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Critical role of the potential *O*-linked glycosylation sites of CXCR4 in cell migration and bone marrow homing of hematopoietic stem progenitor cells

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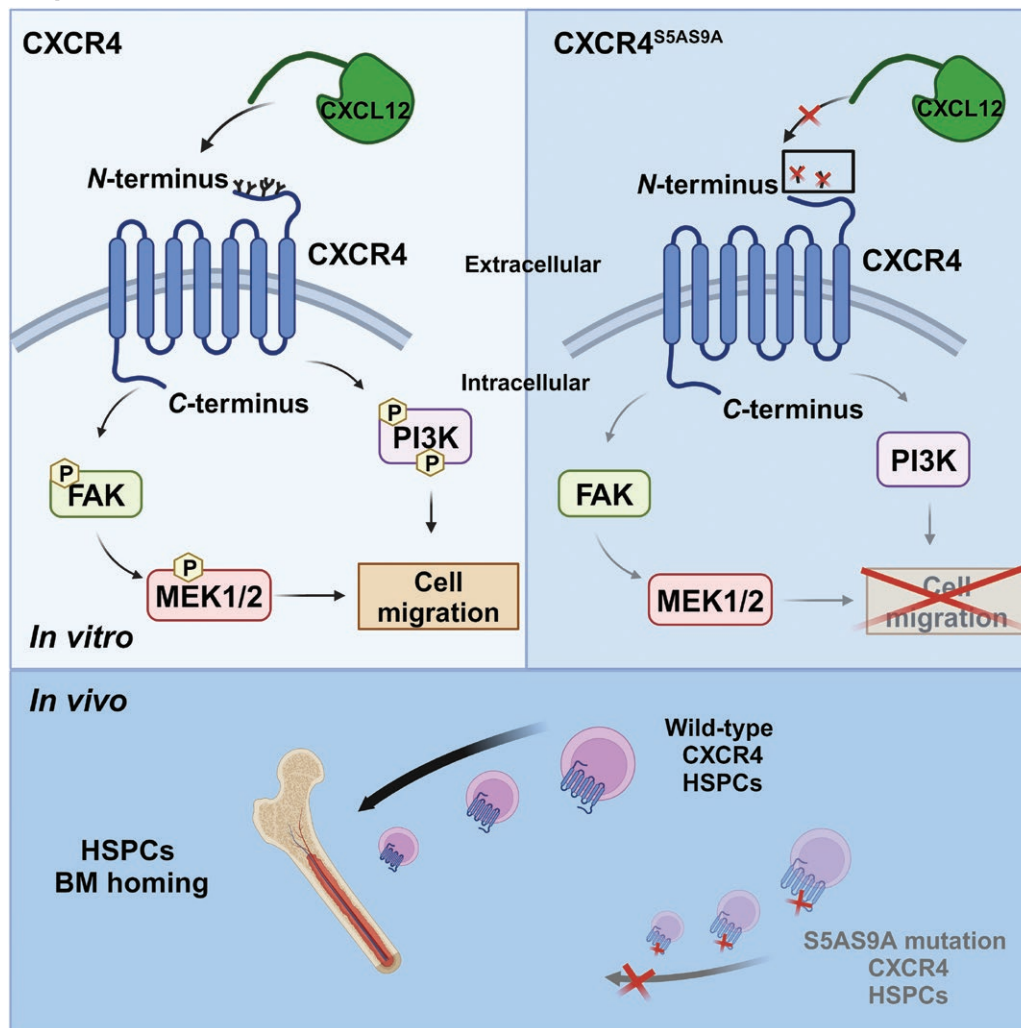
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Abstract

The C-X-C chemokine receptor type 4 (CXCR4) and its ligand, C-X-C motif chemokine ligand 12 (CXCL12), are critical for the homing of hematopoietic stem progenitor cells (HSPCs) to bone marrow (BM). Our previous study revealed that carbohydrate chains on HSPCs are vital in the homing and engraftment of HSPCs. However, the relationship between the glycosylation of CXCR4 and HSPCs homing remains unclear. In this study, we analyzed the glycosylation sites of the *N*-terminal 38 amino acids of mouse CXCR4, which is indispensable for CXCL12 binding. Among these, simultaneous mutations of possible glycosylation sites, Serine-5 and Serine-9 of mouse CXCR4 lost cell migration activity through CXCL12 in cultured cells and mouse HSPCs. Furthermore, Serine-5 and Serine-9 mutations in HSPCs caused a deficiency in the homing to the BM. Our findings suggest that the glycosylation of mouse CXCR4 is essential for homing HSPCs to the BM, which can be used to screen cord blood HSPCs suitable for transplantation.

Key words: CXCR4; CXCL12; mouse; glycosylation; hematopoietic stem cell homing.

Graphical Abstract



Significance statement

Our study focuses on the relationship between the *O*-linked glycosylation of CXCR4 and HSPC homing to the BM, which has not yet been studied. We found that the mutation of the potential *O*-linked glycosylation sites reduced its binding to the ligand CXCL12, weakened the downstream signaling of CXCR4, and impaired the cell migration and homing to the BM. Our results will aid in the development of efficient HSPC transplantation methods for clinical therapy.

Introduction

Bone marrow (BM) is the primary site of hematopoiesis.¹ Hematopoietic stem progenitor cells (HSPCs) are undifferentiated and self-renewing cells. In a healthy adult, approximately 10–100 billion new blood cells are produced daily to maintain steady-state levels of circulating blood cells in the periphery.^{2,3} For hematopoiesis, HSPCs should be maintained in the BM niche, which protects HSPCs from sustained stimulation or overstimulation.⁴ HSPCs can generate all hematopoietic lineages, including leukocytes, erythrocytes, and platelets. When HSPCs are transplanted into a lethally irradiated recipient, the HSPCs settle in the BM (homing) and produce donor-derived blood cells.⁵ Notably, some hematological diseases, such as aplastic anemia, hemophilia, and multiple myeloma, due to defects in blood cell production, may benefit from treatment with HSPC replacement through BM transplantation (BMT).⁶

Various molecules are required for the viable process of HSPCs in the BM. For example, lethally irradiated E-/P-selectin double knockout mice show a lower survival rate than control mice in a minimal number of cell transplants. In addition, homing is compromised further when vascular cell adhesion molecule-1 (VCAM-1) is functionally blocked.⁷ VCAM-1 is an essential molecule mediating cell adhesion,⁸ and an endothelial ligand of very late antigen-4 (VLA-4, $\alpha 5 \beta 1$ -integrin) and VLA-5 ($\alpha 5 \beta 1$ -integrin) binds to VCAM-1 and fibronectin in the BM,^{9,10} respectively. HSPC homing and engraftment are impaired when the function of these molecules is blocked^{11,12} or hematopoietic $\beta 1$ integrin¹³ is genetically deleted. Furthermore, leukocyte function-associated antigen-1 (LFA-1, $\alpha L \beta 2$ -integrin) and lymphocyte Peyer's patch cell adhesion molecule-1 (LPAM-1, $\alpha 4 \beta 7$ -integrin) in HSPCs, which interact with intercellular adhesion molecule-1 (ICAM-1) and mucosal addressin cell adhesion molecule-1

(MAdCAM-1) in the BM, respectively, also mediate homing and engraftment.^{12,14}

Chemokine (C-X-C motif) receptor 4 (CXCR4) is a cell surface molecule that is essential in hematopoiesis and the development and organization of the immune system.¹⁵⁻¹⁷ C-X-C motif chemokine 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF-1), is a ligand for CXCR4 and is widely expressed in the hematopoietic system.¹⁸ CXCL12 and vascular ligands specifically trigger the activation of transplanted HSPC engraftment.¹⁹ CXCR4 is highly expressed on HSPCs, whereas CXCL12 is expressed throughout the BM.²⁰ CXCR4 expressing on HSPCs can bind circulating CXCL12, which plays a leading role in the homing of HSPCs to the BM.²¹ Furthermore, an exogenous expression of CXCR4 on CD34⁺ cells improves CXCL12-mediated chemotaxis and actin polymerization, increasing the homing ability.²²

Protein glycosylation affects several cell-cell interactions, including interactions between endothelial cells and leukocytes.²³⁻²⁸ Protein glycosylation is classified into N-linked glycosylation and O-linked glycosylation, with N-linked glycosylation occurring on asparagine (Asn) residues within the Asn-X-Serine (Ser) or Asn-X-Threonine (Thr) sequences (where X is an amino acid other than proline or aspartic acid) and O-linked glycosylation occurring on the side chain hydroxyl group of a Ser or Thr residue without any consensus surrounding sequence. Our previous study showed that HSPCs from β -1,4-galactosyltransferase-1 (β 4GalT-1)-deficient mice have impaired N-glycans and core 2 O-glycans and are severely impaired in homing to the lethally irradiated recipient mice.²⁸ These studies suggest that cell surface glycosylation of HSPCs is crucial for homing after BM transplantation.

In previous studies, N-linked glycosylation occurs on Asn-11 of human CXCR4,²⁹⁻³¹ and the site is conserved in humans and mice. This relatively free-conforming N-terminus (1-38 residues) of CXCR4 is essential for the first step in the binding of CXCR4 and CXCL12,³² and substitution of a few amino acids, for example, S5A, Y7A, or Y21A, at the N-terminus of CXCR4 leads to a reduced binding ability.^{33,34} However, O-linked glycosylation in the CXCR4 has not been studied.

Materials and methods

Cell migration assay

Cell migration abilities were measured using wound healing assay³⁵ and transwell assay.³⁶ In the wound healing assay, we created a scratch in the cell monolayers of murine embryo fibroblast cell line, NIH/3T3, or human embryonic kidney cell line, HEK293, grown in conventional culture plates using 200 μ L micropipette tips. For the wound healing assay, 2.5×10^5 NIH/3T3 cells or 4×10^5 HEK293 cells per 1.9 cm² well were used. After incubating the cells overnight in the complete DMEM, the cells were treated with or without 200 nM of CXCR4 antagonist AMD3100³⁷⁻³⁹ (Abcam) for 2 hours before migration. Cells were washed with phosphate-buffered saline (PBS) twice, and then incubated with or without the addition of 100 ng/mL CXCL12 (R&D SYSTEMS) in DMEM containing 2% (for HEK293) or 3% (for NIH/3T3) fetal bovine serum (FBS) for 24 hours. After 24 hours, we measured the percentage of scratch area filled by cells.

In the transwell assay, 2×10^5 NIH/3T3 cells or KIT⁺ BM cells, pre-treated with or without AMD3100, were added into the upper chamber of the 8 μ m (NIH/3T3) or 5 μ m (KIT⁺ BM cells) transwell insert (Corning) with 200 μ L DMEM

containing 3% FBS (NIH/3T3) or serum free Ham's F-12 medium (KIT⁺ BM cells), and 500 μ L medium with or without 100 ng/mL CXCL12 (NIH/3T3) or 200 ng/mL CXCL12³³ (KIT⁺ BM cells) was added to the lower chamber. After 24 hours (NIH/3T3) or 4 h (KIT⁺ BM cells), the upper chamber was removed, and the cells on the lower chamber were fixed and stained with 0.1% crystal violet for 30 minutes at 37 °C. Cells that had migrated through the filter were counted and averaged from three randomly selected optical microscopic fields. The contrast of the pictures was enhanced at saturated pixels 8.0% by ImageJ and the background was subtracted by photoshop. All pictures were edited using the same method. Every experiment was repeated at least 3 times.

Ligand binding assay

CXCR4 and CXCR4^{S5AS9A}-overexpressing HEK293 (2×10^5 cells/well) cells were pre-cultured for 24 hours in a 24-well plate and then treated with 100 ng/ μ L CXCL12 for 10 minutes, 30 minutes, 2 hours, 4 hours, or 8 hours. The cells were recovered and stained with the Alexa Flour 488-conjugated anti-CXCL12 antibody (Supplementary Table S6) at 4 °C for 30 minutes. Populations of CXCL12⁺ cells were analyzed using a FACSARIA IIIu cell sorter (BD Biosciences) and FlowJo software (BD Biosciences).

Mice

C57BL/6 mice were purchased from Japan SLC Inc. C57BL/6-CD45.1 (CD45.1) was provided by Dr. Kuroiwa. *Cxcr4* mutant mice, *Cxcr4*^{-/-}, *Cxcr4*^{S5A/S5A}, *Cxcr4*^{S9A/S9A}, and *Cxcr4*^{S5AS9A/S5AS9A} used in this study, were generated by CRISPR/Cas9 genome editing as described below.⁴⁰

Pseudopregnant ICR female mice were purchased from CLEA Japan. All the mice in this study were housed in specific pathogen-free (SPF) conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University following the previous study.⁴¹

All animal experiments were conducted according to the Fundamental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and were approved by the Animal Experimentation Committee of Kyoto University (MedKyo24329).

Polyvinyl alcohol-based serum-free culture of bone marrow cells and fetal liver cells

Polyvinyl alcohol (PVA)-based serum-free culture of BM cells and fetal liver (FL) cells was performed as described previously with slight modification.⁴² KIT⁺ cells were isolated by anti-mouse c-Kit MicroBeads (Miltenyi Biotech). Magnetic column-enriched KIT⁺ BM/FL cells were seeded at 2×10^6 cells/well onto 24-well plates coated with human fibronectin (Corning) in 2 mL culture medium. Expression and ratio of KIT⁺ cells used for transplantation were checked by flow cytometry before transplantation.

Construction and production of lentiviral vectors

The self-inactivating (SIN) lentiviral vector with HIV-1 LTR (5'LTR and 3'LTR/ Δ U3) and lentivirus packing signal (Ψ) was purchased from Takara Bio, and cloned to carry the mouse *Cxcr4* or *Cxcr4*^{S5AS9A} gene, and a fluorescent gene ZsGreen and puromycin gene driven by a human elongation factor-1 alpha (EF-1 α) promoter.

Viral supernatant was generated from Lenti-X 293T cells (Takara Bio) transfected with a lentiviral vector using a *TransIT*-293 transfection reagent (Takara Bio) transfection protocol. For each 10-cm culture dish, 11 μ L of 0.5 μ g/ μ L lentiviral transfer vector plasmid and 7 μ L packaging plasmid (lentiviral high titer packing mix, Takara Bio) were diluted by 1500 μ L serum-free DMEM. Subsequently, 45 μ L *TransIT*-293 transfection reagent was added and incubated at 25°C for 15–30 minutes. After dropping LV mixture into Lenti-X 293T cells, cells were incubated at 37 °C under 5% CO₂ for LV production.

Subsequently, the viral supernatant was collected 48 hours after transfection and concentrated using the Lenti-X Concentrator (Takara Bio). NucleoSpin RNA Virus (Takara Bio) was used to isolate viral RNA for titrating LV stocks (Lenti-X qRT-PCR Titration Kit, Takara Bio).

Viral transfection

Mouse *Cxcr4*^{-/-} KIT⁺ FL cells were transduced via the RetroNectin-bound virus (RBV) method according to the manufacturer's instructions (RetroNectin, Takara Bio), and 24-well plates were coated with RetroNectin Reagent at 40 μ g/cm² prior to cell seeding. In addition, 125–250 μ L/cm² virus stocks (virus infectivity titer: 2.5–3.5 $\times 10^{10}$ copies/mL) were added to the plate and incubated for 6 hours at 37°C under 5% CO₂ to facilitate their adsorption to the plate. After washing with PBS, 3 $\times 10^5$ cells/well KIT⁺ FL cells were seeded.

In vivo homing assay using *Cxcr4*^{-/-} KIT⁺ FL cells expressing exogenous CXCR4

Mouse *Cxcr4*^{-/-} KIT⁺ FL cells overexpressing CXCR4 or CXCR4^{SSAS9A} by lentivirus vector (LV) were cultured in vitro, and medium was changed every other day. Proteins of cells were collected for Western blot to determine CXCR4 protein expression level 2 days after infection of LV. Wild-type or CXCR4^{SSAS9A} overexpressing KIT⁺ FL cells were transplanted to recipient mice 7 days after infection of LV. Before transplantation, the expression level of CXCR4 was determined one more time using flow cytometry. The recipient mice were irradiated with 9.5 Gy gamma radiation (Nordion, Ontario, Canada). After 24 hours of radiation, recipient mice were transplanted with 1 $\times 10^6$ cells resuspended in Hanks' Balanced Salt Solution (HBSS)⁴³ through retro-orbital sinus injection under anesthesia. 24 hours after transplantation, BM cells from recipient mice were prepared for flow cytometry.⁴⁴

In vivo homing assay and survival rate using BM or FL cells from *Cxcr4* mutant mice

The recipient mice (CD45.1) were irradiated with 9.5 Gy gamma radiation. After 24 hours of radiation, the cultured KIT⁺ BM cells (5 $\times 10^5$) from *Cxcr4*^{-/-} mice and *Cxcr4*^{SSAS9A} mice or FL cells (1 $\times 10^6$ or 5 $\times 10^5$) from *Cxcr4*^{+/-}, *Cxcr4*^{+/-}, *Cxcr4*^{SSAS9A}, *Cxcr4*^{SSAS9A/SSAS9A}, and *Cxcr4*^{SSAS9A/SSAS9A} mice were resuspended in 100 μ L of HBSS buffer and transplanted to recipient mice through retro-orbital sinus injection under anesthesia. For the survival rate experiment, 1 $\times 10^5$, 5 $\times 10^4$, and 2 $\times 10^4$ cultured KIT⁺ FL cells from *Cxcr4*^{+/-}, *Cxcr4*^{-/-}, *Cxcr4*^{SSAS9A} and *Cxcr4*^{SSAS9A/SSAS9A} mice were transplanted. Specimens of the femur were prepared from recipient mice 24 hours after transplantation. The abilities of homing were analyzed by flow cytometry.⁴¹

Graphical illustrations

All graphical illustrations were created using BioRender.com.

Statistics

Statistical analyses were performed using Prism 10 for Mac (GraphPad Software Inc). Data comparison between 2 conditions was performed utilizing the Student's *t*-test. Unless otherwise stated, data are displayed as the mean $\pm$ standard error of the mean (SEM). All figures indicate statistical significance using these designations: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and NS for non-significance.

Other materials and methods are available in Supporting Information.

Results

CXCR4-overexpressing cell migration was enhanced by the addition of CXCL12, whereas mutations at potential O-linked glycosylation sites did not increase cell migration activity

CXCR4 interacts with CXCL12 in 2 independent steps: the CXCL12 interacts with the CXCR4 N-terminus, and subsequently, the CXCL12 N-terminus interacts with the CXCR4 transmembrane (TM) region to trigger receptor activation^{45,46} (Supplementary Figure S1A). CXCR4/CXCL12 interaction mediates cell migration, proliferation, and survival of CXCR4-expressing cells.⁴⁷ To examine the migration ability of mouse CXCR4 by binding to CXCL12, CXCR4 was overexpressed in HEK293 and NIH/3T3 cells, which originally showed low CXCR4 expression levels, by transfecting the expression vector (Figure 1A). After G418 selection, 3 to 6 clones were selected to check the CXCR4 protein expression level (Supplementary Figure S2), and these clones were used for the following experiments.

Wound healing assay using HEK293 cells (Figure 1B-C) and NIH/3T3 cells (Supplementary Figure S1B-C), representing non-directional chemokinesis, was adopted to compare the migration ability of parental cells with that of CXCR4-overexpressing cells (CXCR4). The migration ability of both CXCR4-overexpressing HEK293 and NIH/3T3 cells was enhanced by adding CXCL12; however, the migration ability of parental cells was not increased (Figure 1B-C, Supplementary Figure S1B-C). Furthermore, pre-treatment with AMD3100 inhibited the increased migration ability similar to that of cells cultured without CXCL12. Capitalizing on the property of strong cell adhesion, a transwell assay, representing directional chemokinesis, using NIH/3T3 cells (Figure 1J-K) was also performed. CXCR4-overexpressing NIH/3T3 showed a higher migration ability toward the CXCL12-containing medium, which was canceled by the AMD3100 treatment.

The N-terminus (1-38 amino acids) of CXCR4 is essential for the interaction with CXCL12.⁴⁸ In CXCR4 mutants lacking the entire N-terminus (codons 2-25), CXCL12 binding was reduced by 30%-60% compared with the wild-type.⁴⁹ Asn-11 is the only N-linked glycosylation site in these 38 amino acids.³⁰ Potential O-linked glycosylation sites were analyzed using the NetOGlyc 4.0 Server (<https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/>).⁵⁰ As a result, Ser-5, Ser-9, and Ser-18 were regarded as the potential O-linked glycosylation sites (Table 1). We introduced amino acid point mutations at these positions to investigate the requirement

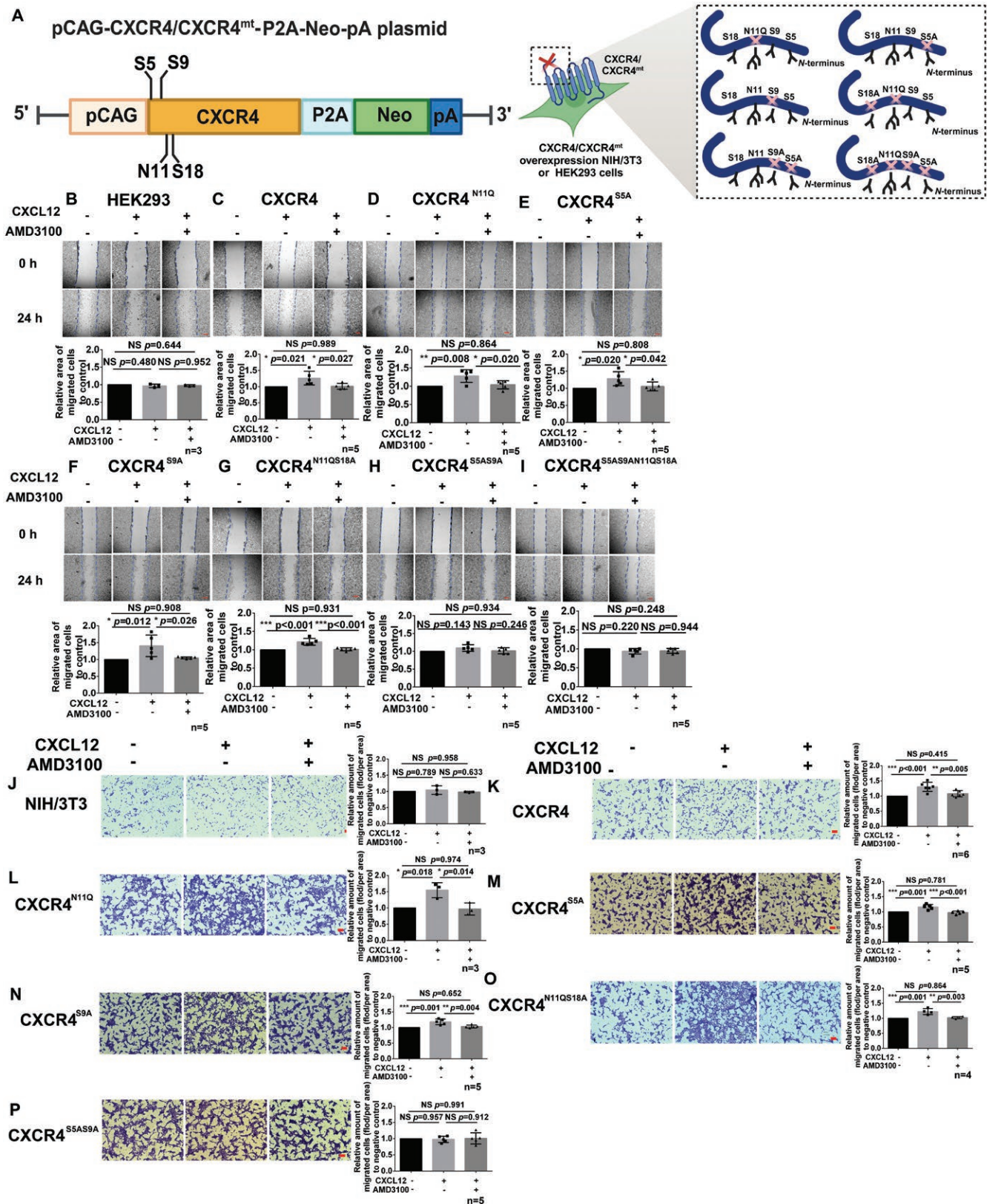


Figure 1. Migration ability toward CXCL12 of HEK293/NIH/3T3 cells overexpressing wild-type or mutant CXCR4. (A) Wild-type *Cxcr4* and six kinds of *Cxcr4* mutant genes were overexpressed by the CMV/CAG promoter (pCAG-CXCR4/CXCR4^{mt}-P2A-Neo-pA) in HEK293 or NIH/3T3 cells. The cells were pre-treated with or without the CXCR4 inhibitor AMD3100 for 2 h and cultured in DMEM containing 2% FBS (HEK293) or 3% FBS (NIH/3T3) with or without CXCL12 for 24 h. (B-I) The results of the wound healing assay using HEK293 cells (magnification, × 40, scale bars: 300 μm). (J-P) The results of transwell assay using NIH/3T3 cells (magnification, × 100, scale bars: 100 μm). Values are normalized to the control, which excludes CXCL12 or AMD3100, and set to 1 in each parallel experiment. Statistical analysis is performed using a one-way ANOVA with post-hoc Tukey HSD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS not significant.

Table 1. Prediction of the possible O-linked glycosylated sites on CXCR4 (Mouse) protein using NetOGlyc 4.0 Server.

Amino acid position	Score	Comment	Amino acid position	Score	Comment
S5	0.7476	#POSITIVE	S234	0.008266	
T8	0.311602		T245	0.0113363	
S9	0.718451		T246	0.0129078	
S13	0.381586	#POSITIVE	S265	0.0134341	
S18	0.54712		S268	0.0103332	
S23	0.253625		S283	0.0549798	
T43	0.0347217		S290	0.0149051	
T51	0.00496028		T292	0.00672927	
S71	0.00732109		S316	0.0291751	
T73	0.00333473		S317	0.0181138	
S81	0.00796462		S324	0.0383714	
T90	0.0065588		S326	0.111634	
T117	1.84031e-05		S329	0.0578078	
S122	1.59088e-05		S330	0.248753	
S123	0.00559367		S335	0.126013	
S131	0.00753846		S343	0.443103	
T142	0.0411		S344	0.393707	
S144	0.0391621		S346	0.292033	
T168	0.00783604		T347	0.113014	
S178	0.174302		S349	0.124547	
S183	0.104621		S351	0.0507851	
S198	0.0352937		S352	0.0210294	
S222	0.00947376		S353	0.0601778	
S229	0.00633763		S356	0.00865054	
S232	0.0254058		S357	0.0126305	

The score exceeding 0.5 is considered as positive site.

of these four amino acids in the activation of cell migration by CXCL12. Six kinds of mutant *Cxcr4* gene (CXCR4^{N11Q}, CXCR4^{S5A}, CXCR4^{S9A}, CXCR4^{N11QS18A}, CXCR4^{S5AS9A}, and CXCR4^{S5AS9AN11QS18A}) were overexpressed in HEK293 and five kinds of mutant *Cxcr4* gene (CXCR4^{N11Q}, CXCR4^{S5A}, CXCR4^{S9A}, CXCR4^{N11QS18A} and CXCR4^{S5AS9A}) in NIH/3T3 cells (Figure 1A). The expression level of mutant CXCR4 protein in each clone was determined by Western blot using an antibody for the C-terminal of mouse CXCR4, which might not be influenced by amino acid replacement (Supplementary Figure S2).

The migration ability of these clones was analyzed using the same method as that for the CXCR4-overexpressing cells. The results of the wound healing assay showed that the migration ability of CXCR4^{N11Q}-, CXCR4^{S5A}-, CXCR4^{S9A}-, or CXCR4^{N11QS18A}- overexpressing cells was enhanced by the addition of CXCL12 (Figure 1D–G, Supplementary Figure S1D–G). The enhanced effects were canceled by AMD3100 treatment. However, the migration capacity was not increased in cells expressing the CXCR4^{S5AS9A} and CXCR4^{S5AS9AN11QS18A} (Figure 1H, 1I, and Supplementary Figure S1H).

The results of transwell assay using CXCR4^{N11Q}-, CXCR4^{S5A}-, CXCR4^{S9A}-, or CXCR4^{N11QS18A}- overexpressing cells (Figure 1L–O) enhanced the migration ability as CXCR4 (Figure 1K), whereas the overexpression of CXCR4^{S5AS9A} (Figure 1P) did not. These results suggested the importance of Ser-5 and Ser-9 of CXCR4 for migration activity through the interaction of CXCR4 with CXCL12.

Mutation of Ser-5, Ser-9, and Asn-11 showed a lower binding level to lectins

The wound healing assay and transwell assay showed Ser-5 and Ser-9 to be the critical sites for cell migration; therefore, we analyzed the O-linked glycosylation on Ser-5 and Ser-9.

First, we compared the molecular weights of wild-type CXCR4 and mutant CXCR4 using Western blot. We established the CXCR4^{N11Q}-, CXCR4^{S5AN11Q}-, CXCR4^{S9AN11Q}-, and CXCR4^{S5AS9AN11Q}-overexpressing HEK293 cells and examined their molecular weights (Supplementary Figure S3A). The molecular weight of CXCR4^{N11Q} was smaller than that of the wild-type CXCR4; therefore, we supposed that N-linked glycosylation might affect the electrophoretic mobility. The molecular weight of CXCR4^{S5AN11Q} and CXCR4^{S9AN11Q} showed no difference compared with that of CXCR4^{N11Q}; however, the molecular weight of CXCR4^{S5AS9AN11Q} was smaller than that of CXCR4^{N11Q} (Supplementary Figure S3B). For confirmation, wild-type CXCR4 and CXCR4^{S5AS9A} were loaded into two adjacent wells (Supplementary Figure S3C), and the molecular weight of CXCR4^{S5AS9A} was slightly smaller than that of wild-type CXCR4. These results suggested that Ser-5 and Ser-9 of mouse CXCR4 might be O-glycosylated.

Furthermore, we performed lectin blot using Artocarpus integrifolia lectin (Jacalin),⁵¹ Peanut agglutinin (PNA),⁵² and Sambucus Nigra (Elderberry Bark) Lectin (SNA, EBL)⁵³ (Supplementary Figure S4A). In this experiment, we used the CXCR4^{N11Q} mutant to exclude the influence of N-glycan on Asn-11 because N-glycan would also bind to lectins, and

compared to the CXCR4^{S5AS9AN11Q}. The PNA result revealed that CXCR4^{N11Q} and CXCR4^{S5AS9AN11Q} could not bind to PNA without sialidase treatment. After treatment with sialidase, CXCR4^{S5AS9AN11Q} showed reduced binding to PNA, although the expression level of CXCR4 was higher. These results suggested that Galβ1,3GalNAc might bind to Ser-5 and Ser-9, further connecting to a sialic acid (Supplementary Figure S4B). Jacalin supported this result (Supplementary Figure S4C) and the CXCR4^{S5AS9AN11Q} appeared a lower binding to SNA, which implied there might be an α2,6-sialic acid linking to Galβ1,3GalNAc (Supplementary Figure S4D).

To confirm the O-linked glycosylation structure of mouse CXCR4, we also tried to purify the CXCR4 protein from Expi-293 cells using the FLAG tag, because Expi-293 expression system was designed to produce up to 6 times more proteins in a week, compared with other transient 293 expression systems (Asada et al unpublished methods). We used liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to analyze the O-linked glycosylation on Ser-5 and Ser-9. However, the CXCR4 was not stable enough to be purified even when combined with an antagonist. In the final purified samples, many proteolysis products were mingled with CXCR4, leading to the failure of CXCR4 peptide identification (results not shown).

Migration signaling pathways were not activated in CXCR4^{S5AS9A}-overexpressing HEK293 cells

We hypothesized that the low migration activity of CXCR4^{S5AS9A}-overexpressing HEK293 cells was owed to a lower binding of CXCR4-CXCL12. CXCR4-CXCL12 shows a strong affinity level similar to that of ordinary antibodies (Kd = 3.6 nmol/L)⁵⁴; therefore, we thought that the binding of CXCR4 to CXCL12 could be examined using flow cytometry. Before examining CXCL12 binding, the CXCR4 protein expression level was determined using a Western blot to be almost the same between CXCR4^{S5AS9A}-overexpressing cells and CXCR4-overexpressing cells (Figure 2A). Flow cytometry showed that the population of CXCL12⁺ cells in CXCR4-overexpressing cells significantly exceeded that in CXCR4^{S5AS9A}-overexpressing cells at 30 minutes of CXCL12 treatment. In 2 hours, the level reached a peak and was markedly higher in CXCR4-overexpressing cells than in CXCR4^{S5AS9A}-overexpressing cells. From 4 to 8 hours post-treatment, the same tendency continued; however, CXCL12 binding started decreasing (Figure 2B). These results suggested that the double mutations at Ser-5 and Ser-9 impaired mouse CXCR4's binding ability to CXCL12. The lower CXCR4^{S5AS9A}-CXCL12 affinity than CXCR4-CXCL12 suggested that signaling pathways associated with migration, such as focal adhesion kinase (FAK), MEK1/2, and phosphoinositide 3-kinase (PI3K) might also be affected. Western blot results showed that FAK, MEK1/2, and PI3K phosphorylation levels were significantly increased in CXCR4 overexpressing cells treated with CXCL12. Conversely, pre-treatment with AMD3100 did not increase the phosphorylation levels of these molecules, indicating that these phosphorylations were dependent on CXCL12. However, in HEK293 cells overexpressing CXCR4^{S5AS9A}, the phosphorylation levels of these molecules did not increase despite the addition of CXCL12 (Figure 2C). These results suggested that the S5AS9A double mutation in mouse CXCR4 inhibited the binding to CXCL12 and could not activate the migration signal (Figure 2D).

Homing activity of HSPCs overexpressing Ser-5 and Ser-9 double mutant *Cxcr4* gene was not enhanced

An essential function of CXCR4 is to regulate the homing and engraftment of HSPCs in the BM microenvironment.^{19,55} Therefore, we examined whether the exogenous expression of CXCR4 or CXCR4^{S5AS9A} in HSPCs enhanced the homing activity. We used the LV to overexpress CXCR4 or CXCR4^{S5AS9A} in *Cxcr4*^{-/-} background KIT⁺ FL cells. The empty vector, without the mouse *Cxcr4* gene, was used as a control. The fluorescence intensity of ZsGreen increased at 24, 48, and 72 hours after transfection (data not shown). In this experiment, we harvested LV supernatants at 48 h after transfection.

To eliminate the effects of endogenous CXCR4, *Cxcr4*^{-/-} KIT⁺ FL cells were then transfected with wild-type *Cxcr4* or *Cxcr4*^{S5AS9A} gene by LV and transplanted into recipients. *Cxcr4*^{-/-} mice died perinatally⁵⁶⁻⁵⁸ (Supplementary Table S2); therefore, KIT⁺ cells from *Cxcr4*^{-/-} FL cells were used in this experiment (Figure 3A). The empty vector, *Cxcr4* or *Cxcr4*^{S5AS9A} gene was overexpressed in the *Cxcr4*^{-/-} KIT⁺ FL cells using LV. Immediately before transplantation, the percentage of Lineage⁻ SCA-1⁺ KIT⁺ (KSL) cells was measured, and almost the same levels among these cells (Figure 3B and C). CXCR4 expression levels were also examined using flow cytometry and Western blot (Figure 3D and E) and CXCR4 expression levels of wild-type CXCR4 and CXCR4^{S5AS9A} KIT⁺ FL cells were almost the same. One million cultured KIT⁺ FL cells were transplanted into recipient mice irradiated with gamma-ray. BM cells were collected from recipient mice 24 hours after transplantation, and the percentage of KSL cells in BM cells was examined. As shown in Figure 3F, the homing activity by the mouse *Cxcr4* gene was upregulated, but not by the *Cxcr4*^{S5AS9A} gene, compared with the empty vector. These results indicated that the exogenous CXCR4^{S5AS9A} expression in KIT⁺ FL cells could not improve the poor homing of CXCR4-deficient KIT⁺ FL cells.

Mutation of Ser-5 and Ser-9 in the endogenous CXCR4 caused defects in the ex-vivo migration, homing activity, and engraftment efficiency of HSPCs

Cxcr4^{S5A/S5A}, *Cxcr4*^{S9A/S9A}, and *Cxcr4*^{S5AS9A/S5AS9A} mice were generated through CRISPR/Cas9 genome editing to examine the effect of Ser-5 and Ser-9 mutation of the *Cxcr4* gene in the homing ability more precisely. *Cxcr4*^{S5AS9A/S5AS9A} genome-edited mice could be born normally, which was quite different from *Cxcr4*^{-/-} mice (Supplementary Table S2). BM cells were collected from adult mice femur. After being enriched with anti-KIT antibody beads, BM cells were cultured in a PVA-based HSPC medium and the transwell assay was performed using KIT⁺ BM cells cultured for 5 days. The percentage of KSL cells was measured before the transwell assay and was found to be nearly identical across the three genotypes (Figure 4A). KIT⁺ BM cells of all genotypes showed no significant migration unless CXCL12 was added. *Cxcr4*^{+/+} KIT⁺ BM cells showed a significant increase in migration with CXCL12, as well as *Cxcr4*^{-/-} KIT⁺ BM cells, while *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ BM cells showed a slight increase with CXCL12 (Figure 4B and C). These increases were completely inhibited by AMD3100, indicating that these KIT⁺ BM cell migrations were dependent on CXCL12. Migration of *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ BM cells was significantly reduced compared to that of *Cxcr4*^{+/+} and *Cxcr4*^{-/-} KIT⁺ BM cells, indicating that the decreased cell migration ability was due to the S5AS9A mutation.

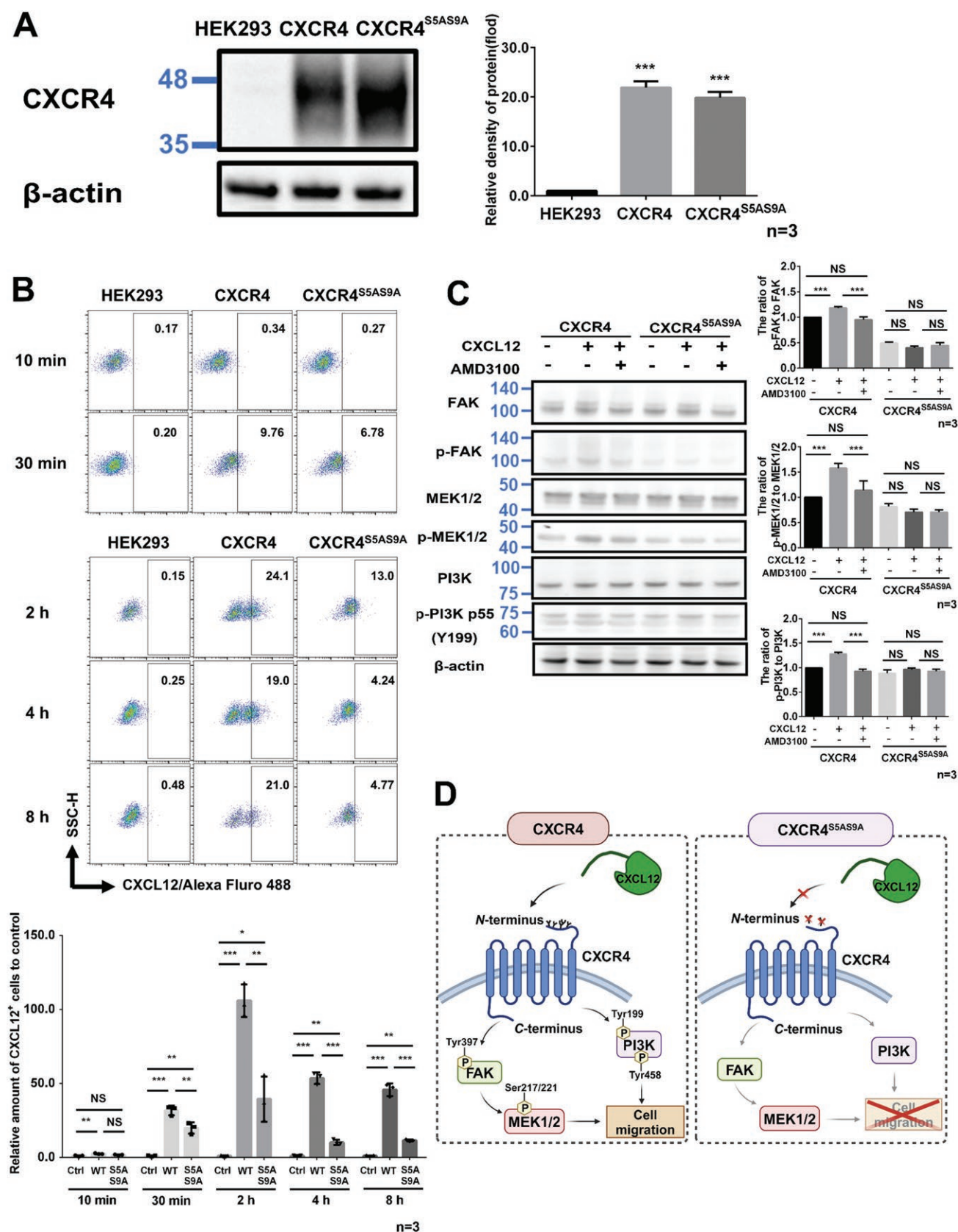


Figure 2. CXCL12 binding ability and downstream signaling pathways of CXCR4 and CXCR4^{S5AS9A}-overexpressing HEK293. (A) CXCR4 expression levels of CXCR4- and CXCR4^{S5AS9A}-overexpressing HEK293 were determined by Western blot analysis. Values are normalized to the CXCR4 expression level of parental HEK293 cells as 1 in every parallel experiment. (B) CXCL12⁺ cells stained with CXCL12 antibody were analyzed using flow cytometry. Parental HEK293 cells were used as a control. Values are normalized to the number of CXCL12⁺ cells of HEK293 cells as 1 in every parallel experiment. Ctrl, parental HEK293; WT, wild-type CXCR4-overexpressing HEK293 cells; S5AS9A, CXCR4^{S5AS9A}-overexpressing HEK293 cells. (C) Total and phosphorylated FAK, MEK1/2, and PI3K were detected using Western blot, and band intensities were quantified using β-actin as a control. CXCR4 and

CXCR4^{S5AS9A}-overexpressing HEK293 cells were pre-treated with or without AMD3100 for 2 h and cultured in DMEM containing 2% FBS with or without CXCL12 for 6 (for FAK) or 12 h (for MEK1/2 and PI3K). Cell proteins were extracted by RIPA buffer. Values are normalized to the ratio of phosphorylation to non-phosphorylation levels without CXCL12 and AMD3100 treatment as 1 in every parallel experiment. (D) Schematic diagram of migration signalling pathways of CXCR4-CXCL12 and CXCR4^{S5AS9A}-CXCL12. Statistical analysis is performed using a one-way ANOVA with post-hoc Tukey HSD. * $p < 0.05$, *** $p < 0.001$, NS not significant.

For transplantation, FL cells were collected from E14.5 embryos derived from an intercross of *Cxcr4*^{+/-} mice. KIT⁺ FL cells were enriched and cultured ex-vivo for 5 days and genotyped before transplantation, following the method described in the previous study⁴² (Figure 5A). Cultured KIT⁺ FL cells (CD45.2 background) were transplanted into C57BL/6-CD45.1 recipient mice irradiated with gamma-ray. The quality of cultured cells was examined using flow cytometry to confirm the ratio of KSL cells just before transplantation (data not shown). The homing ability of *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ FL cells was significantly lower than that of *Cxcr4*^{+/+} cells and almost the same as that of *Cxcr4*^{-/-} cells (Figure 5D and E). The level of CXCR4 expression in *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ FL cells was half that of wild-type cells and comparable to *Cxcr4*^{+/-} cells (Figure 5B and C). The homing activity of *Cxcr4*^{S5AS9A/S5AS9A} cells was also significantly less than that of *Cxcr4*^{+/-} KIT⁺ FL cells (Figure 5F). The single mutation of Ser-5 or Ser-9 did not affect the homing ability (Figure 5G), suggesting that the homing ability was retained if either amino acid was maintained.

We also checked the homing ability of *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ BM cells compared to *Cxcr4*^{+/-} cells (Figure 6). The result indicated that the homing ability of *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ BM cells decreased to almost half of that of *Cxcr4*^{+/-} cells, similar to *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ FL cells.

Hematopoietic reconstitution in recipient mice was examined to test the relationship between Ser-5 and Ser-9 mutations and BM engraftment efficiency. Cultured KIT⁺ FL cells of each genotype were transplanted into lethally irradiated recipient mice, and their survival was monitored for 4 weeks⁵⁷ (Supplementary Table S1, Figure 5H). Notably, all mice without transplantation died within 10 days. At the highest cell number injection (1×10^5 cells), almost all recipient mice were rescued from lethal irradiation and were alive after 4 weeks (except the 80% survival of *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ FL cells). However, when 5×10^4 KIT⁺ FL cells were transplanted into recipient mice, the survival ratio of recipient mice transplanted with *Cxcr4*^{-/-} or *Cxcr4*^{S5AS9A/S5AS9A} cells was reduced to 40%, whereas that of recipient mice with *Cxcr4*^{+/-} and *Cxcr4*^{+/-} cells was 80%. At the lowest cell number injection (2×10^4 cells), 20% of recipient mice with *Cxcr4*^{+/-} cells survived; however, other recipient mice died within 3 weeks. These results suggested that the double mutation in Ser-5 and Ser-9 of mouse CXCR4 influenced homing ability and was closely associated with BM engraftment.

Discussion

Notably, numerous molecules affect the homing of HSPCs, such as integrins,^{59,60} selectins,⁶¹ and chemokines.⁶² In particular, CXCL12, the predominant HSPC chemoattractant through its receptor CXCR4,⁶³ has been reported to mediate HSPC homing and engraftment.^{15,64}

In this study, we examined the potential glycosylation sites of CXCR4. We found that double mutations of Ser-5 and Ser-9 of CXCR4 affected cell migration in both cultured cells (Figure 1 and Supplementary Figure S1) and HSPCs (Figure

4). Furthermore, the double mutation of Ser-5 and Ser-9 reduced its binding to CXCL12 and weakened the downstream signaling of CXCR4 (Figure 2). These results indicated that possible O-linked glycosylation sites on Ser-5 and Ser-9 of CXCR4 are indispensable for cell migration. Although we used the cultured cells of human HEK293, mouse NIH/3T3, and mouse HSPC cells in this study, the use of human HSPC was limited by resource availability and technical challenges. We compared the molecular weight between wild-type CXCR4 and several mutant CXCR4. We found that the molecular weight of CXCR4^{S5AS9A/N11Q} was smaller than that of CXCR4^{N11Q} (Supplementary Figure S3B), suggesting that there might be O-glycans attached to Ser-5 and Ser-9. We also performed lectin blot experiments (Supplementary Figure S4), which suggested that the O-linked glycosylation on Ser-5 and Ser-9 might be Neu5Acα2,6Galβ1,3GalNAc. However, we were unable to verify whether O-linked glycans are attached to Ser-5 and Ser-9 of CXCR4.

One limitation of this study is that we were unable to determine whether the impairment of mouse CXCR4 function is due to O-linked glycosylation deficiency at Ser-5 and Ser-9 or the amino acid exchange that is independent of O-linked glycosylation. This limitation persists even though we substituted serine with alanine, the amino acid most similar to serine in both physical and chemical properties. However, when we aimed to analyze the O-glycans on Ser-5 and Ser-9, we faced technological challenges in purifying CXCR4. In the subsequent study, we intend to improve the CXCR4 purification method.

Subsequently, we investigated the effects of point mutations in mouse CXCR4 in two ways: transplantation of CXCR4^{S5AS9A}-overexpressing KIT⁺ FL cells from *Cxcr4*^{-/-} embryos (Figure 3) and transplantation of CXCR4 point mutant cells derived from embryos and BM (Figure 5, 6). Collectively, we revealed that the S5AS9A mutation affects homing ability and leads to an engraftment defect. Our results were consistent with a previous report on the transplantation of *Cxcr4*^{-/-} FL cells to lethally irradiated mice. In the previous report,⁶⁵ short-term radioprotection of lethally irradiated mice by limiting number of *Cxcr4*^{-/-} FL cells was impaired compared with that of *Cxcr4*^{+/-} FL cells. Furthermore, the homing efficiency of *Cxcr4*^{-/-} FL cells decreased compared with that of *Cxcr4*^{+/-} FL cells, but not completely lost. Therefore, our results indicate that the S5AS9A mutation in mouse CXCR4 causes homing and engraftment defects similar to *Cxcr4*^{-/-} FL cells.

A difference from *Cxcr4*^{-/-} mice was that *Cxcr4*^{S5AS9A/S5AS9A} mice were not embryonic lethality. A previous report showed that one-third of *Cxcr4*^{-/-} mice died at around E18.5, and the rest died 2 h after birth,¹⁶ whereas our *Cxcr4*^{S5AS9A/S5AS9A} mice survived normally until the weaning period. Perinatal death is thought to be due to the heart and ventricular septal defect.⁶⁶ One possible explanation of the difference between *Cxcr4*^{-/-} and *Cxcr4*^{S5AS9A/S5AS9A} mice is that S5AS9A mutation of CXCR4 only reduces affinity for CXCL12 but may not entirely impair CXCR4-CXCL12 interaction.⁴⁹ Another possibility is that the mutations of Ser-5 and Ser-9 may not affect the formation of CXCR4-CXCR7 heterodimers. CXCR7 is another receptor

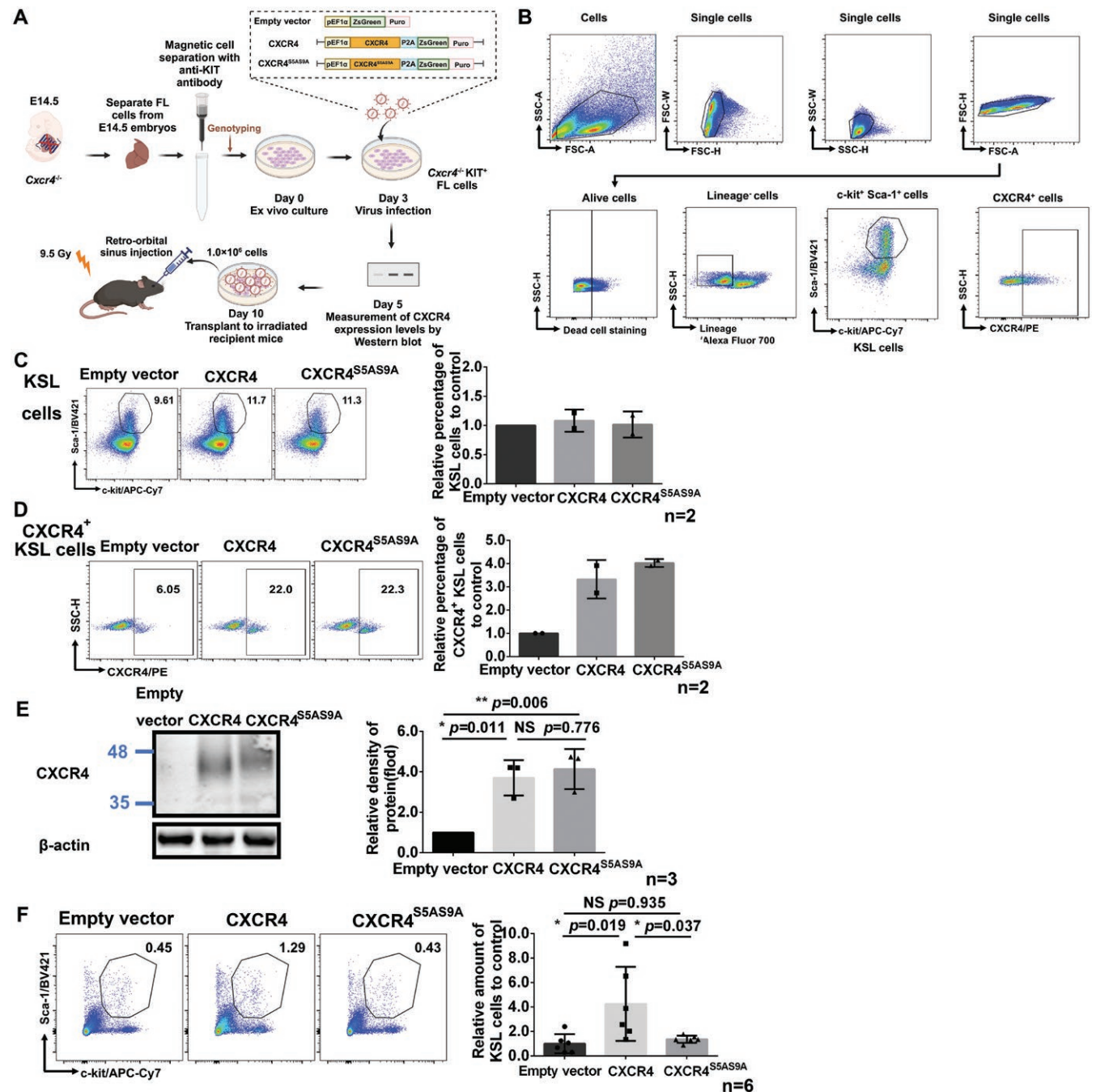


Figure 3. In vivo homing activity of *Cxcr4*^{-/-} background KIT⁺ FL cells expressing exogenous *Cxcr4* and *Cxcr4*^{S5AS9A} genes. (A) Schematic illustrating CXCR4 overexpressing *Cxcr4*^{-/-} KIT⁺ FL cells. FL cells were collected from E14.5 fetal livers, and their genotypes were determined using fetal tail tips. KIT⁺ FL cells were enriched using a magnetic column with an anti-KIT antibody. After 3 days of culture in PVA-based HSPC medium, *Cxcr4*^{-/-} FL cells were infected with LV expressing *Cxcr4* and *Cxcr4*^{S5AS9A} genes. (B) Gating strategies for identifying KSL cells and CXCR4⁺ cells. Number of CXCR4⁺ KSL cells were determined by flow cytometry. (C) Before transplantation, KSL cell percentage was measured using flow cytometry. (D, E) After being infected for 2 days (the fifth day of ex vivo culture), cells or cell proteins were collected for CXCR4 expression level measurement using flow cytometry (D) or Western blot (E), respectively. (F) Seven days after LV infection (the tenth day of ex vivo culture), cells were transplanted to lethally irradiated recipient mice through retro-orbital sinus injection. KSL cells from recipient BM cells were investigated using flow cytometry 24 h after transplantation. Values are normalized to those of control experiment with empty vector as 1 in every parallel experiment. Statistical analysis is calculated with a one-way ANOVA with post-hoc Tukey HSD. **p* < 0.05, ***p* < 0.01, NS not significant.

of CXCL12^{67,68} and forms a heterodimer with CXCR4.⁶⁹ Although CXCR7 does not trigger Gαi protein-dependent signaling, it enhances CXCL12-induced signaling through CXCR4 to be involved in angiogenesis⁷⁰ and cardiogenesis.⁷¹ We suppose that, in *Cxcr4*^{-/-} mice, the CXCR4-CXCR7 heterodimer could not be formed, resulting in angiogenesis

and cardiogenesis failure, which might cause perinatal death by the heart and ventricular septal defects. In *Cxcr7*^{-/-} mice, the CXCR4-CXCR7 heterodimer also could not be formed, showing similar heart defects as those of *Cxcr4*^{-/-} mice, but normal hematopoiesis by CXCL12-induced signaling through CXCR4.⁷² In contrast, in our *Cxcr4*^{S5AS9A/S5AS9A} mice, mutant

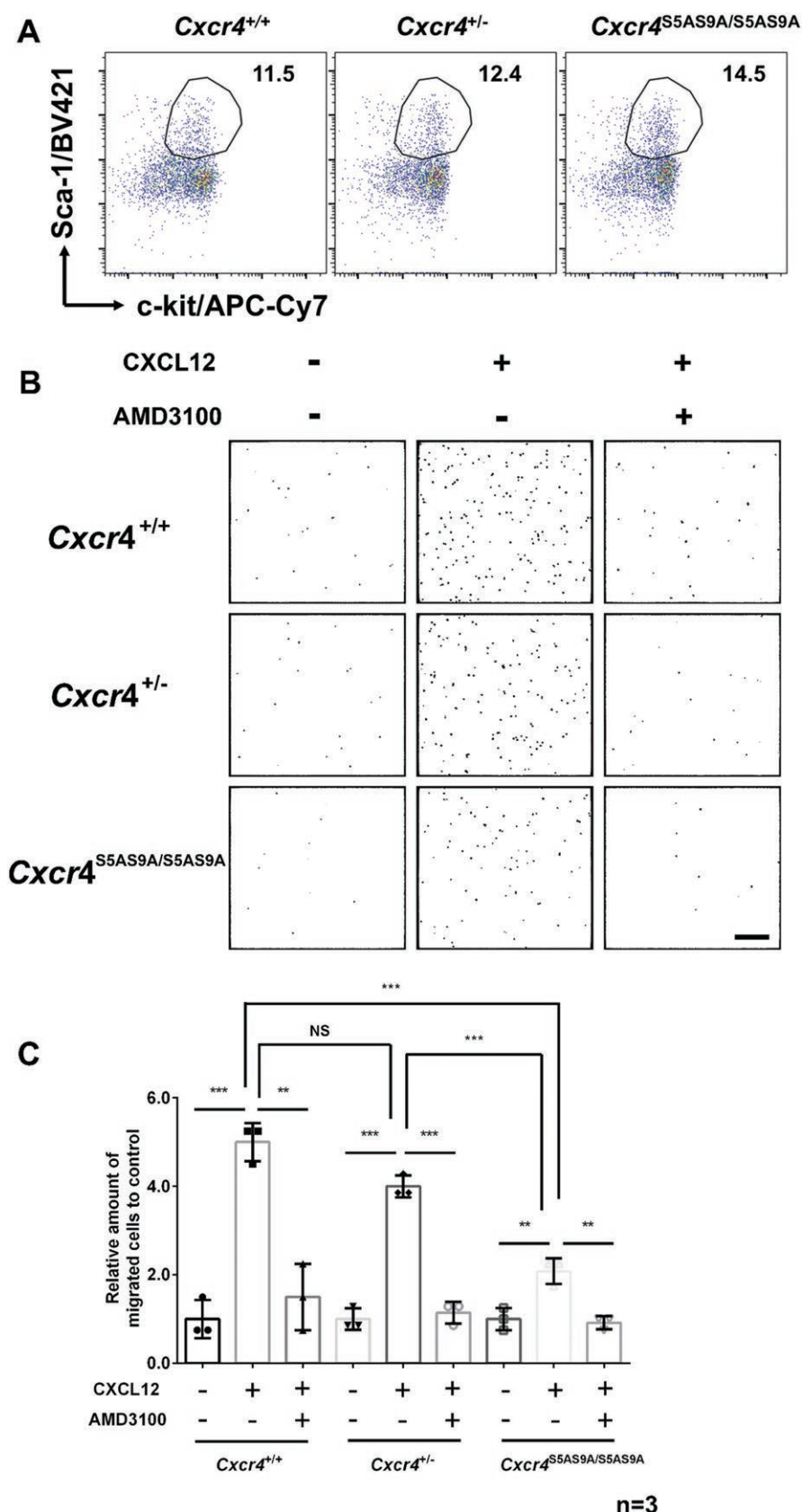


Figure 4. Ex vivo migration ability of KIT⁺ BM cells from *Cxcr4*^{S5AS9A/S5AS9A} genome-edited mice (A) BM cells from two different mice of the same genotype were collected and combined for the transwell assay. The percentage of KSL cells from 5-day ex-vivo cultured KIT⁺ BM cells from *Cxcr4*^{+/+}, *Cxcr4*^{+/-} and *Cxcr4*^{S5AS9A/S5AS9A} were examined. (B) The results of transwell assay using KIT⁺ BM cells (magnification, × 40, scale bars: 200 μm). 2 × 10⁵ KIT⁺ BM cells, pre-treated with or without AMD3100, were resuspended in 200 μL serum-free Ham's F-12 medium and added to the upper chamber of the transwell insert. 500 μL Ham's F-12 medium with or without 200 ng/mL CXCL12 was added to the lower chamber and incubated for 4 h. (C) Relative amounts of migrated cells compared to control. Cells treated without CXCL12 and AMD3100 in every genotype group was used as control. Values are normalized to those cells without CXCL12 and AMD3100 treatment in every genotype as 1 in every parallel experiment. Statistical analysis is performed using one-way ANOVA with post-hoc Tukey HSD. ***p* < 0.01, ****p* < 0.001, NS not significant.

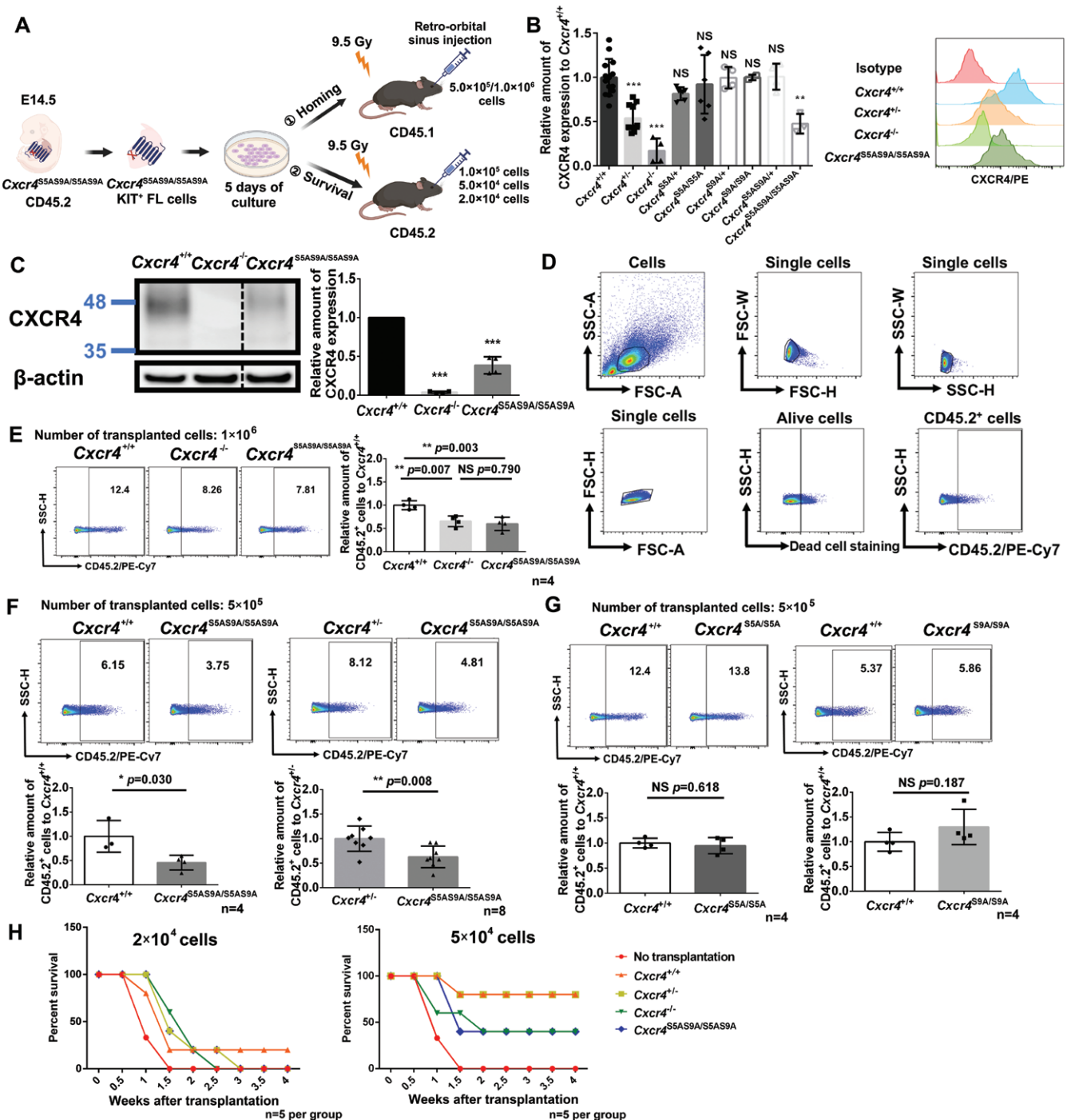


Figure 5. In vivo homing activity of FL cells from *Cxcr4*^{S5A/S5A} genome edited embryos. (A) Experimental diagram: *Cxcr4*^{S5A/S5A} genome-edited mice in CD45.2 background were generated, in which Ser-5 and Ser-9 were replaced by alanine in CXCR4 protein. FL cells were collected from E14.5 fetal livers, and KIT⁺ FL cells were enriched using a magnetic column with an anti-KIT antibody. KIT⁺ FL cells were plated in PVA-based HSPC media and cultured for 5 days ex-vivo before transplantation. For the homing assay, 5×10^5 or 1×10^6 KIT⁺ FL cells were transplanted to recipient mice (CD45.1) lethally irradiated before 24 h. BM cells were collected from recipient mice and analyzed using flow cytometry 24 h after transplantation. For survival experiments, 2×10^4 , 5×10^4 , and 1×10^5 KIT⁺ FL cells were transplanted to lethally irradiated recipient mice (CD45.2). (B) CXCR4 protein expression levels of KIT⁺ FL cells from *Cxcr4*^{+/+}, *Cxcr4*^{-/-}, *Cxcr4*^{S5A/+}, *Cxcr4*^{S5A/S5A}, *Cxcr4*^{S9A/+}, *Cxcr4*^{S9A/S9A}, *Cxcr4*^{S5A/S9A}, and *Cxcr4*^{S5A/S5A} embryos were examined by flow cytometry and relative CXCR4 expression levels to KIT⁺ FL cells from *Cxcr4*^{+/+} were shown. CXCR4 expression levels of *Cxcr4*^{+/+}, *Cxcr4*^{S5A/+}, *Cxcr4*^{S5A/S5A}, *Cxcr4*^{S9A/+}, *Cxcr4*^{S9A/S9A}, and *Cxcr4*^{S5A/S9A} KIT⁺ FL cells showed no significant difference to those of *Cxcr4*^{+/+} cells. However, the CXCR4 expression level of *Cxcr4*^{S5A/S5A} KIT⁺ FL cells was approximately half that of *Cxcr4*^{+/+} cells and similar to that of *Cxcr4*^{-/-} cells. (C) CXCR4 protein expression levels of KIT⁺ FL cells from *Cxcr4*^{+/+}, *Cxcr4*^{-/-}, and *Cxcr4*^{S5A/S5A} embryos were examined by Western blot analysis and relative CXCR4 expression levels to KIT⁺ FL cells from *Cxcr4*^{+/+} were shown. CXCR4 expression level of *Cxcr4*^{S5A/S5A} KIT⁺ FL cells was approximately half that of *Cxcr4*^{+/+} cells. (D) Gating strategies for identifying CD45.2⁺ cells. (E) 1×10^6 KIT⁺ FL cells from *Cxcr4*^{+/+}, *Cxcr4*^{-/-}, and *Cxcr4*^{S5A/S5A} mice were transplanted to recipient mice, and the homing activity was analyzed using flow cytometry. Values are normalized to the amount of CD45.2⁺ cells in *Cxcr4*^{+/+} cell transplanted mice as 1 in every parallel experiment. (F) Homing activity of 5×10^5 *Cxcr4*^{S5A/S5A} KIT⁺ FL cells was also compared with *Cxcr4*^{-/-} KIT⁺ FL cells, which showed the same CXCR4 protein expression level to *Cxcr4*^{S5A/S5A} KIT⁺ FL cells. Values are normalized to the amount of CD45.2⁺ cells in *Cxcr4*^{+/+} (left panel) or *Cxcr4*^{-/-} (right panel) cell transplanted mice as 1 in every parallel experiment. (G) Homing activity of *Cxcr4*^{S5A/S5A} and *Cxcr4*^{S9A/S9A} KIT⁺ FL cells. KIT⁺ FL cells from *Cxcr4*^{+/+} mice was used as control. Values are normalized to the amount of CD45.2⁺ cells in *Cxcr4*^{+/+} cell transplanted mice as 1 in every parallel experiment. (H) Survival ratio of recipient mice during 4 weeks after transplantation. Statistical analysis is calculated using a one-way ANOVA with post-hoc Tukey HSD (B) or an unpaired two-tailed *t*-test (C). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, NS not significant.

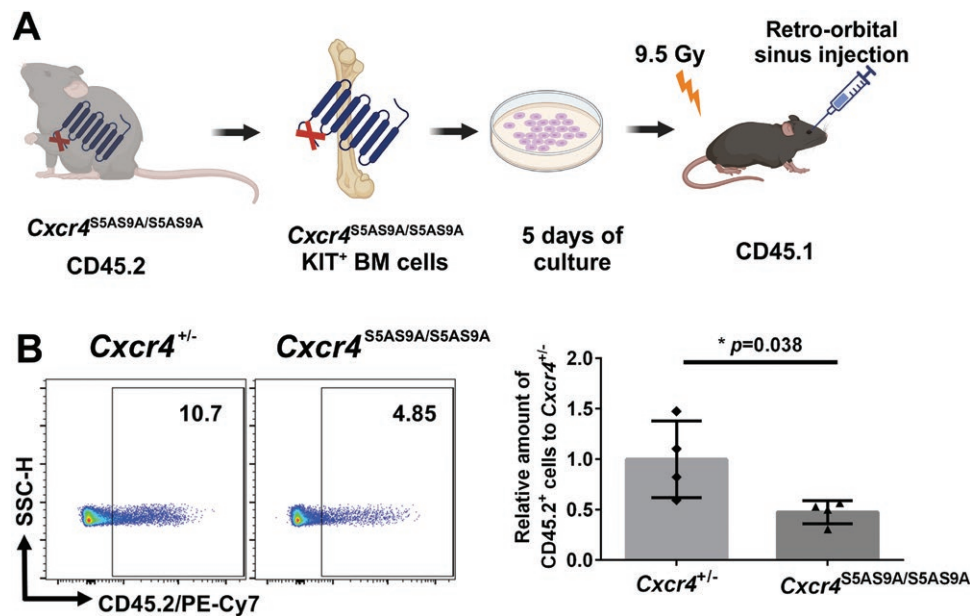


Figure 6. In vivo homing activity of KIT⁺ BM cells of *Cxcr4*^{+/-} and *Cxcr4*^{S5AS9A/S5AS9A}. (A) Experimental diagram: Homing activity of *Cxcr4*^{+/-} and *Cxcr4*^{S5AS9A/S5AS9A} KIT⁺ BM cells. (B) 5×10^5 KIT⁺ BM cells from *Cxcr4*^{+/-} and *Cxcr4*^{S5AS9A/S5AS9A} mice were transplanted to recipient mice (CD45.1), which were lethally irradiated 1 day before transplantation. BM cells were collected from recipient mice and analyzed using flow cytometry 24 h after transplantation. KIT⁺ BM cells from *Cxcr4*^{+/-} mice were used as control. Values are normalized to the amount of CD45.2⁺ cells in *Cxcr4*^{+/-} cell translated mice, which are set to 1 in each parallel experiment. Statistical analysis is calculated using an unpaired two-tailed *t*-test. **p* < 0.05.

CXCR4 could form a heterodimer with CXCR7 and activate the signals of angiogenesis and cardiogenesis. This might be the reason why *Cxcr4*^{S5AS9A/S5AS9A} mice did not die at birth like *Cxcr4*^{-/-} and *Cxcr7*^{-/-} mice.

Several human diseases are related to O-linked glycosylation deficiency. For example, mutations in the glycosyltransferase genes that synthesize the O-mannose-glycans of α -dystroglycan cause certain muscular dystrophies.⁷³ The mutations in the EOGT gene, which causes extracellular O-GlcNAc modification, were regarded to be one of the causes of Adams-Oliver syndrome (AOS).⁷⁴

In conclusion, we indicate possible O-linked glycosylation sites (Ser-5 and Ser-9) of mouse CXCR4 are crucial for HSPC homing and engraftment in the BM. To the best of our knowledge, this is the first report showing important function of glycosylation of mouse CXCR4. We suspect that engraftment failure occurred occasionally in BM and umbilical cord blood (UCB) transplantation might be caused by an abnormal glycosylation of human CXCR4, which has Ser-5 and Ser-9 as well. However, there are currently no reports examining CXCR4 glycosylation in human HSPCs. If any abnormal glycosylation of CXCR4 is identified, screen methods using lectins or antibodies to select the BM and UCB cells suitable for HSPC transplantation could be developed for clinical therapy.

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Author contributions

Conceptualization, X.P., C.N. and M.A.; Investigation, X.P., T.M., O.I., K.S. and H.A.; Writing—original draft, X.P.; Writing—review & editing, C.N. and M.A.; Project administration, C.N. and M.A.; Supervision, C.N. and M.A.; Funding Acquisition, M.A.

Conflicts of interest

The authors declare no competing interests.

Supporting material

Supporting Information is available at *Stem Cells* online.

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Data availability

The data that support the findings of this study are publicly available in Mendeley Data (doi: 10.17632/ggmzmf6hdn.2; <https://data.mendeley.com/drafts/ggmzmf6hdn>)

Supplementary material

Supplementary material is available at *Stem Cells* online.

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