Tracing muscle mesenchymal progenitors in fracture healing

Jiawei Lu[1], Qi He[1], Michael Duffy[1], Jaimo Ahn[2], Nathaniel A Dyment[1], Joel Boerckel[1], Ling Qin[1]
Department of Orthopaedic Surgery, [1]University of Pennsylvania, Philadelphia, Pennsylvania; [2]Univeristy of Michigan, Ann Arbor, Michigan, USA

Disclosures: The authors declare that they have no conflict of interest

INTRODUCTION: Although bone is a highly regenerative tissue, about 10% of fractures exhibit either delayed union or nonunion. It has been long recognized in clinic care that compromised fracture healing often occurs at sites with less muscle coverage and that muscle flaps in fracture patients with severe soft tissue injury assist in skeletal healing. Recent animal studies using muscle graft transplants clearly demonstrate that muscle fibro-adipogenic progenitors (FAPs), a muscle interstitial mesenchymal cell population, migrate to the fracture healing site and contribute to chondrocytes and osteoblasts in the callus (1). However, it is unclear whether this observation is physiologically relevant. Common FAP markers, such as Pdgfra and Sca1, are also expressed in periosteal mesenchymal progenitors. In this study, we found that *Prg4-CreER* specifically labels FAPs but not bone marrow and periosteal cells at the fracture site, allowing us to perform lineage tracing to study the in vivo cell fate of FAPs in fracture healing.

METHODS: *Animals*- All animal work was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. *Prg4-CreER Rosa-tdTomato* (*Prg4ER/Td*) mice were generated by breeding *Prg4-CreER* and *Rosa-tdTomato* mice. Mice at 2 months of age received tamoxifen (Tam) injections (75 mg/kg/day) for 3-5 days followed by bone injury 2 weeks later. For fracture injury, mouse tibiae were subjected to closed transverse fracture via a blunt guillotine with a pre-inserted intramedullary pin. For drill-hole injury, mouse tibiae were subjected to a monocortical non-critical size (0.8 mm diameter) defect using a drill bit. *Flow cytometry*- Hindlimb muscles were digested by 0.1% collagenase and 4.8 IU/mL dispase. Cells were stained with APC anti-CD45, APC anti-Ter119, APC anti-CD31, Brilliant Violet 421 anti-Pdgfra, and Brilliant Violet 605 anti-Sca1 antibodies and analyzed by BD LSRFortessa B. *Histology*- Tissues were processed for frozen sections followed by wheat germ agglutin (WGA), anti-Col2, anti-Osterix, and anti-Perilipin antibodies as well as secondary antibody staining. *Statistics*- Data are expressed as means ± standard error of the mean and analyzed by t-tests or one-way ANOVA.

RESULTS: Examining a published muscle single cell RNA-sequencing (scRNA-seq) dataset (2) revealed that FAPs express Prg4 (Fig. 1A). Thus, we constructed *Prg4ER/Td* mice to study the fate of FAPs in bone injury. Tam injections at 2 months of age induced Td signal not only in the articular cartilage and synovium as expected (Fig. 1B, B1), but also in the muscle surrounding bone (Fig. 1B2, C). No Td+ cells were detected inside the cortical bone, at the periosteum (Fig. 1B3), or in the bone marrow (Fig. 1B4). Flow analysis of Td+ cells from muscle showed that they constitute 24.0±0.8% FAPs marked by Sca1 and Pdgfra (Fig. 1D). Fracture damages muscle as well. On day 3 post fracture, we observed a sharp increase of Td+ cells in the muscle next to the fracture site and inside fracture hematoma (Fig. 2A). These Td+ cells became Col2+ chondrocytes and Osterix+ osteoprogenitors on day 7 and 14, respectively (Fig. 2B,C). On day 28, a substantial amount of bone lining cells and bone embedding osteocytes were Td+ in the callus (Fig. 2D). One month later, the regenerated cortical bone and its surface periosteum contained many Td+ cells, while the old cortical bone at the site distal to the fracture remained negative for Td (Fig. 2E). In another bone injury model (drill-hole), which has much less muscle injury, Td+ cells were mostly observed in the fibrosis tissue next to the healing site but not inside callus nor in bone marrow on day 7 post injury (Fig. 3).

DISCUSSION: In this work, we first identified Prg4 as a novel marker for muscle FAPs. Since Td signal is not detected in the periosteum, *Prg4ER/Td* mice represent a unique model to study the action of muscle FAPs in bone regeneration. Cell fate tracing revealed that Prg4+ FAPs contribute substantially to bone healing post fracture, give rise to chondrocytes and osteogenic cells in the callus, and eventually become bone forming cells at the repaired site.

SIGNIFICANCE: Our work advances previous fracture studies, which have mostly focused on bone mesenchymal progenitors, by demonstrating the important contribution of muscle mesenchymal progenitors to bone repair and their trans-differentiation into bone lineage cells in the cortical bone and periosteum. This novel insight advances our knowledge of muscle-bone interaction and sheds light not only on current treatments but also on emerging therapies targeting muscle FAPs for delayed and non-healing fractures.

REFERENCES: (1) Julien, A. et al. *Nat. Commun*. 2021, 12: 2860; (2) Giordani, L. et al. *Mol. Cell* 2019, 74: 609-621.

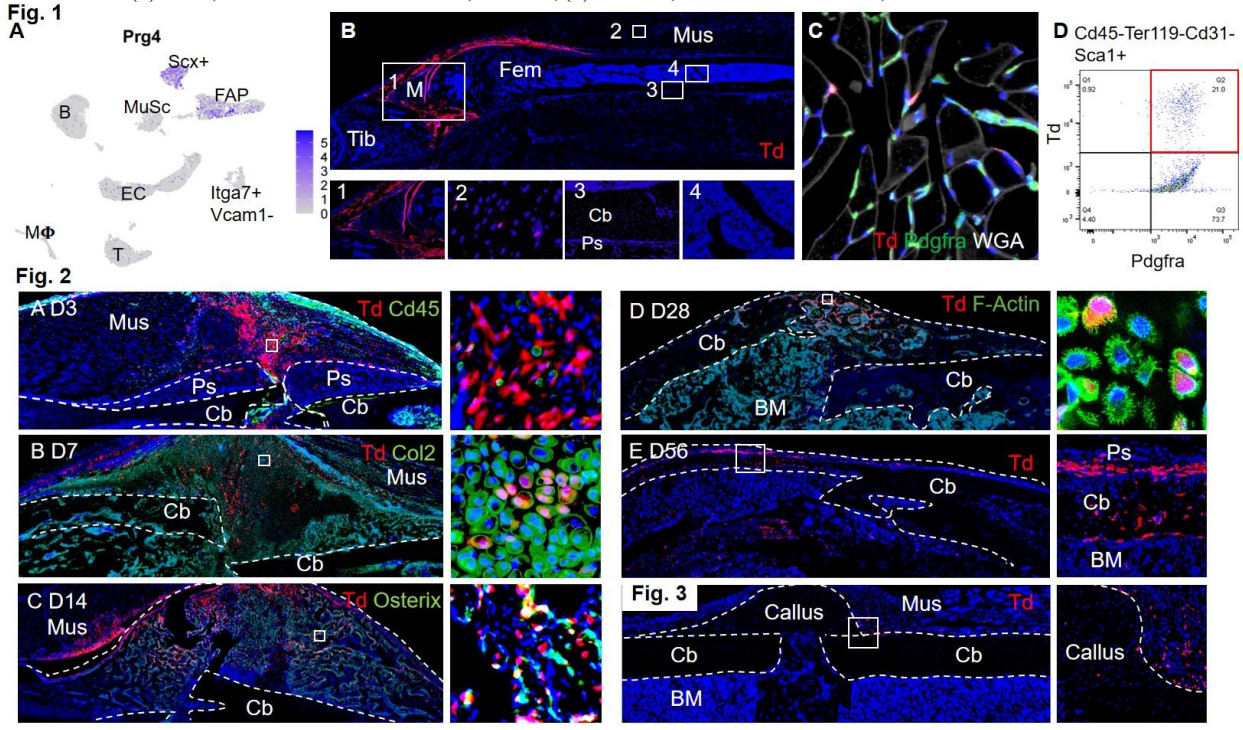

Figure 1. **Prg4 marks muscle FAPs but not bone cells. (A)** UMAP plot shows Prg4 expression in muscle cells. MuSc: muscle stem cell; B: B cell; T: T cell; mφ: macrophage; Scx+: Scleraxis+ cell. **(B)** Fluorescent image of a hindlimb from *Prg4ER/Td* mice that received 5 Tam injections and euthanized at 2 days after the last injection. Squares in the top panel are magnified at the bottom. Mus: muscle; M: meniscus Cb: cortical bone; Ps: periosteum; Fem: femur, Tib: tibia. **(C)** Fluorescent image of muscle with WGA and Pdgfra staining. **(D)** Flow analysis of Td+ cells in muscle FAPs (Sca+Pdgfr+). **Figure 2**: Muscle FAPs contribute to fracture healing. Fluorescent images of tibiae at day 3 **(A)**, 7 **(B)**, 14 **(C)**, 28 **(D)**, 56 **(E)** post fracture are shown. Right panels are magnified images of squares in the left panels. **Figure 3**: **Muscle FAPs do not significantly contribute to bone healing after drill-hole injury**.