SDF-1/CXCR4 Axis in Tie2-lineage Cells Including Endothelial Progenitor Cells Regulates Bone Fracture Healing

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Introduction: CXC chemokine receptor 4 (CXCR4) is a specific receptor for stromal derived-factor 1 (SDF-1). SDF-1/CXCR4 interaction is reported to play an important role in vascular development. (1) SDF-1/CXCR4 interaction contributes to the regulation of endothelial progenitor cell (EPC) recruitment in ischemic tissues. (2) On the other hand, the therapeutic potential of EPCs in fracture healing has been demonstrated with mechanistic insight of angiogenesis and osteogenesis enhancement at sites of fracture. (3,4) However, a relationship between a SDF-1/CXCR4 pathway and bone fracture healing is still unclear. Building from these backgrounds, we speculated that a mechanism of SDF-1/CXCR4 signaling in bone fracture healing by EPC recruitment is pivotal. Thus, in the present study, we used a bone fracture model of the Tie2-CreER CXCR4 conditional knockout mouse, in which CXCR4 is knocked out specifically in Tie2 expressing endothelial lineage cells. The purpose of this study is to investigate the influence of the SDF-1/CXCR4 pathway in Tie2-lineage cells (including EPCs) in the bone formation in vivo and in vitro using CXCR4 conditional knockout mouse and to probe the development of future bone therapy.

Methods: The institutional animal care and committees of RIKEN Center for Developmental Biology approved all animal procedures.

1Animal model: We made CXCR4 gene knockout mice using the Cre/loxP system(5). Tie2-CreER transgenic mice were crossed with CXCR4flox/flox mice. To disrupt CXCR4 in endothelial lineage cells postnatally, tamoxifen was injected 6weeks-old male mice intraperitoneally. To confirm the tamoxifen-induced disruption of CXCR4, mononuclear cells were collected from bone marrow and peripheral blood and flow cytometry were performed using antibodies for Tie2 and CXCR4. To simulate clinical situation of fracture, we applied a reproducible model of closed femoral fracture. We set two groups of CXCR4 knockout (CXCR4KO) and wild type(WT) mice group.(n=20 for each group)

2In vitro study of mEPC derived from CXCR4KO and WT mice: Migration activity and colony formation assay were evaluated using BM-derived mEPCs. BM corrected from all bones of CXCR4KO mice or WT mice aged 6 weeks was separated by Histopaque-1083 (Sigma) density gradient centrifugation.

3Incorporation of EPCs in vivo: To evaluate EPC incorporation into the fracture site, EPCs derived from CXCR4KO and WT mice were intravenously injected to the WT fractured mice just after creation of bone fracture. To detect EPC incorporation into the fracture site, transplanted EPCs were labeled with Dil.

4Stimulation of SDF-1: To evaluate gain-of-function of the SDF-1/CXCR4 axis on therapeutic neovascularization and bone healing during fracture repair, we set another two groups of the SDF-1
intraperitoneally injected WT group (WT+SDF1) and SDF-1 injected CXCR4KO group (CXCR4KO+SDF1), and studied radiographical and histological assessments after fracture creation comparing them with existing WT and CXCR4KO groups. (n=20 for each group)

**Results:**
(1) Confirmation of conditional knockout of CXCR4 on Tie2 expressing cells (EPC enriched population): Flow cytometry analysis demonstrated that there exists rarely Tie2+/CXCR4+ cells in peripheral blood and bone marrow cells in CXCR4KO mouse, indicating successful generation of the conditional CXCR4 knockout. (Fig1)
(2) Morphological and functional fracture healing: Before bone fracture creation, micro CT and skeletal preparation revealed no significant difference between WT and CXCR4KO groups. (Fig.1) Radiological examinations demonstrated that relative callus area and union rate at week 2 and 3 were significantly greater in the WT group than in the CXCR4KO group. Moreover, bone density, width and number of trabecula and biomechanical three point bending test at week 4 in the CXCR4KO group exhibited significantly lower values than the WT group. (Fig.2)
(3) Blood flow and Immunohistochemical staining: Laser doppler perfusion imaging analysis demonstrated that CXCR4KO group mice represented a reduction of blood flow recovery at fracture site at week 1, 2. Quantitative analysis of capillary density and osteoblastic density around peri-fracture at week 1 showed significant decrease in the CXCR4KO group compared to the control WT group. (Fig.3)
(4) Molecular analysis of the fractured tissue: Real time RT-PCR analysis at one week showed that the gene expressions of angiogenic (vascular endothelial growth factor (VEGF), CD31, VE-Cadherin) and osteogenic markers (Osteocalcin (OC), Collagen1A1, bone morphogenetic protein 2 (BMP-2)) were lower in the CXCR4KO group than the WT group.
(5) In vitro character of EPCs derived from CXCR4KO: The migration activities of mEPCs were evaluated using a Transwell culture plate, and mEPCs migrated toward SDF-1a containing medium were evaluated as the activity. EPCs which derived from CXCR4KO mice demonstrated severe reduction of migration activity and EPC colony forming activity when compared with those derived WT mice.
(6) Incorporation of EPCs: Staining for EPCs revealed significantly abundant incorporation of labeled EPCs derived from WT mice into fracture sites. In contrast, very few EPCs which derived from CXCR4KO mice incorporated into the fractured site at day 7.
(7) Stimulation of SDF-1: In the SDF-1 injected study, radiographical and histological assessment demonstrated that the fracture in the WT+SDF-1 group was healed significantly faster from fracture with enough callus and vascular formation than other groups. Blood flow recovery at fracture site was also promoted in the WT+SDF-1 group at week 1.

**Discussion:** In previous study, the other group reported that circulating bone marrow-derived osteoblast progenitor cells are recruited in the bone formation through the SDF-1/CXCR4 pathway. (6) However, a relationship between SDF-1/CXCR4 pathway on EPC and bone fracture healing is not mentioned yet. In the present study, using Tie2-Cre CXCR4 knockout mouse, we demonstrated the significance of SDF-1/CXCR4 signal on EPCs to bone fracture healing. The bone fracture healing delayed and formed callus diminished in CXCR4 deficient mice compared with wild type mice. An attenuation of angiogenesis and osteogenesis at fracture site in CXCR4 deficient mice could be considered a cause of this impair in fracture repair. Moreover, the promotion of EPC SDF-1/CXCR4 axis leads to the acceleration of bone fracture healing and might be served as a novel therapeutic application for bone injuries.
**Significance:** We demonstrated that EPC SDF-1/CXCR4 axis plays an important role in bone fracture healing using Tie2-CreER CXCR4 conditional knockout mice. Our results also indicated that mobilization and incorporation of EPCs in bone fracture healing process was through SDF-1/CXCR4 pathway. Moreover, the promotion of EPC CXCR4/SDF-1 axis leads to the acceleration of bone fracture healing and might be served as a novel therapeutic application for genetic bone disease and bone injuries.