CXCR4-Mediated Bone Marrow Progenitor Cell Maintenance and Mobilization Are Modulated by c-kit Activity

Min Cheng, Junlan Zhou, Min Wu, Chan Boriboun, Tina Thorne, Ting Liu, Zhifu Xiang, Qiatang Zeng, Toshikazu Tanaka, Yao Liang Tang, Raj Kishore, Michael H. Tomasson, Richard J. Miller, Douglas W. Losordo, Gangjian Qin

Rationale: The mobilization of bone marrow (BM) progenitor cells (PCs) is largely governed by interactions between stromal cell-derived factor (SDF)-1 and CXC chemokine receptor (CXCR)4. Ischemic injury disrupts the SDF-1–CXCR4 interaction and releases BM PCs into the peripheral circulation, where the mobilized cells are recruited to the injured tissue and contribute to vessel growth. BM PCs can also be mobilized by the pharmacological CXCR4 antagonist AMD3100, but the other components of the SDF-1–CXCR4 signaling pathway are largely unknown. c-kit, a membrane-bound tyrosine kinase and the receptor for stem cell factor, has also been shown to play a critical role in BM PC mobilization and ischemic tissue repair.

Objective: To investigate the functional interaction between SDF-1–CXCR4 signaling and c-kit activity in BM PC mobilization.

Methods and Results: AMD3100 administration failed to mobilize BM PCs in mice defective in c-kit kinase activity or in mice transplanted with BM cells that expressed a constitutively active c-kit mutant. Furthermore, BM levels of phosphorylated (phospho)–c-kit declined after AMD3100 administration and after CXCR4 deletion. In cells adhering to culture plates coated with vascular cell adhesion molecule 1, SDF-1 and stem cell factor stimulated c-kit phosphorylation. SDF-1–induced c-kit phosphorylation also required the activation of Src nonreceptor tyrosine kinase; pretreatment of cells with a selective Src inhibitor blocked both c-kit phosphorylation and the interaction between c-kit and phospho-Src.

Conclusions: These findings indicate that the regulation of BM PC trafficking by SDF-1 and CXCR4 is dependent on Src-mediated c-kit phosphorylation. (Circ Res. 2010;107:1083-1093.)

Key Words: CXCR4 • c-kit • stem cells • bone marrow • mobilization

The mobilization or release of progenitor cells (PCs) from the bone marrow (BM) to the peripheral blood (PB) is highly regulated and occurs both under normal conditions and in response to stress. PB PCs have an essential role in blood cell formation and homeostasis and in the response of various tissues to noxious insults. Thus, therapies that enhance PC mobilization are being investigated as novel strategies for promoting tissue repair. Ample evidence suggests that PCs are retained in the BM by interactions between the CXC chemokine stromal cell–derived factor (SDF)-1 and CXC chemokine receptor (CXCR)4. CXCR4 is expressed by mononuclear cells (MNCs), and SDF-1 is expressed by osteoblasts, endothelial cells, and a subset of reticular cells scattered throughout the BM. Ischemic injury disrupts the SDF-1–CXCR4 interaction and releases BM PCs into the peripheral circulation; then, the mobilized cells are recruited to...
the injured tissue and contribute to vessel growth.\textsuperscript{11,12} BM PCs can also be mobilized by the pharmacological CXCR4 antagonist AMD3100,\textsuperscript{13–15} but the downstream components of the SDF-1–CXCR4 signaling pathway are largely unknown.

Like CXCR4, c-kit is expressed predominantly in BM PCs, and the ligand for c-kit, stem cell factor (SCF), is constitutively produced by BM endothelial cells and fibroblasts.\textsuperscript{16} c-kit is a class III receptor tyrosine kinase, and administration of a c-kit-neutralizing antibody (ACK2) to wild-type (WT) mice released BM cells to the peripheral circulation and enhanced the engraftment of intravenously injected BM cells.\textsuperscript{17} On the other hand, PC mobilization is markedly blunted in c-kit\textsuperscript{w/w–v} mutant mice, which are defective in c-kit phosphorylation. AMD3100 administration failed to mobilize BM PCs in c-kit\textsuperscript{w/w–v} mice or in mice transplanted with BM cells that expressed a constitutively active c-kit mutant, and BM levels of phosphorylated (phospho)–c-kit declined after both AMD3100 administration and induction of a CXCR4 knockout mutation. In cells adhering to plates coated with vascular cell adhesion molecule (VCAM)-1, SDF and SCF increased phospho–c-kit levels, and AMD3100 treatment suppressed SDF-1–induced, but not SCF-induced, c-kit phosphorylation. Furthermore, pretreatment of cells with a selective Src inhibitor blocked both c-kit phosphorylation and the interaction between c-kit and phospho-Src. Collectively, these results suggest that the regulation of BM PC trafficking by SDF-1 and CXCR4 is linked to Src-mediated c-kit phosphorylation.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

#### Mice

c-kit\textsuperscript{W/W–v} (WBB6F1/J-Kit\textsuperscript{W/W–v}/J) and WT mice (male, 10 to 12 weeks old) were purchased from The Jackson Laboratory. CXCR4\textsuperscript{WT} and CXCR4\textsuperscript{WT/AC–GFP} transgenic mice were provided by Dr Richard J. Miller (Northwestern University).\textsuperscript{24,25} The Mx1-cre\textsuperscript{–} CXCR4\textsuperscript{WT} mice were bred by mating CXCR4\textsuperscript{WT} and Mx1-cre (The Jackson Laboratory) mice, and CXCR4 gene deletion was induced with 3 intraperitoneal injections of 250 μg of poly(I)-poly(C) (GE Healthcare) administered at 2-day intervals.\textsuperscript{26} All surgical procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University.

#### Isolation of BM and PB MNCs

BM and PB MNCs were isolated as described previously\textsuperscript{27} and as detailed in the Online Methods section.

#### PC Colony-Forming Assay

PB and BM MNCs were isolated, and the PC colony-forming assay was performed in 35-mm dishes with a semisolid methylcellulose medium containing SCF and other recombinant cytokines (MethoCult GF M3434, StemCell Technologies, Canada), as directed by the protocol of the manufacturer; 2×10\textsuperscript{4} PB MNCs and 2×10\textsuperscript{4} BM MNCs were seeded in each dish, and colonies were counted 12 days later. To ensure that c-kit\textsuperscript{W/W–v} PC levels would not be underestimated, Flt3-ligand (R&D Systems; final concentration, 100 ng/mL) was added to the medium to compensate for cells that may not respond well to SCF.

#### BM Clearance/Repopulation Assay

AMD3100 (5 mg/kg) was subcutaneously injected into WT and c-kit\textsuperscript{W/W–v} mice to mobilize PCs from the BM. Two hours later, 40×10\textsuperscript{4} BM MNCs that had been isolated from eGFP-transgenic mice (C57BL/6-Tg(Actb-eGFP); The Jackson Laboratory) were injected into the tail vein and allowed to repopulate the BM for 3 hours;\textsuperscript{26} then, BM MNCs were isolated from the recipients, and eGFP expression was evaluated by flow cytometry.

#### Flow Cytometry

Flow cytometric analyses of gene expression in isolated BM and PB MNCs were performed as described previously.\textsuperscript{26}

#### Transplantation of Retrovirally Transduced c-kit\textsuperscript{WT} and c-kit\textsuperscript{D816V} BM MNCs

MIG-HyKIT\textsuperscript{WT} and MIG-HyKIT\textsuperscript{D816V} retroviral vectors were generated by cotransfecting 293FT cells with a packaging plasmid, pIK6.1 MCV.ecopac.UTd, and 1 of 2 backbone plasmids: pMSCV-HyKIT\textsuperscript{WT}/IRES-eGFP (pMIG-HyKIT\textsuperscript{WT}) or pMSCV-HyKIT\textsuperscript{D816V}/IRES-eGFP (pMIG-HyKIT\textsuperscript{D816V}).\textsuperscript{28} Methods for virus generation, BM MNC isolation and ex vivo expansion, viral infection, and BM transplantation are described in the Online Methods section.

#### Tissue Sectioning and Immunofluorescent Staining

Mice were subcutaneously injected with AMD3100 (5 mg/kg) or PBS and euthanized 30 minutes later. Femurs were harvested, fixed, and immunofluorescently stained as described in the Online Methods section.
Cell Adhesion Assay
The BM MNC adhesion assay was performed as described previously.26

Western Blotting and Coimmunoprecipitation
Western blotting and coimmunoprecipitation was performed as described previously,30,31 and additional details can be found in the Online Methods section.

Quantitative Real-Time RT-PCR
Real-time RT-PCR was performed via standard techniques,32 as detailed in the Online Methods section.

Statistics
Data are presented as means±SEM. Comparisons between 2 means were assessed for significance with the unpaired Student’s t test; comparisons between 3 or more means were assessed for significance via ANOVA and Bonferroni multiple comparison procedures. Statistical significance was assigned if $P<0.05$.

Results
AMD3100-Induced PC Mobilization Is Impaired in c-kit<sup>W/W-V</sup> Mice
We initiated our investigation into the potential involvement of c-kit in CXCR4-mediated BM PC trafficking by quantifying the levels of colony forming PCs in the PB and BM of WT mice and mice defective in the kinase activity of c-kit (c-kit<sup>W/W-V</sup> mice). PB PC levels significantly increased and BM PC levels significantly declined 2 hours after AMD3100 injection in WT mice but not in c-kit<sup>W/W-V</sup> mice (Figure 1A and 1B). To determine whether the impaired mobilization observed in c-kit<sup>W/W-V</sup> mice was limited to specific subpopu-
AMD3100 Fails to Mobilize PCs That Express a Constitutively Active c-kit Mutant (c-kit<sup>DR16V</sup>)

Because AMD3100-induced BM PC mobilization is impaired in c-kit–kinase defective mice, we investigated whether increasing the level of activated c-kit would enhance AMD3100-induced mobilization. WT BM MNCs were transduced with retroviral vectors coding for eGFP and WT c-kit (c-kit<sup>WT</sup>) or eGFP and a constitutively active c-kit mutant (c-kit<sup>DR16V</sup>)<sup>28,33</sup> (Figure 2A and 2B) and then transplanted into WT mice after lethally irradiating the endogenous BM. Three weeks later, the numbers of BM MNCs in the 2 recipient strains were similar. Furthermore, the expression of eGFP and of the transgene were driven by the same promoter (CMV), so the high proportion of eGFP<sup>+</sup> BM and PB MNCs (Figure 2C) confirmed that transgene expression persisted for at least 3 weeks after transplantation. The constitutive activation of c-kit in BM MNCs from mice transplanted with c-kit<sup>DR16V</sup>–transduced cells was verified via Western blot (Online Figure II).

On the third week after BM transplantation, the recipient mice were treated with AMD3100 or PBS, and the numbers of eGFP<sup>+</sup> PCs in the BM and PB were determined 2 hours later. AMD3100 treatment significantly increased the number of eGFP<sup>+</sup> PB PCs (Figure 2D), and significantly decreased the number of eGFP<sup>+</sup> BM PCs (Figure 2E), in mice transplanted with MNCs that overexpressed c-kit<sup>WT</sup> but not in mice transplanted with c-kit<sup>DR16V</sup>–expressing MNCs. Thus, either a deficiency (Figure 1A and 1B) or the constitutive activation of c-kit kinase activity (Figure 2D and 2E) impaired AMD3100-induced BM PC mobilization. Collectively, these observations suggest that CXCR4-mediated BM PC mobilization requires a change in the phosphorylation state of c-kit.

SDF-1/CXCR4 Signaling Upregulates c-kit Phosphorylation in BM MNCs In Vitro

Previous reports suggest that c-kit participates in PC mobilization through an α4-integrin–mediated mechanism,<sup>19</sup> and that interactions between α4-integrin and VCAM-1 support the adhesion and retention of MNCs in the BM.<sup>26</sup> Thus, we performed a series of in vitro experiments to determine whether the phosphorylation state of c-kit is altered by α4-integrin–mediated adhesion and CXCR4 signaling. Freshly isolated WT and c-kit<sup>w/w-v</sup> BM MNCs were applied to VCAM-1–coated or uncoated plates, allowed to adhere for 15 minutes, incubated with or without AMD3100 for another 15 minutes, and then c-kit phosphorylation at tyrosine719 was evaluated via Western blotting. The VCAM-1 coating did not induce c-kit phosphorylation in c-kit<sup>w/w-v</sup> cells, but phospho–c-kit levels were notably higher in adherent WT cells (ie, cells from VCAM-1–coated plates) than in nonadherent WT cells (ie, cells from uncoated plates) (Figure 3A). In adherent WT cells, treatment with an α4-integrin–blocking antibody (Figure 3B) reduced phospho–c-kit levels, whereas treatment with the CXCR4 ligand SDF-1 markedly increased phospho–c-kit levels (Figure 3C). Furthermore, both SDF-1 and SCF (ie, c-kit ligand) induced c-kit phosphorylation, but c-kit levels were highest when the cells were incubated with both factors (Figure 3C), and AMD3100 treatment suppressed SDF-1–induced, but not SCF–induced, c-kit phosphorylation (Figure 3D). Notably, similar results were obtained after treating HEK293 cells that transiently expressed CXCR4 and c-kit with SDF-1, SCF, or AMD3100 alone and in combination (Figure 3E and 3F), and AMD3100 treatment did not downregulate c-kit phosphorylation in BM MNCs transduced with the constitutively active c-kit<sup>DR16V</sup> mutant (Figure 3G). Collectively, these observations suggest that MNC adhesion is associated with an increase in phospho–c-kit levels, and that in adherent MNCs, SDF-1 upregulates, and AMD3100 downregulates (in the presence of exogenous SDF-1), c-kit phosphorylation. Thus, AMD3100 appears to induce BM MNC mobilization through a decline in phospho–c-kit levels. Furthermore, SDF-1– and SCF–induced c-kit activation may occur independently and regulate different cellular activities.

Antagonism or Genetic Deletion of CXCR4 Downregulates BM c-kit Phosphorylation In Vivo

To determine whether CXCR4 also regulates BM MNC c-kit phosphorylation in vivo, WT mice were treated with AMD3100 or PBS and euthanized 30 minutes later, and then BM phospho–c-kit levels were evaluated by Western blot; phospho–c-kit levels were distinctly lower in mice administered AMD3100 than in PBS-treated mice (Figure 4A). These
findings were corroborated by breeding Mx1-cre<sup>−/−</sup> mice with CXCR4<sup>Cre</sup> mice<sup>24</sup> to generate Mx1-cre<sup>−/−</sup> CXCR4<sup>Cre</sup> offspring, which carry an inducible CXCR4 knockout mutation that is triggered by poly(I)-poly(C) injection.<sup>26</sup> Two weeks after the CXCR4 knockout mutation was induced, real-time RT-PCR analyses confirmed that CXCR4 expression had fallen to only ≈5% of the levels observed in CXCR4-expressing mice (Figure 4B), and BM phospho–c-kit levels had also declined (Figure 4C and 4D). These observations were corroborated by administering AMD3100 or PBS to CXCR4<sup>BAC</sup>GFP mice, which carry a BAC (bacterial artificial chromosome) coding for GFP expression driven by the human CXCR4 genomic regulatory sequence.<sup>25</sup> The mice were euthanized 30 minutes after treatment, and then sections of the femoral BM (Figure 2).
Figure 3. SDF-1/CXCR4 signaling upregulates c-kit phosphorylation in BM MNCs and in HEK293 cells in vitro. A through D, BM MNCs were isolated from WT and c-kit<sup>w/w-v</sup> mice and applied to uncoated plates or to plates coated with the stromal protein VCAM-1 to mimic adhesion in the BM. Fifteen minutes later, the levels of phospho–c-kit (P-c-kit) and total c-kit were analyzed by Western blot in WT and c-kit<sup>w/w-v</sup> cells (A), in WT cells incubated with or without an anti-α4-integrin antibody (Anti-α4 Ab) (B), and in WT cells incubated with the indicated combinations of SCF, SDF-1, or AMD3100 (C and D). P-c-kit levels were quantified densitometrically, normalized to the total c-kit level, and expressed as the fold difference from the levels measured in WT cells applied to uncoated plates and incubated without treatment. Values are means ± SEM (*P<0.05, **P<0.01, ***P<0.001; n=6 to 8 per treatment). E and F, HEK293 cells were cotransfected with pMyc-CXCR4 and pMSCV-HyKIT<sup>WT-IRES-eGFP</sup> plasmids via standard techniques; 24 hours later, the transfected cells were treated with the indicated combinations of SCF, SDF-1, and AMD3100 for 15 minutes, lysed, and then P-c-kit and total c-kit levels were evaluated via Western blot. Representative results from 4 experiments are shown. G, WT BM MNCs were transduced with retroviral vectors coding for WT (c-kit<sup>WT</sup>) or constitutively active (c-kit<sup>D816V</sup>) c-kit as described for Figure 2, and then the cells were applied to VCAM-1–coated plates and stimulated with SDF-1 for 15 minutes in the presence or absence of AMD3100, and the levels of P-c-kit and total c-kit were analyzed via Western blot. Values are means ± SEM (***P<0.01; N.S., not significant; n=6 per treatment).
proportion of P-c-kit+ cells in the total c-kit+ cell population was quantified (H). Values are means±SEM; n=5 mice per group.

SDF-1/CXCR4-Induced c-kit Phosphorylation Is Mediated by Src Kinase

CXCR4 is a G protein–coupled receptor, and previous reports suggest that signaling through G protein–coupled receptors activates the Src family of protein tyrosine kinases (SFKs)\(^3\); furthermore, the SDF-1/CXCR4 axis has been shown to activate Lyn, an SFK that is expressed in hematopoietic cells.\(^3\) Thus, we investigated whether Src kinase has a role in SDF-1/CXCR4-induced c-kit phosphorylation.

SDF-1 treatment markedly increased Src phosphorylation at tyrosine 416 (which signals Src activation) in HEK293 cells that transiently expressed CXCR4 and c-kit, whereas the proportion of PB MNCs that expressed GFP (ie, the number of circulating, BM-derived cells) increased from \( \approx 60\% \) to \( \approx 89\% \). Thus, AMD3100-induced MNC mobilization is associated with a decline in c-kit expression, as well as c-kit phosphorylation.

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(Figure 5D) in adherent BM MNCs, which suggests that phospho-Src binds to c-kit in vivo, and the levels of c-kit–bound phospho-Src increased after SDF-1 treatment (Figure 5D and 5E); once again, the effects of SDF-1 treatment declined in BM MNCs cotreated with ADM3100 (Figure 5D) or SU6656 (Figure 5E). Collectively, these observations suggest that SDF-1–induced c-kit phosphorylation requires the activation of Src and, consequently, that SDF-1 and CXCR4 regulate BM PC trafficking through a Src- and c-kit–dependent pathway.

**Discussion**

The mobilization of PCs from the BM to the peripheral circulation is a crucial step in the response to ischemic injury. Researchers in other laboratories have shown that BM PC retention is largely governed by interactions between SDF-1 and CXCR4 and that the selective CXCR4 antagonist AMD3100 disrupts these interactions, thereby releasing PCs into the peripheral circulation. For these reasons, AMD3100 and other treatments that enhance PC mobilization are currently being investigated for use in...
cell-based therapeutic approaches designed to promote tissue repair and to treat a number of diseases. Our findings provide the first evidence that SDF-1–CXCR4 binding retains PCs in the BM by transactivating c-kit and that CXCR4 antagonism mobilizes PCs by downregulating c-kit phosphorylation. Furthermore, phospho–c-kit has been shown to activate phosphatidylinositol 3-kinase (PI3K) (through the p85 regulatory subunit), Ras mitogen-activated protein kinase (through growth factor receptor-bound protein 2), and integrins (eg, VLA-4/VCAM-1), which are linked to the regulation of cell adhesion and mobility, and results from other labs suggest that growth factors such as granulocyte colony-stimulating factor (G-CSF) mobilize BM PCs by triggering the enzymatic cleavage of membrane-bound SCF–c-kit and SDF-1–CXCR4 complexes. Thus, SDF-1–CXCR4–c-kit signaling could function as a “final common pathway” of fundamental importance to BM PC retention and mobilization.

Because G-CSF–induced PC mobilization requires an increase in c-kit activation, the decline in c-kit phosphorylation reported here may seem counterintuitive. However, G-CSF–induced mobilization occurs 3 to 5 days after administration and is accompanied by an increase in the number of PCs present in the perivascular niche, whereas AMD3100-induced mobilization occurs within a few hours and, consequently, is unlikely to be preceded by the perivascular accumulation of PCs. Furthermore, the 2 agents appear to mobilize different PC subpopulations, and more PCs are mobilized when G-CSF and AMD3100 administration are combined than when G-CSF is administered alone. Thus, the available evidence suggests that the mechanisms of G-CSF- and AMD3100-induced PC mobilization differ substantially. G-CSF and other slow-acting agents may increase c-kit phosphorylation by upregulating SCF, which (by itself) has only a modest effect on mobilization but potently promotes PC proliferation; if so, G-CSF-induced mobilization may be delayed until an adequate surplus of PCs is available for release to the PB. Conversely, fast-acting agents, such as AMD3100 or the 4-integrin-blocking antibody, may mobilize PCs directly by reducing c-kit phosphorylation in the perivascular niche. This hypothesis is also supported by recent evidence that PCs can be rapidly mobilized by the administration of a c-kit-neutralizing antibody.

Both the loss and the constitutive activation of c-kit kinase activity impaired AMD3100-induced BM PC mobilization. In c-kit kinase–defective mice, BM PC levels were lower than in WT mice, and systemically administered CXCR4 PCs could not repopulate the BM. These observations suggest that c-kit kinase inactivation blocks the retention of CXCR4 PCs in the BM and, consequently, that the cells susceptible to AMD3100-induced mobilization are (in effect) already mobilized. In mice transplanted with BM cells that expressed a constitutively active c-kit mutant, AMD3100 treatment did not alter PC levels in the BM or the PB, which suggests that CXCR4-mediated BM PC mobilization requires c-kit kinase deactivation. Collectively, these observations indicate that PCs are retained in the BM by the kinase activity of c-kit, and that AMD3100 mobilizes CXCR4+ BM PCs by downregulating c-kit kinase activity.

Our observations also indicate that SDF-1/CXCR4–induced c-kit transactivation is mediated by SFKs. CXCR4 is a G protein–coupled receptor, and 2 families of G-protein α subunits (Gαs and Gα1) activate SFKs (c-Src and Hck) by binding directly to the catalytic domain and increasing substrate access to the active site. CXCR4-dependent stimulation of SFKs (Lyn) has also been associated with the activation of PI3K. However, SFKs display considerable redundancy both in their activation pathways and in the downstream effectors that mediate their biological function; therefore, whether the G proteins, PI3K, or other upstream components link the SDF-1/CXCR4 axis to SFK activation and, subsequently, to c-kit phosphorylation and BM PC retention, has yet to be determined.

There are nine known SFKs, several of which, as well as multiple isoforms of the same SFK, are expressed in any given tissue. SU6656 inhibits all SFKs (without directly altering c-kit activity), and the available antibodies are not specific for the phosphorylated forms of individual family members; therefore, we used antibodies against activated SFKs that are found predominantly in the hematopoietic system (ie, Lyn, Hck, and Fgr). Thus, the results reported here cannot identify which specific SFK mediates c-kit activation. We have begun a series of experiments with mice deficient in one or more SFK to characterize the roles of the individual family members.

In conclusion, the results presented here demonstrate that SDF-1/CXCR4 signaling retains PCs in the BM through the Src-mediated activation of c-kit and that CXCR4 antagonism induces PC mobilization by downregulating c-kit phosphorylation. Because BM PCs have an essential role in tissue repair, the mechanism identified by our studies could be an important component of the physiological response to ischemic injury. Furthermore, SDF-1, CXCR4, and c-kit also have a role in normal development and in diseases such as arthritis and cancer; therefore, the implications of our findings may extend to a wide variety of pathological and developmental processes.

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Disclosures
None.
References


**Novelty and Significance**

What Is Known?

- Progenitor cells (PCs) are mobilized from the bone marrow (BM) to the peripheral circulation in response to ischemic injury, and ample evidence suggests that this process is governed by interactions between stromal cell–derived factor (SDF)-1 and CXC chemokine receptor (CXCR)4.
- Within the BM, CXCR4 is expressed by mononuclear cells (including PCs), and SDF-1 is expressed by osteoblasts, endothelial cells, and a subset of reticular cells.
- Like CXCR4, c-kit is expressed predominantly in BM PCs, and the signaling pathways regulated by c-kit activation contribute to BM PC mobilization and trafficking.

What New Information Does This Article Contribute?

- The binding of SDF-1 to CXCR4 upregulates c-kit phosphorylation (ie, c-kit activity) in BM PCs.
- Disruption of the SDF-1–CXCR4 interaction (ie, CXCR4 antagonism) mobilizes PCs by downregulating c-kit phosphorylation.
- SDF-1/CXCR4-induced c-kit phosphorylation is mediated by the Src family of nonreceptor tyrosine kinases (SFKs).

In response to ischemic injury, PCs are mobilized from the BM to the peripheral circulation and recruited to the injured tissue, where they contribute to vessel growth. The retention and release of BM PCs is governed by both SDF-1/CXCR4 signaling and c-kit signaling, but whether these 2 pathways interact has not been investigated previously. The results presented here are the first to show that SDF-1/CXCR4 signaling retains PCs in the BM by transactivating c-kit, that CXCR4 antagonism mobilizes PCs by downregulating c-kit phosphorylation, and that SDF-1/CXCR4–induced c-kit transactivation is mediated by SFKs. Because BM PCs have an essential role in tissue repair, the mechanism identified by our studies could be an important component of the physiological response to ischemic injury and, consequently, may be a viable target for therapies designed to promote BM PC mobilization and the recovery of injured cardiovascular tissue. Furthermore, SDF-1, CXCR4, c-kit, and SFKs also have a role in normal development and in diseases such as arthritis and cancer; therefore, the implications of our findings may extend to a wide variety of pathological and developmental processes.
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CXCR4 mediated bone marrow progenitor cell maintenance and mobilization are modulated by c-kit activity

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Online Supplement

Detailed Methods

Isolation of BM and PB MNCs
BM cells were collected by flushing femurs and tibias with 5 mM EDTA in PBS (PBS-E), and PB was collected via cardiac puncture with heparin-coated needles, then diluted 2-fold with PBS-E. After collection, the BM and PB were overlayed onto Histopaque-1083 (Sigma-Aldrich) and centrifuged at 2145 RPM for 20 minutes at room temperature. BM or PB MNCs were collected from the buffy-coat and thoroughly washed.
Flow cytometry
Cells were blocked with 50% rat serum and mouse Fc blocker (BD Bioscience) for 10 minutes, then stained for 30 minutes with APC-conjugated anti-Lin, PE-Cy7–conjugated anti-c-kit, FITC-conjugated anti-Sca-1, biotin-conjugated anti-CXCR4, PerCP-Cy5.5-conjugated streptavidin, and their corresponding isotype control antibodies (BD Bioscience). Analyses were performed on a multi-parametric flow cytometer (Dako Cyan).

Transplantation of retrovirally transduced c-kit<sup>WT</sup> and c-kit<sup>D816V</sup> BM MNCs
MIG-HyKIT<sup>WT</sup> and MIG-HyKIT<sup>D816V</sup> retroviral vectors were generated by co-transfecting 293FT cells with a packaging plasmid, pIK6.1 MCV.ecopac.UTd, and one of two backbone plasmids: pMSCV-HyKIT<sup>WT</sup>-IRES-eGFP (pMIG-HyKIT<sup>WT</sup>), or pMSCV-HyKIT<sup>D816V</sup>-IRES-eGFP (pMIG- HyKIT<sup>D816V</sup>)<sup>1</sup>; the transfection was performed via the calcium-phosphate method<sup>2</sup>, and virus-containing supernatant was collected 48 hours after transfection.

BM MNCs were isolated from donor C57BL/6 mice 2 days after intraperitoneal injection of 5-fluorouracil (150 mg/kg), stimulated for 2 days in IMDM supplemented with 20% FBS, 10 ng/mL SCF, 6 ng/mL IL-3, 50 ng/mL Flt-3 ligand (R&D Systems), and 10 ng/mL thrombopoietin (Prepro’Tech), then infected with the MIG-HyKIT<sup>WT</sup> or MIG-HyKIT<sup>D816V</sup> retroviruses. Multiplicities of infection (MIG-HyKIT<sup>WT</sup>: 2.3; MIG-HyKIT<sup>D816V</sup>: 1.5) were based on titration in NIH 3T3 cells, and transfection efficiency was evaluated 3 days later via flow-cytometry analysis of GFP expression. The BM of recipient C57BL/6 mice was ablated via lethal irradiation (12Gy), then 3.4×10<sup>6</sup> transfected BM MNCs were injected into the tail vein. The BM of recipient mice was allowed to repopulate for 3 weeks before subsequent analyses were performed.

Tissue sectioning and immunofluorescent staining
Mice were subcutaneously injected with AMD3100 (5 mg/kg) or PBS and sacrificed 15 minutes later. Femurs were harvested, fixed in 4% PFA for 5 hours, incubated in 10% EDTA at 4°C under constant agitation for 48-72 hours (the EDTA solution was changed every 24 hours), fixed again in 4% PFA for 30 minutes, incubated in 30% sucrose at 4°C for 24 hours, frozen in OCT compound, and cut into 6-µm sections with a Microm HM550 (Thermo Fisher Scientific Inc.). Subsequent evaluations were performed in H&E-stained sections or in sections stained for immunofluorescence. For immunofluorescent evaluations, sections were incubated in 0.5% Triton for 5 minutes, in 10% horse serum/PBS for 30 minutes, and in 5% horse serum, 2% BSA and 0.1% Tween 20 with rabbit anti-mouse c-kit (1/50) or anti-mouse phospho–c-kit (1:100) antibodies (Santa Cruz) in a humid chamber at 37°C for 2 hours; then the sections were rinsed with PBS and incubated with secondary antibodies (1:250) at room temperature for 1 hour and with DAPI for 10 min. After staining, sections were rinsed in PBS and mounted.

Cell adhesion assay
Six-well tissue culture plates were coated with 10 µg/mL recombinant murine VCAM-1 (R&D Systems) for 30 min and blocked with 10% goat serum, then 6×10<sup>6</sup> freshly
isolated BM MNCs, suspended in serum-free HEPES medium, were added to each well and incubated in a 5% CO₂ incubator at 37°C for 15 min. Nonadherent and loosely attached cells were removed by tapping each plate and gently washing the wells 3 times with PBS. Anti-α4 integrin PS/2 antibodies (100 ug/mL, purified from cultured hybridoma cells [ATCC] with Montage Antibody Purification kits) 3, AMD3100 (100 ng/mL), recombinant SDF-1 protein (200 ng/mL; R&D systems), SCF (100 ng/mL), or the corresponding delivery vehicles were added to each well, then the cells were incubated at 37°C under 5% (v/v) CO₂ for 15 min, and adhesion was evaluated under a light microscope.

**Western blotting and co-immunoprecipitation**

Cells were lysed in SDS-containing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue) or in cell-lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1% w/v Triton X-100, 10 mM Sodium orthovanadate, 10 mM sodium beta-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% v/v β-mercaptoethanol, and protease-inhibitor tablets [one tablet/10 mL, Roche Applied Science], pH 7.5). Immunoblotting was performed via standard techniques 4-5 with anti-phospho-c-kit (Tyr 719), anti-c-kit, anti-phospho-Src (Tyr 416), anti-Src, anti–SDF-1 (Cell Signaling Technology), and control IgG (Santa Cruz Biotechnology, Inc) antibodies. For co-immunoprecipitation, cells were lysed in cell-lysis buffer containing 0.1% w/v Triton X-100 and the cell lysates were incubated overnight at 4°C with the appropriate antibody, then incubated with protein A/G plus-Agarose (Santa Cruz) for 1 hour at 4°C. After washing, the immunoprecipitates were eluted by boiling for 5 min, and extracts were analyzed by immunoblotting as described above. Band intensities were determined densitometrically with Image J software.

**Quantitative real-time RT-PCR**

Real time RT-PCR was performed via standard techniques 6. Briefly, total RNA was extracted with RNA Stat-60 (Tel-Test, Friendswood, TX), and RNA was reverse transcribed with the Taqman Multiscribe RT kit (Applied Biosystems). Real-time PCR was performed in duplicate with cDNA from 10 ng of RNA by using the Lightcycler hybridization Probes Master Mix (Roche); a negative control (lacking a template) was included for each probe set. Relative gene expression was calculated using the Ct method and normalized to GAPDH. The primer sequences for mouse CXCR4 were 5’-CCTCGCCTTCTTCCACTGTT-3’ (forward) and 5’-CTGGGCAGAGCTTTGAACTTG-3’ (reverse). The primer sequences for mouse SDF-1 were 5’-CCTCCAAACGCATGCTTCA-3’ (forward) and 5’-CCTTCCATTGCAGCATTGGT-3’ (reverse).
Online Figure I: WT and c-kit^{W/W-V} mice express similar levels of SDF-1 in the BM. (A) SDF-1 mRNA levels were assessed in BM lysates from WT mice and c-kit^{W/W-V} mice via quantitative real-time RT-PCR and normalized to 18S (n=6). (B) SDF-1 protein levels were evaluated by Western blotting (left panel), quantified densitometrically, and normalized to the levels of α-actin (right panel) (n=3). N.S., not significant.
Online Figure II: c-kit remains constitutively active in c-kit<sup>D816V</sup>–transduced BM MNCs for at least 3 weeks after transplantation. MNCs were harvested from WT mice, transduced <i>ex vivo</i> with retroviral vectors coding for eGFP-linked WT c-kit (c-kit<sup>WT</sup>) or eGFP-linked constitutively active c-kit (c-kit<sup>D816V</sup>) as described in Figure 2, then transplanted into WT mice after lethally irradiating the endogenous BM. Three weeks after transplantation, BM MNCs were isolated from the recipient mice, and c-kit and phospho–c-kit (P–c-kit) levels were measured in (A) cells treated with SCF-1 (100 ng/ml) or Vehicle (–) and in (B) cells applied to VCAM-1–coated plates and sequentially treated with SDF-1 and AMD3100 or with SDF-1 and Vehicle as described in Figure 3; each treatment lasted for 15 min. c-kit and P–c-kit levels were analyzed by Western blot and quantified densitometrically. P–c-kit levels were normalized to the total c-kit levels and expressed as the fold-difference from the levels measured in vehicle-treated c-kit<sup>WT</sup> MNCs. Values are mean ± SEM (**P<0.01, n=10 per group). SCF significantly upregulated c-kit phosphorylation, and AMD3100 significantly downregulated SDF-1–induced c-kit phosphorylation, in c-kit<sup>WT</sup> MNCs but not in c-kit<sup>D816V</sup> MNCs. Collectively, these observations confirm that c-kit was constitutively activated (i.e., c-kit activity could be neither upregulated nor downregulated) in MNCs from mice transplanted with c-kit<sup>D816V</sup>–transduced BM cells.
Online Figure III: AMD3100 treatment does not alter the proportion of PB MNCs that express c-kit. (A) BM and PB MNCs were isolated from CXCR4\textsubscript{BAC}-eGFP mice, then the proportion of cells that expressed CXCR4 (i.e., eGFP\textsuperscript{+} cells, upper panels; n=3) or c-kit (lower panels; n=4) were evaluated via flow cytometry. (B) CXCR4\textsubscript{BAC}-eGFP mice were subcutaneously injected with PBS or AMD3100; 2 hours later, PB MNCs were isolated and the proportion of cells that expressed CXCR4 (i.e., eGFP\textsuperscript{+} cells) or co-expressed CXCR4 and c-kit was evaluated via flow cytometry (n=4).
Supplemental References


