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Rapid hematopoietic progenitor mobilization by sulfated colominic acid

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Abstract

Hematopoietic progenitor cells (HPCs) can be mobilized from bone marrow (BM) to the blood by G-CSF. In this process, CXCR4 and CD26 play critical roles. Sulfated colominic acid (SCA) inhibits HIV entry, the step which requires CXCR4 and CD26 as co-receptors. Thus, we hypothesized that SCA would modulate HPC trafficking. We first found that SCA mobilized HPCs rapidly via CD26-independent mechanism. In vitro progenitor migration toward chemokine SDF-1 was significantly enhanced by SCA, and it was completely abrogated by CXCR4 inhibition. This likely originated from the inhibition of CXCR4 down-regulation after interaction with SDF-1. Serum SDF-1 level increased after SCA injection, whereas no change was observed in BM and bone. These results suggest that SCA induces HPC mobilization by modulating CXCR4 function resulting in attraction toward increased SDF-1 in the circulation. Furthermore, we confirmed an additive effect with G-CSF in mobilization. SCA may provide an efficacy in clinical mobilization.

Keywords: hematopoietic stem/progenitor cells, SDF-1/CXCL12, CXCR4, mobilization, sulfated colominic acid, G-CSF

Introduction

Hematopoietic stem/progenitor cells (HSCs/HPCs) can be mobilized from the bone marrow (BM) compartment to the blood by various molecules such as cytokines, chemokines and chemotherapeutic agents [1]. Mobilized peripheral blood HSCs/HPCs, instead of BM, have become the principal cellular source for reconstitution of the hematopoietic system following myeloablative and non-myeloablative therapy. Granulocyte-colony stimulating factor (G-CSF) is most widely used agent in clinic for mobilization [2]. The advantages of G-CSF are that 1) the mobilization efficiency is relatively potent and predictable, 2) the safety to donor is established. However, several problems are remained to be solved such as 1) necessity of at least 4 day-administration of high dose of G-CSF, 2) high cost of this recombinant protein, 3) bone pain, headache and fever during administration, 4) a certain population of unpredictable poor mobilizers [3]. It is important to characterize new agents which can induce rapid and efficient mobilization to overcome these problems.

The investigation for mechanistic insights of G-CSF-induced mobilization has been focused on mainly chemokine SDF-1/CXCL12 in the BM and its cognate receptor CXCR4 on HSCs/HPCs. It has been reported that proteolytic or transcriptional down-regulation of SDF-1 in the BM may be the main reason for the loss of the

retention signal resulting in release of HSCs/HPCs to the periphery [4-7]. Moreover, treatment with AMD3100, a specific CXCR4 antagonist, induces rapid and robust HPC mobilization [8]. Interestingly, a membrane-bound extracellular peptidase CD26 (dipeptidylpeptidase IV [DPPIV]) that cleaves dipeptides from the N-terminus of polypeptides including SDF-1, has been reported to be expressed on HPCs and indispensable for G-CSF-induced mobilization [9, 10]. Collectively, these data strongly suggest that the modulation of SDF-1 oriented molecules, such as SDF-1 itself produced in microenvironment and CXCR4/CD26 expressed on HSCs/HPCs, is crucial for mobilization.

Colominic acid (CA) is a homopolymer of N-acetylneuraminic acid (sialic acid) containing α-2,8 ketosidic linkages between the sugar moieties [11]. It has been reported that sulfated colominic acid (SCA, structure is shown in supplemental Fig. 1S) exhibits anti-HIV activity [12], and suppresses scrapie prion protein (PrP^{Sc}) and HIV-1 gp-120-induced neuronal cell death [13]. Since recent accumulating evidences suggest that CXCR4 and CD26 play critical roles in HIV entry as co-receptors [14, 15], it is possible that SCA modulates CXCR4 and/or CD26. Thus, we hypothesized that SCA would regulate HSC/HPC trafficking by modulating SDF-1 oriented molecules.

Materials and methods

Mice and cell lines

C57BL/6, DBA/2 and Balb/c mice were purchased from Charles River Japan (Tsukuba, Japan). C57BL/6-CD45.1 congenic mice were purchased from Charles River Laboratories (Frederick Cancer Research Center, Frederick, MD). CD26-deficient mice were generated by gene targeting [16] and were backcrossed 10 generations into Balb/c background. All animals used in this study were matched for sex and age in each experiment for the comparison purpose (7-12 week-old). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center. FDCP-mix mouse progenitor cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

Mobilization of hematopoietic stem/progenitor cells

SCA and CA were prepared in Marukin Bio, Inc. (Kyoto, Japan) as previously described [12]. Endotoxin in SCA solution was under detection level by Toxicolor LS-20 (Seikagaku, Tokyo, Japan). For mobilization studies, mice were injected intravenously with a single dose of CA or SCA diluted in phosphate-buffered saline (PBS) (100 mg/kg). Mice were bled at various time points post-administration of SCA. Since mobilization effect peaked at 30 min, mice were treated with CA or SCA 30 min prior to harvesting blood in the following mobilization studies. Inhibition of endogenous CD26/DPPIV activity was accomplished by intraperitoneal injection of 30 μ mol Diprotin A (Sigma, St. Louis, MO) 30 min prior to SCA injection. For G-CSF-induced mobilization, mice were subcutaneously injected with recombinant human G-CSF (filgrastim, Kirin Brewery, Tokyo, Japan, 250 μ g/kg/day, every 12 hours, 8 divided doses) in PBS supplemented with 0.1% BSA and were bled 3 hours following the last dose of G-CSF.

Long-term competitive reconstitution

Stem cell activities of blood mobilized by 2 day-G-CSF + single dose of SCA and by 4 day-G-CSF were compared by long-term competitive reconstitution. C57BL/6 mice

(CD45.2) and Ly5.2 congenic mice (CD45.1) were treated with 2 day-G-CSF+SCA and 4 day-G-CSF, respectively. The number of CFU-Cs in the blood obtained from each donor was assessed as described above. As predicted, the average ratio of CFU-Cs in the same volume of blood from each group was 49 : 51, respectively (n=5). The same volume (150 μ l) of blood from a pair of donor mice were mixed and injected into the tail vein of a lethally (13 Gy, 2 split doses) irradiated CD45.1 recipient mouse. The proportion of peripheral blood leukocytes bearing CD45.1 or CD45.2 antigen was determined monthly after transplantation by flow cytometry as previously described [17].

Chemotaxis assay

IMDM supplemented with 0.5% BSA with or without 200 ng/ml rhSDF-1 α (R&D Systems, Minneapolis, MN) was added to the lower chamber of 24-well transwell (pore size, 5 μm; Corning, NY). Cells were treated with PBS or 1 mg/ml SCA for 30 min on ice and washed twice with PBS and once with IMDM+0.5% BSA. We chose this concentration because it was predicted by calculation in the mouse blood immediately after injection of 100mg/kg SCA. Then, 1 x 10^6 cells with or without 10^{-7}M

4F-Benzonyl-TN14003 (a CXCR4 inhibitor) [18] or 400 μ M NSC23766 (a Rac inhibitor) [19] in IMDM+0.5% BSA were loaded to the upper chamber and were allowed to migrate for 3 hours at 37°C. Migrated cells were collected from the lower chamber and counted using a hemocytometer or assayed for CFU-C.

Antibodies, flow cytometry, colony-forming units in culture (CFU-C) assay, ELISA, statistics

These methods can be found in online supplemental materials.

Results

SCA induces rapid hematopoietic progenitor mobilization

First, we injected SCA 100mg/kg i.v. into C57BL/6 mice, and WBC count and the number of CFU-Cs in the blood were assessed. As shown in Fig. 1A, WBC was increased 3.3-fold (peaked at 60 min, n= 6-9, $p < 0.01$), and the number of CFU-C was increased 5.1-fold (peaked at 30 min, n= 9-12, $p<0.01$) following injection, and returned to baseline by 5 hours. It is well known that broad variability exists in mobilization of HSCs/HPCs in different strains of mice [20]. Thus, we compared the mobilizing capability of SCA in C57BL/6 mice which respond poorly to G-CSF and DBA/2 mice which are relatively good mobilizers [20]. As shown in Fig. 1B, HPC mobilization with single administration of 100mg/kg SCA was greater in DBA/2 compared with C57BL/6 mice, displaying a similar phenomenon as G-CSF-induced mobilization. We also confirmed that SCA mobilizes HPCs in dose dependent manner up to 100mg/kg (Fig. 1C). To investigate whether the sulfation is important for SCA-induced HPC mobilization, DBA/2 mice were treated with single-dose (100 mg/kg) of CA or SCA. As shown in Fig. 1D, CA displayed no mobilization, suggesting that the sulfation of CA is critical for mobilization activity.

CD26/Dipeptidyl peptidase (DPPIV) is not required for SCA-induced mobilization

It has been reported that CD26/DPPIV [9, 10] and chemokine SDF-1/ receptor CXCR4 axis [5, 6] play critical roles in G-CSF-induced mobilization. Since CD26/DPPIV and CXCR4 are co-modulated in HIV entry [15] and SCA has anti-HIV activity [12], we hypothesized that SCA might modulate CD26 and/or CXCR4 to induce HPC mobilization. We first focused on CD26/DPPIV. As shown in Figs. 1E and 1F, pre-treatment of DBA/2 mice with a CD26 inhibitor Diprotin A showed no inhibitory effect in HPC mobilization whereas it significantly inhibited the increase of leukocytes ($n=3$-5, $p<0.01$), suggesting different requirement of DPPIV activity for SCA-induced peripheralization of mature and immature hematopoietic cells. Although not as drastic as previously reported probably due to different genetic background [9, 10], G-CSF-induced mobilization in CD26-deficient (CD26-/-) Balb/c mice was reduced compared to that in wild-type (WT) control (Fig. 1G, $n=3$-6, $p<0.05$). Whereas, SCA-induced mobilization in CD26-/- mice was similar to that in WT control (Fig. 1H, $n=3$-6). These results suggest that, in contrast to G-CSF-induced mobilization, CD26 is dispensable for SCA action.

SCA enhances CXCR4 function on hematopoietic progenitors

Next, to assess the functional alteration of CXCR4, we tested the migration potential of SCA-treated FDCP-mix, a mouse progenitor cell line, toward SDF-1 in a transwell. SCA treatment strongly enhanced FDCP-mix migration toward SDF-1 (Fig. 2A, n=8, $p<0.05$) which was completely blocked by the presence of a CXCR4 inhibitor 4F-Benzonyl-TN14003 (Fig. 2A) or a Rac inhibitor NSC23766 (Fig. 2B). These results suggest that SCA may enhance the CXCR4 signaling including the Rac activation in HPCs. Then, we investigated the effects of SCA on primary HPCs. As shown in Fig. 2C, SCA significantly enhanced migration of primary BM CFU-Cs from DBA/2 mice. This effect was completely abrogated by CXCR4 inhibitor. In contrast, in C57BL/6 mice, baseline efficiency of CFU-C migration was lower than DBA/2 and SCA showed no effect (Fig. 2C). This may explain, at least partially, the reason for poor mobilization in C57BL/6 mice. Surface expression of CXCR4 was not significantly increased by SCA treatment in BMMCs, lineage- cells, and LK cells (Fig. 2D). Rather, SCA completely inhibited the down-regulation of CXCR4 upon interaction with SDF-1 (Fig. 2D). These results suggest that SCA enhances CXCR4 function on HPCs by stabilizing its expression on cell surface and inducing continuous maximum signaling in the presence of the ligand.

SCA increases SDF-1 level in peripheral blood but does not decrease in BM or bone

After injection of fucoidan, a natural sulfated fucose polymer extracted from seaweed, SDF-1 level in serum increases whereas its level in BM decreases drastically [21], suggesting that fucoidan sweeps SDF-1 in BM and takes it into the circulation. We then evaluated the alteration of SDF-1 distribution by evaluating its levels in serum, BM, and bone after SCA injection. We used 2 different capture antibodies for ELISA, MAB350 whose binding site on SDF-1 molecule has not been determined (R&D systems) and K15C which detects only non-truncated active SDF-1 [22]. As shown in Fig. 3A, serum SDF-1 level of DBA/2 mice 30 minutes after SCA injection was detected at similar levels between MAB350 and K15C, and was increased approximately 5 times compared to PBS-injected group ($n=5$, $p<0.0001$). An increase of serum SDF-1 level was also observed in C57BL/6 mice, but to the lesser extent (Fig. 3A). This increased SDF-1 did not come from BM cavity and not even bone, since SDF-1 protein levels in BMEF and bone were unchanged during SCA treatment (Figs. 3B and 3C). These results suggest that SCA may induce mobilization by not only enhancing CXCR4

function on HPCs but also by the increased SDF-1 in the circulation.

SCA cooperates with G-CSF in stem and progenitor mobilization

To test the potential clinical application, the mobilizing efficiency of SCA in combination with G-CSF was assessed. C57BL/6 mice were injected with G-CSF for 2 days or 4 days, and 30 min prior to bleeding mice were injected with single dose of vehicle or SCA. As shown in Fig. 4A, G-CSF-induced HPC mobilization was strongly enhanced by the addition of single dose of SCA ($n=5\text{-}6$, $p<0.001$). Since 2 day-G-CSF+SCA showed equivalent progenitor mobilization efficiency to that of full-term (4 days) G-CSF, we compared the stem cell activities in mobilized blood of these two protocols. Same volume of blood from C57BL/6 mice (CD45.2) treated with 2 day-G-CSF+SCA and Ly5.2 mice (CD45.1) treated with 4 day-G-CSF were mixed, and injected into lethally irradiated Ly5.2 (CD45.1) mice (Fig. 4B). As shown in Fig. 4C, the percentage of peripheral blood leukocytes derived from engrafted stem cells originated from CD45.2+ donor was more than 50% for 24 weeks after transplantation. This suggests that 2 day administration of G-CSF together with single dose of SCA mobilizes long-term reconstituting stem cells as efficient as usual G-CSF protocol and may be sufficient for clinical mobilization.

Discussion

Here, we demonstrated that a synthetic polysaccharide SCA rapidly mobilizes HSCs/HPCs by inducing two concurrent events, 1) enhancement of CXCR4 function (inhibition of ligand-induced down-regulation) and 2) increment of SDF-1 concentration in the blood.

Fucoidan can also mobilize HSCs/HPCs [23, 24], and binds L-selectin on hematopoietic cells resulting in up-regulation of surface CXCR4 expression [25]. However, we confirmed that SCA does not bind L-selectin (supplemental Fig. 2S), suggesting that SCA and fucoidan modulate CXCR4 function in different mechanism.

It has been reported that SDF-1 level in BM is sharply decreased after fucoidan treatment, suggesting that increased serum SDF-1 was brought from BM parenchyma [21]. However, following SCA injection, SDF-1 levels in BM and even bone were not altered (Fig. 3). This suggests that increased serum SDF-1 after SCA administration does not originate from BM cavity but perhaps from extramedullary sites. Recently, Kiel et al. have reported that hematopoietic stem cell niche is located not only at osteoblastic area near the endosteum but also at the vascular area [26]. Since our ELISA data showed that SDF-1 levels in BM parenchyma and bone were not altered after SCA treatment, it is likely that stem/progenitor cells located at osteoblastic area

cannot egress from BM even if CXCR4 function of these cells is up-regulated. One possible scenario for SCA mobilization is that stem/progenitor cells located at the vascular area are strongly attracted by rapidly increased serum SDF-1 via enhanced CXCR4 function on the surface of these cells.

According to the cooperative effect between G-CSF and SCA (Fig. 4), it may be possible to shorten the mobilization protocol in clinic. This would be a great benefit for cost effectiveness and the reduction of side effects. Furthermore, addition of SCA to full-term G-CSF may overcome poor mobilization cases in a certain population of healthy donors and in patients with some malignancies such as multiple myeloma. The mechanism of this cooperative action remains to be elucidated.

Modulation of SDF-1-CXCR4 axis is critical in controlling the travel of CXCR4 expressing cells. SCA may be useful not only in HSC mobilization but also in targeting cancer stem cells which express CXCR4 [27].

Acknowledgment

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References

[1] T. Lapidot, and I. Petit, Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells, Exp Hematol 30 (2002) 973-981.
[2] L. B. To, D. N. Haylock, P. J. Simmons, and C. A. Juttner, The biology and clinical uses of blood stem cells, Blood 89 (1997) 2233-2258.
[3] S. Fu, and J. Liesveld, Mobilization of hematopoietic stem cells, Blood Rev 14 (2000) 205-218.
[4] Y. Katayama, M. Battista, W. M. Kao, A. Hidalgo, A. J. Peired, S. A. Thomas, and P. S. Frenette, Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow, Cell 124 (2006) 407-421.
[5] J. P. Levesque, J. Hendy, Y. Takamatsu, P. J. Simmons, and L. J. Bendall, Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide, J Clin Invest 111 (2003) 187-196.
[6] I. Petit, M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R. S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot, G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4, Nat Immunol 3 (2002) 687-694.
[7] C. L. Semerad, M. J. Christopher, F. Liu, B. Short, P. J. Simmons, I. Winkler, J. P. Levesque, J. Chappel, F. P. Ross, and D. C. Link, G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow, Blood (2005).
[8] H. E. Broxmeyer, C. M. Orschell, D. W. Clapp, G. Hangoc, S. Cooper, P. A. Plett, W. C. Liles, X. Li, B. Graham-Evans, T. B. Campbell, G. Calandra, G. Bridger, D. C. Dale, and E. F. Srour, Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist, J Exp Med 201 (2005) 1307-1318.
[9] K. W. Christopherson, 2nd, S. Cooper, and H. E. Broxmeyer, Cell surface peptidase CD26/DPPIV mediates G-CSF mobilization of mouse progenitor cells, Blood 101 (2003) 4680-4686.
[10] K. W. Christopherson, S. Cooper, G. Hangoc, and H. E. Broxmeyer, CD26 is essential for normal G-CSF-induced progenitor cell mobilization as determined by CD26-/- mice, Exp Hematol 31 (2003) 1126-1134.
[11] E. J. McGuire, and S. B. Binkley, The Structure And Chemistry Of Colominic Acid, Biochemistry 3 (1964) 247-251.
[12] D. W. Yang, Y. Ohta, S. Yamaguchi, Y. Tsukada, Y. Haraguchi, H. Hoshino, H. Amagai, and I. Kobayashi, Sulfated colominic acid: an antiviral agent that inhibits the human

immunodeficiency virus type 1 in vitro, Antiviral Res 31 (1996) 95-104.
[13] H. Ushijima, S. Perovic, J. Leuck, P. G. Rytik, W. E. Muller, and H. C. Schroder, Suppression of PrP(Sc)- and HIV-1 gp120 induced neuronal cell death by sulfated colominic acid, J Neurovirol 5 (1999) 289-299.
[14] E. A. Berger, P. M. Murphy, and J. M. Farber, Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease, Annu Rev Immunol 17 (1999) 657-700.
[15] C. Herrera, C. Morimoto, J. Blanco, J. Mallol, F. Arenzana, C. Lluis, and R. Franco, Comodulation of CXCR4 and CD26 in human lymphocytes, J Biol Chem 276 (2001) 19532-19539.
[16] D. Marguet, L. Baggio, T. Kobayashi, A. M. Bernard, M. Pierres, P. F. Nielsen, U. Ribel, T. Watanabe, D. J. Drucker, and N. Wagtmann, Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26, Proc Natl Acad Sci U S A 97 (2000) 6874-6879.
[17] Y. Katayama, A. Hidalgo, B. C. Furie, D. Vestweber, B. Furie, and P. S. Frenette, PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin, Blood 102 (2003) 2060-2067.
[18] H. Tamamura, M. Fujisawa, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka, and N. Fujii, Identification of a CXCR4 antagonist, a T140 analog, as an anti-rheumatoid arthritis agent, FEBS Lett 569 (2004) 99-104.
[19] Y. Gao, J. B. Dickerson, F. Guo, J. Zheng, and Y. Zheng, Rational design and characterization of a Rac GTPase-specific small molecule inhibitor, Proc Natl Acad Sci U S A 101 (2004) 7618-7623.
[20] A. W. Roberts, S. Foote, W. S. Alexander, C. Scott, L. Robb, and D. Metcalf, Genetic influences determining progenitor cell mobilization and leukocytosis induced by granulocyte colony-stimulating factor, Blood 89 (1997) 2736-2744.
[21] E. A. Sweeney, H. Lortat-Jacob, G. V. Priestley, B. Nakamoto, and T. Papayannopoulou, Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells, Blood 99 (2002) 44-51.
[22] A. Amara, O. Lorthioir, A. Valenzuela, A. Magerus, M. Thelen, M. Montes, J. L. Virelizier, M. Delepierre, F. Baleux, H. Lortat-Jacob, and F. Arenzana-Seisdedos, Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine, J Biol Chem 274 (1999) 23916-23925.
[23] P. S. Frenette, and L. Weiss, Sulfated glycans induce rapid hematopoietic progenitor cell mobilization: evidence for selectin-dependent and independent mechanisms, Blood 96 (2000) 2460-2468.
[24] E. A. Sweeney, G. V. Priestley, B. Nakamoto, R. G. Collins, A. L. Beaudet, and T.

Papayannopoulou, Mobilization of stem/progenitor cells by sulfated polysaccharides does not require selectin presence, Proc Natl Acad Sci U S A 97 (2000) 6544-6549.
[25] Z. Ding, T. B. Issekutz, G. P. Downey, and T. K. Waddell, L-selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration, Blood 101 (2003) 4245-4252.
[26] M. J. Kiel, O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison, SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells, Cell 121 (2005) 1109-1121.
[27] J. A. Burger, and T. J. Kipps, CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment, Blood 107 (2006) 1761-1767.

Figure legend

Fig. 1 SCA induces progenitor mobilization via CD26-independent mechanism.

(A) C57BL/6 mice were injected i.v. with 100mg/kg SCA and bled at the indicated times. Number of treated mice is listed within the bar. Blood was assayed for CFU-C (bars) or WBC (line). (B) C57BL/6 or DBA/2 mice were treated with PBS (white bar) or 100mg/kg SCA (black bar) and bled after 30 minutes, n=3-7. (C) DBA/2 mice were treated with the indicated dose of SCA, n=3-7. (D) DBA/2 mice were treated with PBS or 100mg/kg colominic acid (CA) or 100mg/kg SCA, n=7-12. (E, F) Inhibition of CD26 by a CD26 inhibitor Diprotin A. 30 minutes after pretreatment with PBS or 30 μmol Diprotin A (i.p.), DBA/2 mice were treated with PBS (white bar) or 100mg/kg SCA (black bar) and bled after 30 minutes. Blood was assayed for (E) WBC and (F) CFU-C. n=3-5. (G) Wild-type (CD26+/+) and CD26-deficient (CD26-/-) mice were treated with either PBS containing 0.1% BSA (white bar) or 250 μg/kg/day (2 divided doses) G-CSF (black bar) for four days. Three hours after the final dose of G-CSF, mice were bled to assess CFU-C numbers. n=3-6. (H) CD26+/+ and CD26-/- mice were injected i.v. with either PBS (white bar) or 100mg/kg SCA (black bar), and bled after 30 minutes. Blood was assayed for CFU-C. n=4-6. * P<0.05, ** P<0.01 compared to 0 min (A) or control groups, or between indicated two groups.

Fig. 2 SCA enhances CXCR4 function on progenitors.

(A, B) FDCP-mix cells treated with PBS or SCA ± (A) 4F-benzoyl-TN14003 (a CXCR4 inhibitor) or (B) NSC23766 (a Rac inhibitor) were tested in a transwell for their migration toward 200ng/ml SDF-1. n=3-8. (C) Bone marrow mononuclear cells (BMMCs) of DBA/2 or C57BL/6 mice were treated with PBS or SCA ± 4F-benzoyl-TN14003 and tested in a transwell for their migration toward 200ng/ml SDF-1. Input and migrated cells were assayed for CFU-C. n=3-8. (D) Surface CXCR4 expression in BMMCs of DBA/2 mice. BMMCs pretreated with PBS or 1mg/ml SCA were incubated with PBS or 200ng/ml SDF-1α. Histograms show immunofluorescence detection of isotype control (black histogram), or CXCR4 (red line; incubated with PBS, green line; incubated with SDF-1α) in lineage$^-$c-kit$^+$ (LK) fraction. Bar graphs show geometric mean fluorescence intensity after subtraction of isotype control value. *P<0.05 between indicated two groups. NS, not significant.

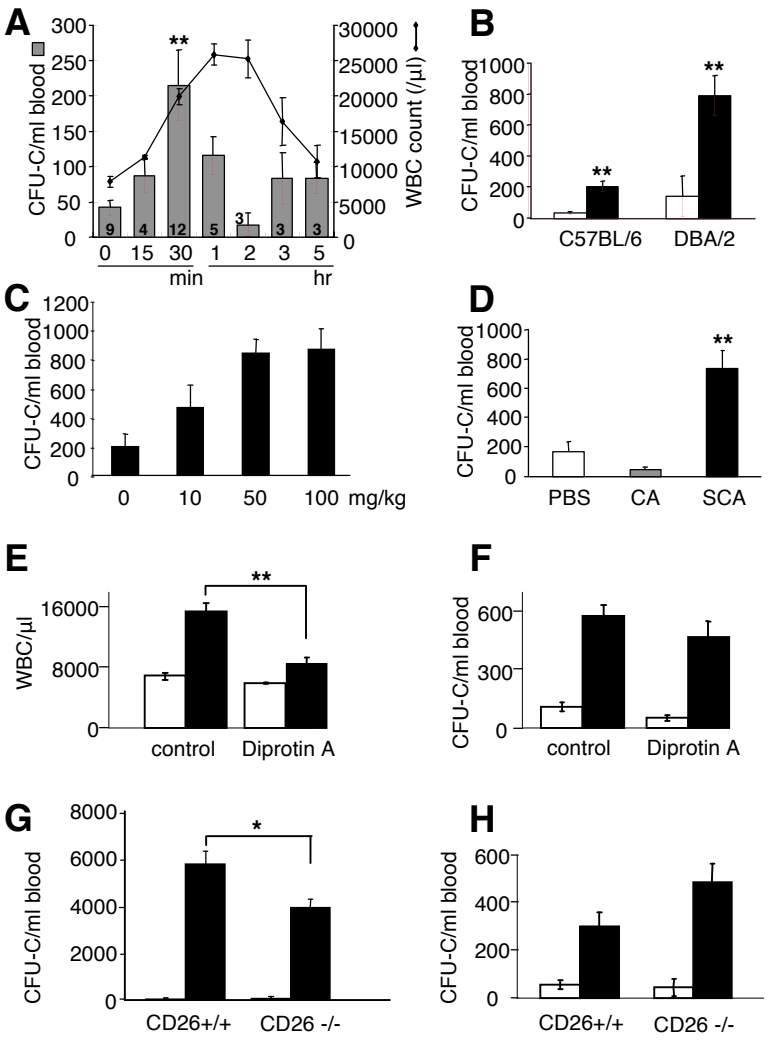
Fig. 3 SDF-1 levels after SCA treatment.

SDF-1 levels were measured by ELISA in (A) serum from DBA/2 and C57BL/6 mice, (B) bone marrow extracellular fluid (BMEF) from DBA/2 mice, (C) bone from DBA/2

mice after treatment with PBS or 100mg/kg SCA. n=4-5 per group, *P<0.05, **P<0.01, ***P<0.0001.

Fig. 4 SCA cooperates with G-CSF in progenitor and stem cell mobilization. (A) C57BL/6 mice were treated with 250 μg/kg/day (2 divided doses) G-CSF for the indicated days. Three hours after the final dose of G-CSF, mice were bled to assess CFU-C numbers. PBS or 100mg/kg SCA was injected 30 minutes prior to bleeding. n=5-6, **P<0.001 compared with the PBS injected group at each time point. (B) Competitive reconstitution. Lethally irradiated Ly5.2 (CD45.1+) mice were injected with blood mixture consisted of the same volume of 2 day-G-CSF+SCA-mobilized blood from C57BL/6 (CD45.2+) mice and 4 day-G-CSF-mobilized blood from Ly5.2 (CD45.1+) mice. Recipient mice were bled monthly and the percentage of CD45.1/CD45.2 was assessed. (C) Chimerism of peripheral blood leukocytes. n=5 at up to 12 weeks, n=3-4 at later periods.

Fig. 1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CFU-C/ml blood
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Diprotin A
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CFU-C/ml blood
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CD26+/+
CD26 -/-
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CFU-C/ml blood
CD26+/+
CD26 -/-

Fig. 2

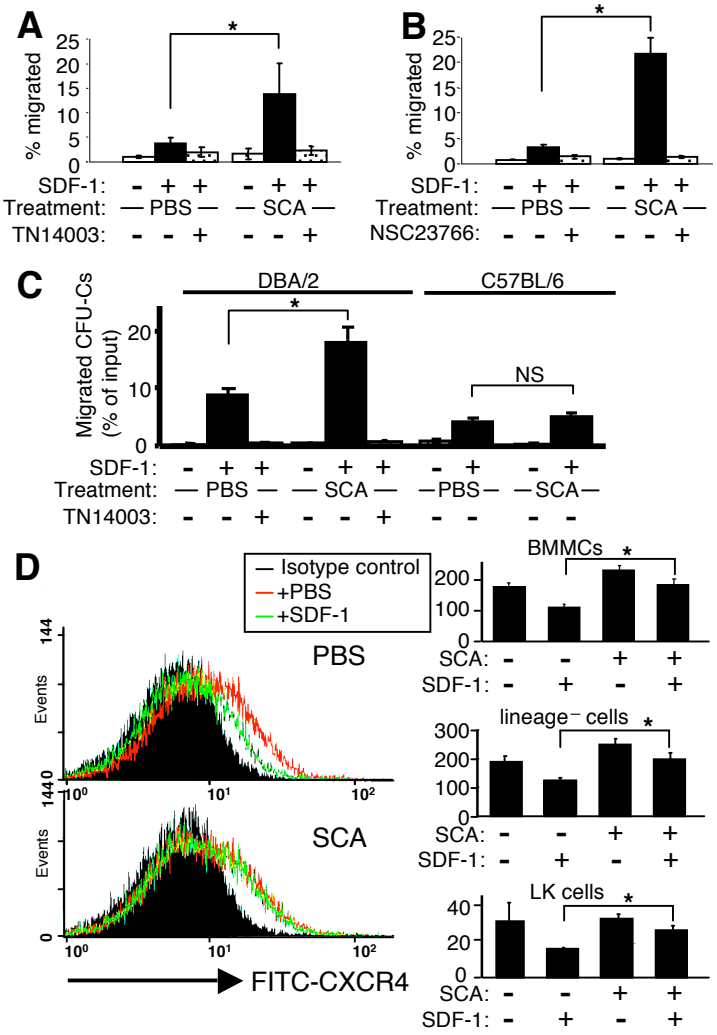

A
% migrated
25
20
15
10
5
0
*
SDF-1: - + + - + +
Treatment: — PBS — — SCA —
TN14003: - - + - - +
B
% migrated
25
20
15
10
5
0
*
SDF-1: - + + - + +
Treatment: — PBS — — SCA —
NSC23766: - - + - - +
C
DBA/2
C57BL/6
Migrated CFU-Cs (% of input)
20
10
0
*
NS
SDF-1: - + + - + + - + - +
Treatment: — PBS — — SCA — —PBS— —SCA—
TN14003: - - + - - + - - - -
D
— Isotype control
—+PBS
—+SDF-1
PBS
SCA
Events
144
0
1440
10⁰
10¹
10²
FITC-CXCR4
BMMCs
200
100
0
*
SCA: - - + +
SDF-1: - + - +
lineage⁻ cells
300
200
100
0
*
SCA: - - + +
SDF-1: - + - +
LK cells
40
20
0
*
SCA: - - + +
SDF-1: - + - +

Fig. 3

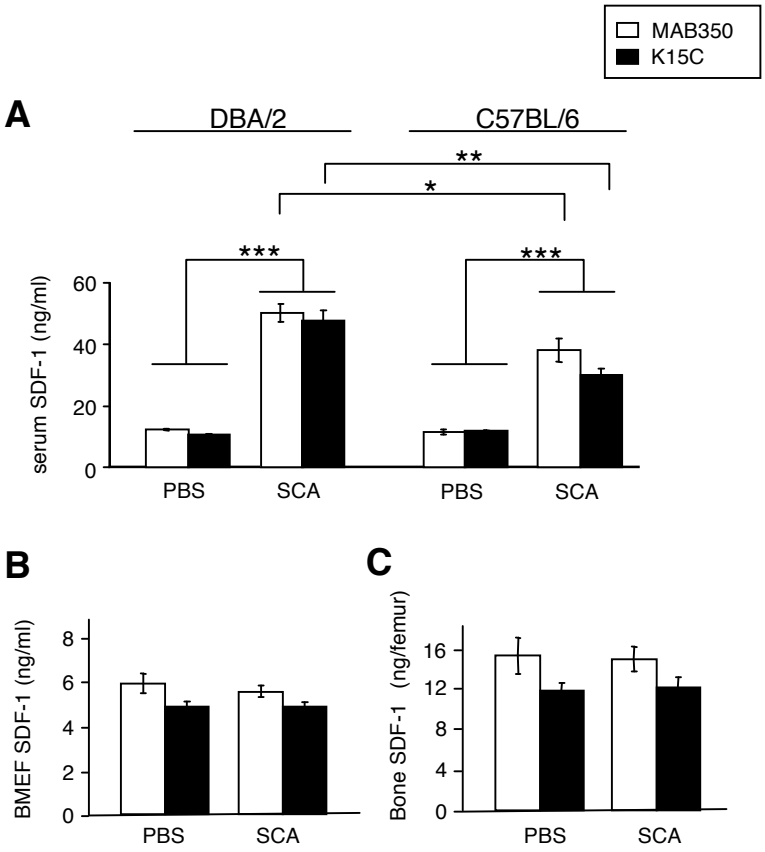
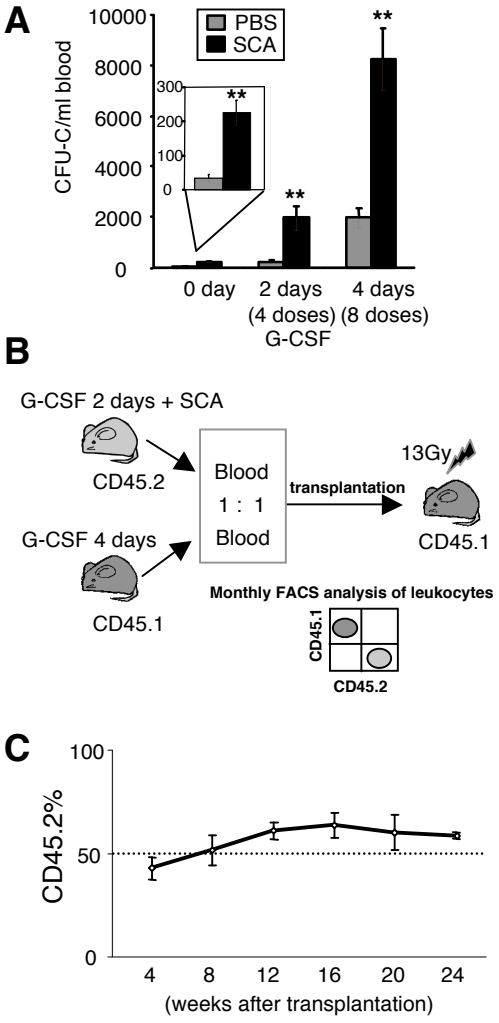
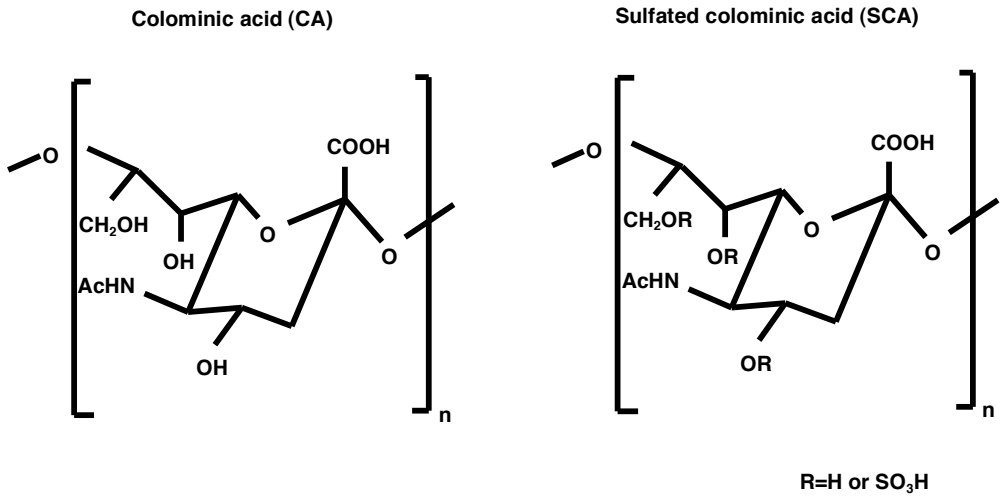

MAB350
K15C
A
DBA/2
C57BL/6
**
*
***
***
serum SDF-1 (ng/ml)
60
40
20
0
PBS
SCA
PBS
SCA
B
BMEF SDF-1 (ng/ml)
8
6
4
2
0
PBS
SCA
C
Bone SDF-1 (ng/femur)
16
12
8
4
0
PBS
SCA

Fig. 4


A
PBS
SCA
CFU-C/ml blood
10000
8000
6000
4000
2000
0
300
200
100
0
**
**
**
0 day
2 days
(4 doses)
4 days
(8 doses)
G-CSF
B
G-CSF 2 days + SCA
CD45.2
Blood
1 : 1
Blood
transplantation
13Gy
CD45.1
G-CSF 4 days
CD45.1
Monthly FACS analysis of leukocytes
CD45.1
CD45.2
C
CD45.2%
100
50
0
4
8
12
16
20
24
(weeks after transplantation)

Supplemental materials

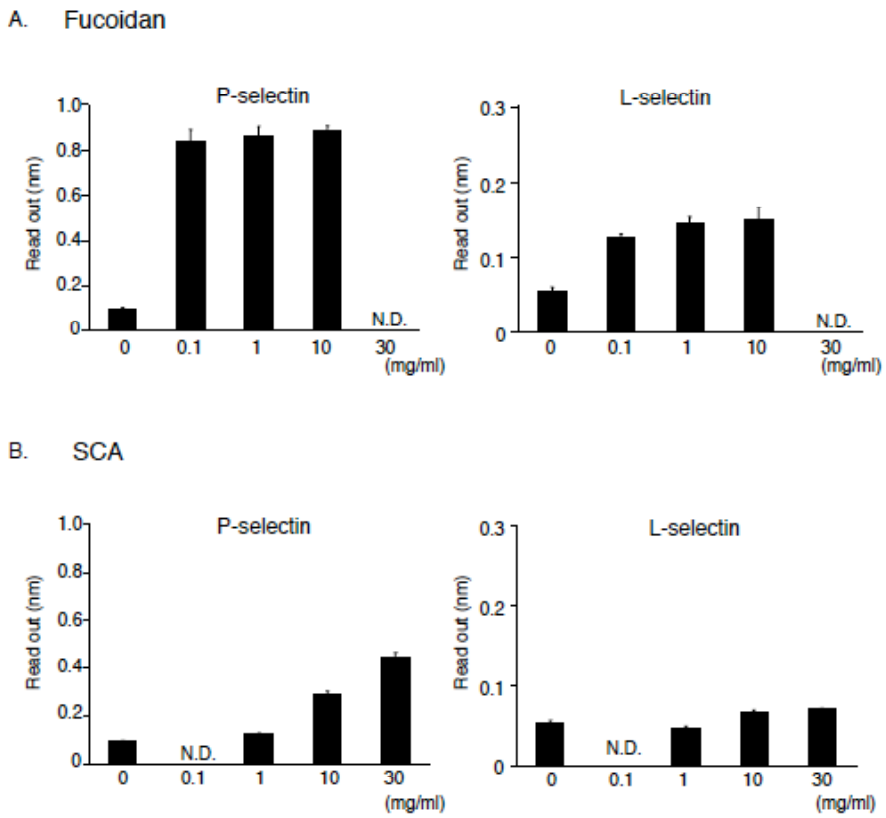


Supplemental Fig. 1S Structures of colominic acid (sialic acid polymer) and sulfated colominic acid.

Selectin binding assay

An immuno 96 microwell plate (Nunc, Roskilde, Denmark) was coated with fucoidan (Sigma, St. Louis, MO) or SCA at indicated concentration diluted in PBS overnight at 4℃. The plates were blocked with 5% BSA/PBS(Ca/Mg free) [BSA/PBS(-)] at room temperature for 2 hr, and incubated with 2 μ g/ml of recombinant mouse P- or L-selectin/Fc chimera (R&D Systems, Minneapolis, MN) diluted in PBS (Ca/Mg+) at 4℃ for 1.5h with gentle shaking. After washing thrice with BSA/PBS(-), the plate was incubated with biotinylated goat anti-human Fc (Jackson ImmunoResearch, West Grove, PA) diluted in PBS(-) at room temperature for 1 hr. The plate was washed thrice with BSA/PBS(-), incubated with NeutrAvidin-HRP (Pierce Biotechnology, Rockford, IL) diluted in PBS(-) at room temperature for 20min, washed thrice with PBS(-), and then

incubated with TMB substrate solution (Sigma) at room temperature for 30min. Binding reactivity was determined by measuring the optical density at 450nm in a microplate reader.



Supplemental Fig. 2S SCA does not bind L-selectin.

(A) Fucoidan efficiently bound both P- and L-selectins. (B) SCA bound P-selectin in a dose-dependent manner, however, it did not bind L-selectin. One representative result from 2 independent experiments which displayed similar results is shown. Mean ± SE of triplicates. ND, not done.

Supplemental methods

Antibodies

For flow cytometry, fluorescein isothiocyanate (FITC), Phycoerythrin (PE)-CXCR4, FITC- and PE-rat IgG2b, PE-Sca-1, allophycocyanin (APC)-c-kit, biotin-mouse lineage panel (Ly-76, CD3e, Mac-1, CD45R/B220 and Gr-1), biotin- CD45.1 and FITC-CD45.2 were from BD Pharmingen (San Diego, CA). APC-Cy7-streptoavidin was purchased from eBioscience (San Diego, CA). For SDF-1 ELISA, anti-SDF-1 mAb MAB350 and polyclonal biotinylated anti-SDF-1 antibody were from R&D Systems (Minneapolis, MN). K15C, monoclonal anti-SDF-1 antibody which specifically blocks binding site to CXCR4, is described elsewhere [1].

Isolation of cells and colony-forming units in culture (CFU-C) assays

Blood was harvested by retro-orbital sampling of mice anesthetized with isoflurane and collected in polypropylene tubes containing EDTA. Blood samples were diluted 10 times with PBS and white blood cell (WBC) counts were obtained using a hemocytometer after hemolysis in 3% acetic acid. Peripheral blood mononuclear cells

(PBMCs) were isolated from 40 μL of blood using Lymphosepar II (IBL, Tokyo, Japan). Bone marrow was harvested from mouse femur by flushing into 1×Hanks Balanced Salt Solution (HBSS), and bone marrow mononuclear cells (BMMCs) were isolated by underlaying 65% Percoll (Amersham Biosciences AB, Uppsala, Sweden) followed by centrifugation at 4℃ at 1200rpm for 30 minutes. Isolated cells were washed twice before use. CFU-Cs were assessed by inoculating PBMCs or BMMCs into Methocult M3534 media (StemCell Technologies, Vancouver, Canada) according to manufacturer's recommendation.

Flow cytometry

Surface expression of CXCR4 in BMMCs was evaluated by flow cytometry. Cells were treated with PBS or 1mg/ml SCA for 30 min, washed twice in IMDM, incubated with PBS or 200ng/ml rhSDF-1α (R&D systems) at 37℃ for 1 hr, washed twice in PBS containing 2mM EDTA and 0.5% bovine serum albumin (BSA) (PEB), and stained with FITC-isotype matched control or FITC-anti-mouse CXCR4 together with PE-Sca-1, APC-c-kit and biotin-mouse lineage panel followed by the staining with APC-Cy7-streptoavidin. Analysis was performed on FACSAria flow cytometer (Becton

Dickinson, Mountain View, CA).

SDF-1 ELISA

Blood serum and bone marrow extracellular fluid (BMEF) were obtained as previously described [2]. Bone protein was extracted as described previously [3]. Briefly, mouse femur bone was harvested after flushing out BM and frozen in liquid nitrogen, and then pulverized into powder. Protein was extracted from the bone powder in extraction medium (0.05M EDTA, 4M Guanidine-HCl, 30mM Tris, 1mg/ml BSA, pH 7.4) containing protease inhibitor cocktails (SIGMA), and 1mM phenylmethansulfonyl fluoride (PMSF) (SIGMA) by rotating for 24 hours. Samples were dialyzed against PBS with Slide-A-Lyzer (MWCO 3500, Pierce Biotechnology) with an extensive change of PBS for 72 hours. For SDF-1 ELISA, Immuno 96 microwell plates (Nunc, Roskilde, Denmark) coated with 100 μl of anti-SDF-1 mAb MAB350 (R&D systems) at 2 μ g/ml or K15C at 20 μg/ml (optimized in our lab) diluted in PBS were used. The following procedure was performed exactly as described elsewhere [4].

Statistics

The data are presented as means ± SEM. Mann-Whitney's U-test was used for

non-parametric comparison. $P<0.05$ was considered statistically significant.

References for supplemental materials

[1] A. Coulomb-L'Hermin, A. Amara, C. Schiff, I. Durand-Gasselin, A. Foussat, T. Delaunay, G. Chaouat, F. Capron, N. Ledee, P. Galanaud, F. Arenzana-Seisdedos, and D. Emilie, Stromal cell-derived factor 1 (SDF-1) and antenatal human B cell lymphopoiesis: expression of SDF-1 by mesothelial cells and biliary ductal plate epithelial cells, Proc Natl Acad Sci U S A 96 (1999) 8585-8590.

[2] A. Hidalgo, A. J. Peired, L. A. Weiss, Y. Katayama, and P. S. Frenette, The integrin alphaMbeta2 anchors hematopoietic progenitors in the bone marrow during enforced mobilization, Blood 104 (2004) 993-1001.

[3] Y. Katayama, M. Battista, W. M. Kao, A. Hidalgo, A. J. Peired, S. A. Thomas, and P. S. Frenette, Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow, Cell 124 (2006) 407-421.

[4] I. Petit, M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R. S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot, G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4, Nat Immunol 3 (2002) 687-694.