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Expression patterns and role of SDF-1/CXCR4 axis in boar spermatogonial stem cells

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ABSTRACT

The signaling of chemokine stromal cell-derived factor (SDF)-1 and its receptor C-X-C motif chemokine receptor 4 (CXCR4) is involved in the cellular proliferation, survival, and migration of various cell types. Although SDF-1/CXCR4 has been implicated in the maintenance of the spermatogonial population during mouse testis development, their expression patterns and functions in boar testis remain unclear. In the present study, the expression pattern of SDF-1 and CXCR4 was determined during pre-pubertal and post-pubertal stage boar testes and *in vitro* cultured porcine spermatogonial stem cells (pSSCs). The role of these proteins in colony formation in cultured pSSCs was also investigated. Interestingly, SDF-1 expression was observed in PGP 9.5-positve spermatogonia in all developing stages of boar testis; however, CXCR4 expression was only detected in spermatogonia from 5-day-old boar testis. In addition, SDF-1 and CXCR4 expression was observed in cultured pSSCs from 5-day-old boar testes, and inhibition of the CXCR4 receptor signaling pathway by AMD3100 significantly decreased the colony formation of pSSCs. These results suggest that SDF-1 and CXCR4 are useful markers for detecting stage-specific spermatogonia in boar testis. Our results reveal the role of the SDF-1/CXCR4 axis in pSSC *in vitro* culture.

1. Introduction

The mammalian testis is formed in a specialized microenvironment niche that regulates the spermatogenesis and homeostasis of testicular cells. The niche for spermatogonial stem cells (SSCs) is the basal compartment of the seminiferous tubules that includes SSCs, Sertoli cells, and peritubular myoid cells [1]. SSCs are stem cells in the testis that generate haploid functional spermatozoa during a male's life after puberty [2]. Sertoli cells are epithelial cells that support SSCs and differentiating germ cells by providing nutrients and mediating external signals to support spermatogenesis [3]. Peritubular myoid cells provide structural integrity to seminiferous tubules and are involved in regulating spermatogenesis and testicular function [4].

Biomarkers of testicular cells in the boar testes have been reported in several studies. Markers for boar SSCs and undifferentiated spermatogonia include protein gene product 9.5 (PGP9.5) [5], lectin *Dolichos biflorus* agglutinin [6], promyelocytic leukemia zinc finger and Nanog [7], glial cell line-derived neurotrophic factor receptor-alpha 1 [8], stage-specific embryonic antigen-1 [9], undifferentiated embryonic cell transcription factor-1 [10], and sex-determining region Y-box 2 [11]. In additions, our previous study described matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 1 (MMP1), glutathione peroxidase 1 (GPX1), chemokine receptor 1 (CCR1), insulin-like growth factor binding protein 3 (IGFBP3), CD14, CD209, and Kruppel-like factor 9 (KLF9) were detected in boar SSCs [12]. Synaptonemal complex protein 3 [10], Sal-like protein 4, and c-Kit [13] were reported as markers for primary spermatocytes, and GATA4 is expressed in Sertoli cells in pre-pubertal boar testis [14]. However, membrane-specific and stage-specific genes expressed following germ cell development require further examination.

Chemokines, which are small pro-inflammatory chemoattractant cytokines that bind to specific G-protein-coupled seven-span transmembrane receptors present on the plasma membranes of target cells, are major regulators of cell trafficking. Stromal cell-derived factor 1 (SDF-1 or CXCL12) is a chemotactic chemokine that binds to C-X-C chemokine receptor type 4 (CXCR4) to regulate the trafficking of various cell types including immune cells, stem cells, and tumor cells [15]. In mouse testis, signaling of SDF-1 and its

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receptor CXCR4 are involved in maintaining the spermatogonial population during postnatal development [16]. In addition, this signaling plays a critical role in the maintenance of cultured SSCs *in vitro* [17]. However, the role of the SDF-1/CXCR4 axis in non-murine testes has not been examined. Therefore, the present study aimed to identify the expression pattern of SDF-1/CXCR4 axis in boar testes, specifically cells in seminiferous tubules, and determine its possible role in cultured boar SSCs *in vitro*.

2. Material and methods

All animals were handled according to the guidelines approved by the Institutional Animal Care and Use Committee at the NIAS (approval no. NIAS2015-120) and Sam-Woo Breeding Farm in Korea.

2.1. pSSC culture and treatment

Boar pSSC cells were obtained during the castration of 5-day-old crossbred piglet testes at the Sam-Woo Breeding Farm, South Korea and cultured as described by Lee et al. [18]. Briefly, 5-day-old crossbred five piglet testes were encapsulated and dissociated with enzyme mixture [collagenase IV and DNase I] for 15 min at 37 °C and then filtered through a 40-μm nylon mesh. Red blood cells were eliminated using RBC Lysis Buffer (Sigma-Aldrich, St. Louis, MO, USA). These testicular cells were seeded onto 0.2% (w/v) gelatin-coated 12-well plates (2×10^5 cells per well) and incubated with Stempro-34 medium (Gibco, Grand Island, NY, USA; 10,640-019) at 31 °C in 5% CO_2. To maintain the pSSC population *in vitro*, every 7 or 8 days upon reaching confluence, pSSCs from testicular fibroblast cells were trypsinized using 0.005% Trypsin-EDTA (Gibco; 25,300-054). Following trypsinization, 0.5×10^5 pSSCs and newly prepared testicular fibroblasts were seeded into 0.2% (w/v) gelatin-coated 12-well plates. Typically, 2–3 passages are required for colony formation. Third passages of pSSC were seed at a density of 2×10^5/well on gelatin-coated 12-well plates and treated with AMD3100 (0, 0.1, 1.0, and 10 μg/mL) with fresh media every day for 7 days. Images of colony formation of AMD3100-treated pSSC cells were obtained using a microscope (Nikon, Tokyo, Japan) 7 days after culture with AMD3100.

2.2. AP staining and antagonist treatment

Alkaline phosphatase (AP) activity in pSSC colonies was measured to verify the stemness characteristics using an Alkaline Phosphatase Kit (Sigma-Aldrich; #86R) according to the manufacturer's instructions. Briefly, cells were rinsed three times with PBS and fixed in citrate-acetone-formaldehyde solution for 30 s. These cells were washed three times with PBS and stained with alkaline dye mixture (naphthol AS-BI and FBB-alkaline,Signa-Aldrich) for 15 min at 18–26 °C. The dye was removed and the cells were rinsed for 2 min in deionized water for imaging to investigate the effect and mechanism of blockade of SDF-1/CXCR4 in *in vitro* pSSC culture. Cultured cells were exposed to 1, 0.1, 1.0, or 10 μg/mL of AMD3100 (antagonist of the CXCR4 chemokine receptor) for 24 h. Next, the colonies were counted from bright-field images and the number of colonies was quantified using n = 5 or more pSSC cultures for each condition.

2.3. Immunohistochemistry and immunocytochemistry

Boar testes taken from 5-, 30-, 60-, 90-, 120, 150-, or 180-day-old boars were obtained from the National Institute of Animal Science (NIAS). After testes collection, samples were rinsed five times with PBS and fixed in Bouin's solution (Sigma-Aldrich; HT10132) overnight at 4 °C. The samples were cut into small piece (1 × 1 cm) and dehydrated through an alcohol gradient for 120 min after washing with PBS. Dehydrated tissues were cleaned in xylene for 2 h, infiltrated with melted paraffin for 2 h at 65 °C, and embedded in paraffin blocks. Testes were sectioned at a thickness of 5 μm using a microtome (Leica, Wetzlar, Germany). These sections were placed on glass slides for immunohistochemistry and tissue sections were rehydrated using xylene and 50–100% ethanol. Antigen unmasking was performed by boiling the sections in 10 mM sodium citrate buffer for 15 min. Non-specific binding was blocked using 3% BSA in PBS for 30 min at 22 °C. Samples were incubated overnight at 4 °C with primary antibodies including SDF-1 (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), PGP9.5 (1:500 dilution; Serotec, Hercules, CA, USA), and CXCR4 (1:100 dilution; Abcam, Cambridge, UK); antibody information is presented in Table 1. After washing each tissue section five times with PBS for 5 min, tissue sections were incubated with fluorescent-conjugated secondary antibodies diluted 1:300 in 1% BSA in PBS for 1 h at 25 °C. Secondary antibodies included Alexa Fluor® 568 Donkey anti-Rabbit IgG, Alexa Fluor® 488 Goat anti-Mouse IgG, Alexa Fluor® 568 Donkey anti-Mouse IgG, and Alexa Fluor® 488 Goat anti-Rabbit IgG from Life Technologies (Carlsbad, CA, USA). Tissue sections were incubated with or without 1 μg/mL 6-diamidino-2-pheylindole (DAPI) in PBS for 10 min and coverslips were applied with mounting solution (DAKO; Carpinteria, CA, USA; S3025).

In vitro-cultured pSSC colonies were rinsed with PBS. Samples were fixed in 4% paraformaldehyde for 10 min followed by membrane permeabilization with PBS containing 0.05% Triton X-100 in PBS for 10 min for immunocytochemistry. Non-specific protein binding was blocked with 1% BSA in PBS for 30 min at 25 °C, and cells were incubated overnight at 4 °C with the following primary antibodies: SDF-1 (1:100 dilution), PGP9.5 (1:500 dilution), and CXCR4 (1:100 dilution). Cells were then washed 5 times with PBS and incubated for 1 h at RT with Alexa Fluor® 568 Donkey anti-Rabbit IgG and Alexa Fluor® 488 Goat anti-Mouse IgG secondary antibody. DAPI was added for nuclear staining at a concentration of 1 μg/mL for 10 min and mounting solution was used to fix pSSC colonies. Samples were observed using a fluorescence microscope (Nikon).

2.4. Isolation of RNA, RT-PCR and real-time QPCR

Total RNA was extracted from pSSC colonies (passage 3) and testis fibroblasts (pFeeder) using an RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with the Oligo (dT)30 primer according to the manufacturer's instructions. Target gene PCR amplification was carried out for 30 cycles of 30 s at 95 °C, 10 s at 57 °C, and 20 s at 72 °C. Primers were designed using Primer3 (http://frodo.wi.mit.edu). The QPCR was achieved using the following method: a total volume of 20 μL, which contained 10 ng of cDNA and 1 pM of each primer, in a reaction buffer containing iQ SYBR Green Supermix (170–8880; Bio-Rad Laboratories). The cycle threshold (Ct) values were normalized against beta-2-myoglobin (B2M) gene expression. A denaturation

Table 1
List of antibodies for immunostaining.

Antibody	Company	Catalogue number	Diluted
CXCR4	Abcam	ab1670	1:100
SDF1	Santa Cruz Biotech	SC-6193	1:100
PGP9.5	Serotec	7863–1004	1:500
Actin	Santa Cruz Biotech	SC-47778	1:100