# CXCL9 neutralizing antibody promotes fracture repair in aged mice; downregulation of CXCL12 contributes to enhanced bone repair

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**Introduction:** Aging disrupts the molecular coordination required for efficient fracture repair, yet alterations in the signaling networks within skeletal stem and progenitor cells (SSPCs) that underlie this decline remain poorly defined. Through single-cell RNA sequencing (scRNA-seq) of periosteal CD45$^-$ Prrx1$^+$ SSPCs from young and aged mice, we reported elevated *Cxcl9* expression in aged male mice and an inflammatory bias in the aging skeletal niche[1]. These findings align with prior reports identifying elevated CXCL9 as a biomarker of inflammatory aging[2], a risk factor for osteoporotic hip fractures in men[3], and a predictor of frailty and increased mortality in osteoporotic fracture patients[4]. However, it is not known whether CXCL9 acts in isolation or as part of a broader dysregulation of the chemokines coordinating progenitor recruitment and angiogenesis. Most notable among these is CXCL12, which functions in the recruitment of mesenchymal progenitors, angiogenic coupling, and early callus organization[4]. However, it is thought that subsequent downregulation of CXCL12 is required for efficient repair, placing it at a pivotal intersection of inflammation and regeneration in the fracture niche[5]. Because SSPCs rather than immune populations largely secrete chemokines in bone, we reasoned that aging might reprogram local SSPC-derived chemokine expression patterns. Therefore, we analyzed our scRNA-seq dataset to identify age-associated transcriptional shifts in chemokine signaling. In parallel, we pharmacologically neutralized CXCL9 to assess the impact of elevated CXCL9 on fracture healing in aged mice.

**Materials and Methods:** In IACUC-approved studies, bilateral mid-diaphyseal fractures were created in young (3-4 months old) and aged (24-25 months old) male mice, as previously described[1]. Male mice were used to ensure consistency, as fractures in aged females are technically challenging due to bone fragility. For scRNA-seq analysis, periosteal cells isolated from intact and fractured femurs three days post-injury were immediately FACS-sorted to isolate CD45-Ter119- and CD45+Ter119- populations for scRNA-seq. Data were analyzed in Seurat with focus on Prrx1$^+$ mesenchymal progenitors **(Fig. 1a)**. A separate cohort of uninjured young and aged mice was used to quantify serum levels of CXCL9, CXCL12, VEGFα, TNFα, and IFN-γ via a multiplex Luminex assay. In fracture healing experiments, aged mice were administered either anti-IgG (control) or anti-CXCL9 neutralizing antibody (10 mg/kg i.p.) on days 1, 3, 6, 9, and 12 post-fracture. Healing was evaluated by histological analysis at day 14. Statistics: Data were analyzed using Student's t-test and represented as mean ± SD.

**Results:** Analysis of scRNA-seq data from periosteal CD45$^-$ Prrx1$^+$ SSPCs (Fig. 1a) revealed increased *Cxcl12* expression at baseline in aged periosteum **(Fig. 1b)**, coinciding with increased *Cxcl9* expression, but unchanged *Vegf*, as we previously published[1]. Three days post-fracture, young periosteum displayed diffuse induction of *Cxcl12*, while aged periosteum exhibited strong, spatially clustered *Cxcl12* expression primarily in Lepr+ and osteogenic cell clusters **(Fig. 1b)**. The CXCL12 receptor, *Cxcr4,* was not expressed in Cd45- cells but was broadly expressed in CD45+ cells across all groups (not shown), indicating proinflammatory mesenchyme-immune crosstalk mediated by aged periosteal cells. At the baseline, serum CXCL9 and CXCL12 levels were significantly higher in aged mice than in young (p 0.05 vs control) **(Fig. 1c)**. Consistent with the single-cell data, serum TNFα and IFNγ levels were unchanged (not shown). Antibody-mediated neutralization of CXCL9 in aged mice enhanced fracture healing at day 14, leading to greater callus mineralization and more Osterix-expressing cells in the callus **(Fig.2b-d)**. Consistent with enhanced osteogenesis, this improvement was accompanied by reduced serum CXCL12 levels in treated animals (*p 0.05 vs control), whereas serum VEGF, IFN-γ, and TNF were not affected by the CXCL9 antibody **(Fig. 2e)**.

**Discussion:** Aged mice showed concurrent increases in CXCL9 and CXCL12 in serum and in periosteal SSPCs, without corresponding changes in TNFα or IFNγ, suggesting a chemokine-biased, rather than cytokine-driven, inflammatory profile during aging. The temporal and spatial regulation of CXCL12 is essential during fracture repair[5]. Although CXCL12 supports early recruitment and angiogenesis, its prolonged elevation in aged bone likely reflects dysregulated activation rather than effective repair. The reduction of CXCL12 following neutralization of CXCL9 in aged mice may indicate a broader normalization of chemokine dynamics, contributing to improved coordination of the healing response. Consistent with this, anti-CXCL9 treatment was associated with increased expression of osteoblast marker genes and enhanced callus mineralization. These findings suggest that CXCL9 influences the chemokine balance in aging bones during fracture healing, and that CXCL9 blockade can restore efficient bone healing by modulating the chondrogenic and osteogenic phases of endochondral bone healing, as well as CXCL12.

**Significance:** These findings highlight CXCL9 as a modulator of the chemokine environment in aging bone. By altering CXCL12 dynamics, elevated CXCL9 may contribute to the reduced regenerative capacity characteristic of aged bone. Transient CXCL12 aids repair, but its persistence in aged bone may impede healing. While preliminary, these results suggest that targeting CXCL9 could help restore chemokine balance and improve bone regeneration in fragility fractures.

**References:** 1. King *et al*., Bone 2025; 2. Sayed *et al*., Nat Aging 2021; 3. Phan *et al*., JBMR 2022; 4. Seo DH *et al*., Osteoporosis Int. 2024; 4. Toupadakis *et al*., JOR 2012; 5. Myers *et al*., JBMR 2014

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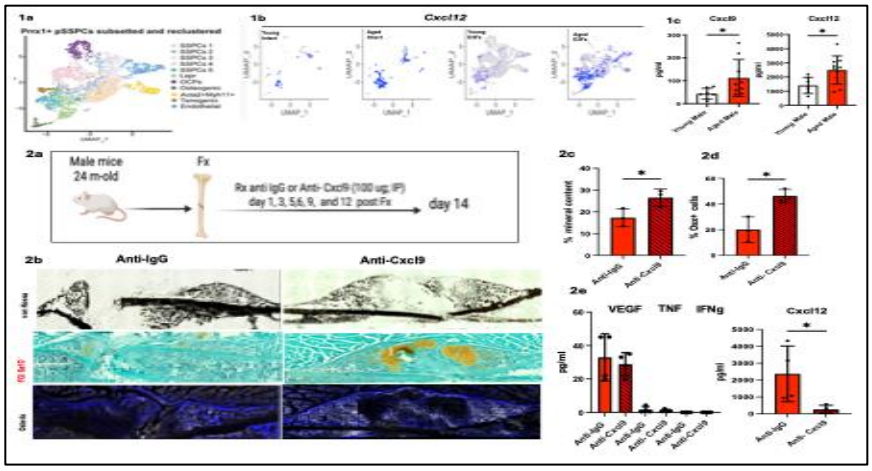


**Figure 1a.** Subsetted and reclustered Prrx1+ pSSPC clusters. **1b.** Feature plot showing *Cxcl12* expression in Prrx1+ cell clusters. **1c.** Serum analysis Cxcl9 and Cxcl12 in uninjured young and aged mice. n=6-9 mice/group. **2a.** CXCL9 neutralizing antibody experimental design n= 4 male mice/condition. **2b.** Representative cryosections stained with von Kossa (mineralized matrix), Fast Green/Saf-O (staining cartilage yellow/orange), anti-Osterix immunofluorescence (white) and DAPI (blue). **2c**. Quantified callus mineral content. **2d**. Osterix+ cells. **2e** Cytokine/chemokine serum levels of analysis. Representative images are shown. *p 0.05 vs control.