone marrow niche: Enigmatic niche brings challenge for HSC expansion. *Nat Med* 18:864–865.
31. Chitteti BR, YH Cheng, B Poteat, S Rodriguez-Rodriguez, WS Goebel, N Carlesso, MA Kacena and EF Srouf. (2010). Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function. *Blood* 115:3239–3248.
32. Cheng YH, BR Chitteti, DA Streicher, JA Morgan, S Rodriguez-Rodriguez, N Carlesso, EF Srouf and MA Kacena. (2011). Impact of maturational status on the ability of osteoblasts to enhance the hematopoietic function of stem and progenitor cells. *J Bone Miner Res* 26:1111–1121.
33. Mendez-Ferrer S, TV Michurina, F Ferraro, AR Mazloom, BD Macarthur, SA Lira, DT Scadden, A Ma'ayan, GN Enikolopov and PS Frenette. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466:829–834.
34. Nakamura Y, F Arai, H Iwasaki, K Hosokawa, I Kobayashi, Y Gomei, Y Matsumoto, H Yoshihara and T Suda. (2010). Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. *Blood* 116: 1422–1432.
35. Ding L, TL Saunders, G Enikolopov and SJ Morrison. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481:457–462.
36. Wang SJ, P Greer and R Auerbach. (1996). Isolation and propagation of yolk-sac-derived endothelial cells from a hypervascular transgenic mouse expressing a gain-of-function *fps/fes* proto-oncogene. *In Vitro Cell Dev Biol Anim* 32:292–299.

37. Lu LS, SJ Wang and R Auerbach. (1996). *In vitro* and *in vivo* differentiation into B cells, T cells, and myeloid cells of primitive yolk sac hematopoietic precursor cells expanded >100-fold by coculture with a clonal yolk sac endothelial cell line. *Proc Natl Acad Sci U S A* 93:14782–14787.
38. O'Connell K, G Landman, E Farmer and M Edidin. (1991). Endothelial cells transformed by SV40 T antigen cause Kaposi's sarcomalike tumors in nude mice. *Am J Pathol* 139:743–749.
39. O'Connell KA and M Edidin. (1990). A mouse lymphoid endothelial cell line immortalized by simian virus 40 binds lymphocytes and retains functional characteristics of normal endothelial cells. *J Immunol* 144:521–525.
40. O'Connell KA and AA Rudmann. (1993). Cloned spindle and epithelioid cells from murine Kaposi's sarcoma-like tumors are of endothelial origin. *J Invest Dermatol* 100:742–745.
41. Bastaki M, EE Nelli, P Dell'Era, M Rusnati, MP Molinari-Tosatti, S Parolini, R Auerbach, LP Ruco, L Possati and M Presta. (1997). Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. *Arterioscler Thromb Vasc Biol* 17:454–464.
42. Salvucci O, L Yao, S Villalba, A Sajewicz, S Pittaluga and G Tosato. (2002). Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. *Blood* 99:2703–2711.
43. Ponomaryov T, A Peled, I Petit, RS Taichman, L Habler, J Sandbank, F Arenzana-Seisdedos, A Magerus, A Caruz, et al. (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest* 106:1331–1339.
44. Peled A, V Grabovsky, L Habler, J Sandbank, F Arenzana-Seisdedos, I Petit, H Ben-Hur, T Lapidot and R Alon. (1999). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. *J Clin Invest* 104:1199–1211.
45. Levesque JP and IG Winkler. (2008). Mobilization of hematopoietic stem cells: state of the art. *Curr Opin Organ Transplant* 13:53–58.
46. Kortessidis A, A Zannettino, S Isenmann, S Shi, T Lapidot and S Gronthos. (2005). Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. *Blood* 105:3793–3801.
47. Itkin T, A Ludin, B Gradus, S Gur-Cohen, A Kalinkovich, A Schajnovitz, Y Ovadya, O Kollet, J Canaani, et al. (2012). FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation and CXCL12 downregulation. *Blood*. [Epub ahead of print]; DOI: 10.1182/blood-2011-11-394692.
48. Tzeng YS, H Li, YL Kang, WC Chen, WC Cheng and DM Lai. Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. *Blood* 117:429–439.
49. Kim KA, M Wagle, K Tran, X Zhan, MA Dixon, S Liu, D Gros, W Korver, S Yonkovich, et al. (2008). R-Spondin family members regulate the Wnt pathway by a common mechanism. *Mol Biol Cell* 19:2588–2596.
50. Yamada W, K Nagao, K Horikoshi, A Fujikura, E Ikeda, Y Inagaki, M Kakitani, K Tomizuka, H Miyazaki, T Suda and K Takubo. (2009). Craniofacial malformation in R-spondin2 knockout mice. *Biochem Biophys Res Commun* 381:453–458.
51. Kazanskaya O, B Ohkawara, M Heroult, W Wu, N Maltry, HG Augustin and C Niehrs. (2008). The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development* 135:3655–3664.
52. Mei J, Y Liu, N Dai, M Favara, T Greene, S Jeyaseelan, M Poncz, JS Lee and GS Worthen. CXCL5 regulates chemokine scavenging and pulmonary host defense to bacterial infection. *Immunity* 33:106–117.
53. Eash KJ, AM Greenbaum, PK Gopalan and DC Link. (2010). CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* 120:2423–2431.
54. Tefferi A. (2000). Myelofibrosis with myeloid metaplasia. *N Engl J Med* 342:1255–1265.
55. Thiele J, C Braeckel, S Wagner, B Falini, D Dienemann, H Stein and R Fischer. (1992). Macrophages in normal human bone marrow and in chronic myeloproliferative disorders: an immunohistochemical and morphometric study by a new monoclonal antibody (PG-M1) on trephine biopsies. *Virchows Arch A Pathol Anat Histopathol* 421:33–39.
56. Mesa RA, CA Hanson, SV Rajkumar, G Schroeder and A Tefferi. (2000). Evaluation and clinical correlations of bone marrow angiogenesis in myelofibrosis with myeloid metaplasia. *Blood* 96:3374–3380.
57. Gupta RR, DJ Yoo, C Hebert, C Niger and JP Stains. (2010). Induction of an osteocyte-like phenotype by fibroblast growth factor-2. *Biochem Biophys Res Commun* 402:258–264.
58. Xiao L, P Liu, X Li, T Doetschman, JD Coffin, H Drissi and MM Hurley. (2009). Exported 18-kDa isoform of fibroblast growth factor-2 is a critical determinant of bone mass in mice. *J Biol Chem* 284:3170–3182.
59. Montero A, Y Okada, M Tomita, M Ito, H Tsurukami, T Nakamura, T Doetschman, JD Coffin and MM Hurley. (2000). Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation. *J Clin Invest* 105:1085–1093.
60. Eswarakumar VP, I Lax and J Schlessinger. (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* 16:139–149.
61. Zhang X, OA Ibrahimi, SK Olsen, H Umemori, M Mohammadi and DM Ornitz. (2006). Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J Biol Chem* 281:15694–15700.
62. Ornitz DM, J Xu, JS Colvin, DG McEwen, CA MacArthur, F Coulier, G Gao and M Goldfarb. (1996). Receptor specificity of the fibroblast growth factor family. *J Biol Chem* 271:15292–15297.

Address correspondence to:

Prof. Je-Yoel Cho

Department of Veterinary Biochemistry

College of Veterinary Medicine

Seoul National University

1 Gwanak-ro, Gwanak-gu

Seoul 151-742

Korea

E-mail: jeycho@snu.ac.kr

Received for publication March 15, 2012

Accepted after revision July 23, 2012

Prepublished on Liebert Instant Online July 24, 2012