SDF-1/CXCR4 Axis on Endothelial Progenitor Cells Regulates Bone Fracture Healing

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Disclosures: 

Introduction: CXC chemokine receptor 4 (CXCR4) is a specific receptor for stromal derived-factor 1 (SDF-1). SDF-1/CXCR-4 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 for fracture healing has been demonstrated with mechanistic insight of vasculogenesis/angiogenesis and osteogenesis enhancement at sites of fracture.(3,4,5) However, a relationship between a SDF-1/ CXCR4 pathway and bone fracture healing is still unclear. Building from these backgrounds, we speculate 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 on 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: Animal model: The institutional animal care and committees of RIKEN Center for Developmental Biology approved all animal procedures. We made CXCR4 gene knockout mice using the Cre/loxP system(6). Tie2-CreER transgenic mice were crossed with CXCR4floxed/flox mice. To disrupt CXCR4 in endothelial lineage cells postnatally, tamoxifen was injected 6weeks-old 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. To simulate clinical situation of fracture, we applied a reproducible model of closed femoral fracture in 10-week-old mice. We set two groups of CXCR4 knockout (CXCR4KO) and wild type(WT) mice group.(n=20 for each group)

In vitro study of EPC 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. 

In vivo incorporation assay: 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 corporation into the fracture site, transplanted EPCs were labeled with DiI.

SDF-1 stimulation test: To evaluate gain function of the SDF-1/CXCR4 pathway 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: Confirmation of conditional knockout of CXCR4 on Tie2 expressing cells: Analysis of flow cytometry and immunostaining demonstrated that there exists rarely Tie2+/CXCR4+ cells in peripheral blood and bone marrow cells, indicating successful generation of the conditional CXCR4 knockout.(Fig.1)

In vitro character of EPCs derived from CXCR4KO: EPCs which derived from CXCR4KO mice demonstrated severe reduction of migration activity to SDF-1 and EPC colony forming activity when compared with those derived WT mice.(Fig.1)

Morphological fracture healing: Radiological and histological 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.(Fig.2) These results indicated that the fracture healing was delayed in the CXCR4 KO group. Moreover, bone density and trabecular width and number of trabeculae in CXCR4 group exhibited significantly lower values than WT group.

Immunohistochemical staining and blood flow: Quantitative analysis of capillary density and osteoblastic density around peri-fracture at week 1 showed significant decrease in the CXCR4 KO group compared to the WT group. Laser Doppler Perfusion Imaging (LDPI) analysis demonstrated that the control group represented a significantly higher perfusion value at fracture site than the CXCR4 KO group at week 1 and 2.(Fig 3)

Functional bone healing by three point bending test: Biomechanical evaluation by 3-point bending test was performed at week 4. The percentage ratios of ultimate stress and extrinsic stiffness in the fractured femur versus contralateral intact femur in WT group were superior to CXCR4KO group.

Molecular analysis of the fractured tissue: Real time RT-PCR analysis at one week after fracture creation 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 higher in the WT group than the CXCR4 KO group.

**Incorporation of EPCs:** Double immunofluorescent staining for isolectin B4 and Dil demonstrated that double stained cells were found in the mice injected EPCs from WT mice, but not in the mice injected EPCs from CXCR4 KO mice. (Fig. 4)

**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 formation. (Fig. 4)

**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. (7) 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 healings in CXCR4 KO mice were delayed compared to the wild type mice via down-regulation of angiogenesis and osteogenesis. This study also suggested that the promotion of CXCR4/SDF-1 signal on EPCs lead to the acceleration of bone fracture healing for new therapeutic strategies to fracture repair.

**Significance:** We demonstrated that SDF-1/CXCR4 axis in EPCs is a critical factor for bone fracture repair using Tie2-Cre$^\text{ER}$ 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, this study also suggested that the promotion of CXCR4/SDF-1 signals in EPCs leads to the acceleration of bone fracture healing and might be served as a novel therapeutic application for genetic bone disease and bone injuries.

**Acknowledgments:**

**References:**

**Figure 1**
Figure 4

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B

C

**ORS 2014 Annual Meeting**

Poster No: 0051