

# Age-induced Periosteal Changes Adversely Affect Proliferation and Restrict Differentiation During Early Fracture Healing

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**INTRODUCTION:** Aging diminishes skeletal resilience, increasing susceptibility to bone injuries and hampering subsequent regeneration<sup>1</sup>. Skeletal stem and progenitor cells (SSPCs), particularly those in the periosteum (pSSPCs), are central to bone repair, especially during endochondral fracture healing. However, the age-related changes in the periosteal cell milieu are less known despite the periosteum's crucial role in fracture healing. The advent of single-cell RNA-sequencing (scRNA-seq) provides unprecedented insight into cellular heterogeneity and transcriptional changes within a specific tissue. In investigating fracture healing with scRNA-seq, many previous studies were performed on whole bone or bone marrow-derived SSPCs from young mice, or periosteal cells that were first expanded *ex vivo* to increase their numbers, which could alter gene expression and cell function. Here, we utilized scRNA-sequencing on periosteal cells directly isolated from intact and fractured bones of young and aged mice to understand the influences of aging on fracture healing. Our results suggest that pSSPCs lose chondrogenic potential, biasing toward early osteogenic trajectory, and exhibit a reduced regenerative potential, undermining their long-term fracture healing efficacy.

**METHODS:** Mice: The institutional IACUC approved this study to directly compare the impact of age on the periosteal healing response; young (3-4 months old) and aged (24-25 months old) male mice were used. Fracture generation: Bilateral mid-diaphyseal fractures were created as previously described<sup>2</sup>. For scRNA-seq analysis, periosteal cells isolated from intact and fractured femurs three days post-injury were collected on the same day, as previously described<sup>2</sup>. Samples from 5-7 mice were pooled for each age group. After red blood cell lysis, FACS was used to isolate CD45-Ter119- (CD45-) and CD45+Ter119- (CD45+) for scRNA-seq. Seurat was used for clustering and gene expression analysis. Parallel experiments were conducted to examine *in vivo* periosteal responses in intact and fractured femurs from young and aged mice using Edu and ALP staining, as previously described<sup>2</sup>. Statistics: Statistical significance was determined using two-way ANOVA followed by a Tukey test. Data is represented as mean ± SD.

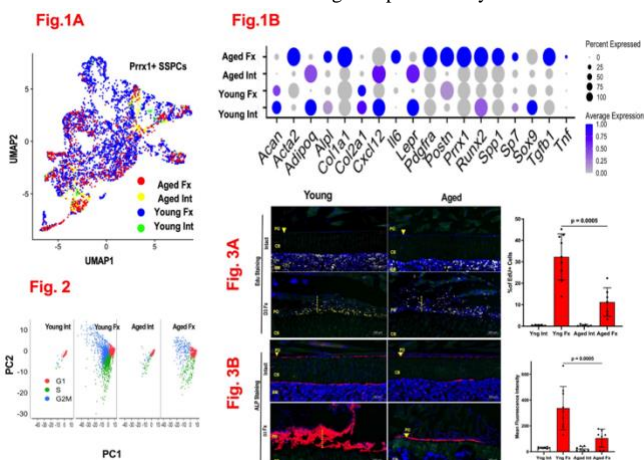
**RESULTS:** Our scRNA-seq of CD45+ periosteal cells identified similar proportions of lymphocytes, neutrophils, and macrophages among the intact young and aged groups. However, three days after fracture, in aged mice a high proportion of neutrophils were present (69.7% young vs 78.7% aged) and these mice recruited less than half as many macrophages (20.7% young vs 10.1% aged). In our scRNA-seq of CD45- periosteal cells, we identified clusters of cells expressing markers for endothelial cells, skeletal muscle, and osteoblasts. Furthermore, we also identified clusters marked by expression of *Acta2*, *Lepr*, *Cxcl12*, *Pdgfra*, *Ctsk*, and *Grem1*, which all robustly co-express *Prrx1*, a well-known marker of pSSPCs. These cells were subclustered as *Prrx1+* pSSPCs, representing 4.0% and 6.3% in young and aged intact mice, respectively. Three days after fracture, the *Prrx1+* pSSPCs population surged to 60.6% and 42.5% in young and aged mice (Fig.1A). Within the *Prrx1+* pSSPCs, there was a marked increase in chondrogenic genes (*Acan*, *Col2a1*) in young mice after injury; however, in aged mice expression of these genes were reduced. In contrast, inflammation-related genes (*Tnf*, *Il6*) and osteogenic genes were markedly increased (*Runx2*, *Col1a1*, *Spp1*) in aged mice after fracture (Fig.1B). In assessing which phase of the cell cycle the *Prrx1+* pSSPCs were in, we observed that 14.5% of the uninjured young mice were in the cell cycle (G2M or S phase); post-injury, the proportion increased to 60.0%. In contrast, among *Prrx1+* pSSPCs in aged mice, 29.3% were cycling without injury, while only 42.5% were cycling after injury. (Fig.2). These data were corroborated by Edu staining, where we observed a significant increase in Edu+ cells in periosteum in young fracture compared to intact bone, but the difference was less pronounced in aged mice (Fig.3A; p<0.001). Similarly, we found a significant increase in Alkaline Phosphatase staining in the periosteum in response to injury in young mice; however, it was less notable in aged mice (Fig.3B), which aligned with our scRNA-seq results.

**DISCUSSION:** Our findings shed light on the age-associated differences among periosteal hematopoietic cells and mesenchymal (*Prrx1+* pSSPCs) during early fracture healing. The comprehensive scRNA-seq profiling of periosteal cells from young and aged mice shows a remarkable similarity in the proportions of specific immune cells between periosteal cells from uninjured young and aged mice. However, following fracture, our data showed that aged mice have an apparent inability to robustly recruit macrophages, which are crucial players in the inflammatory phase of fracture healing and subsequent tissue regeneration. In the CD45- dataset, the heightened cycling activity of *Prrx1+* pSSPCs in young mice post-fracture underscores their inherent regenerative capability. In contrast, the more muted post-injury cycling response of *Prrx1+* pSSPCs in aged mice points towards an age-associated decline in the regenerative capacity. Furthermore, our scRNA-seq data presents intriguing insights into the differentiation fates of the *Prrx1+* population. While aged mice manifested a robust osteogenic response, their chondrogenic potential was conspicuously blunted. Shifts in differentiation can influence the overall quality and efficacy of bone regeneration, especially if a robust cartilaginous callus is not formed first. Conversely, this could also indicate a "premature" differentiation, signaling a disruption in cellular regulatory mechanisms; this premature differentiation could potentially deplete the SSPC pool over time, jeopardizing bone regenerative capacity. Ongoing studies will elucidate mechanisms underlying these age-related changes, including the role of intrinsic aging on genome stability and the cumulative effects of an altered microenvironment on periosteal cells.

**SIGNIFICANCE:** Our study gives insight into the cellular heterogeneity and nuanced transcriptional changes in the aged periosteum at baseline and during early fracture healing, when there is rapid proliferation and differentiation of progenitor cells. Understanding age-associated periosteal changes will help elucidate potential targets for enhancing bone healing, which is becoming more critical as our population ages and the risk for fragility fractures increases.

**REFERENCES:** (1) Goodnough and Goodman, *Current Osteoporosis Rep.*, 2022. (2) Doherty et al., *Bone* 2023.

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**Figure 1. A.** UMAP visualization of merged *Prrx1+* single-cell skeletal stem/progenitor cells (pSSPCs). **B.** Dot plot illustrating the expression of osteogenic, chondrogenic, and inflammatory genes in *Prrx1+* pSSPCs.

**Figure 2.** Principal component (PC) analysis plot depicting cell-cycle attributes of *Prrx1+* pSSPCs.

**Figure 3. A.** Frozen sections of intact and three-day post-fracture mouse femurs labeled with EdU and stained with DAPI. EdU was administered one day before sacrifice. The accompanying bar graph indicates the ratio of EdU+ cells to DAPI+ cells within the periosteal area. **B.** Frozen sections from intact and three days after fracture stained for ALP activity and DAPI. The bar graph quantifies red fluorescence intensity, normalized to the region of interest (ROI), within a defined ROI. Sample sizes are n=8-9 mice for both intact and fractured bones in each age group. PO - periosteum; CB - Cortical bone; BM - Bone marrow. Dotted lines indicate thickened PO; yellow arrowhead indicates PO. All presented data are shown as mean +SD.