The Role of Interstitial Muscle Cells in Fracture Healing and Heterotopic Ossification

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INTRODUCTION:

Muscle-resident cells are capable of converting to osteoblasts and producing bone during both fracture repair and heterotopic ossification. These cells are commonly referred to as fibroadipogenic progenitors (FAPs). How FAPs differentiate to osteoblasts, and the physiologic importance of their contributions to bone formation are incompletely understood. To study FAPs *in vivo*, we genetically engineered a new mouse strain that allowed us to specifically track and modulate FAPs during regenerative and pathologic mineralization processes. Using this new allele, *Clec3b*^{CreERT2}, we tested the hypothesis that FAPs gain multi-lineage differentiation ability in response to musculoskeletal injury, and that their participation in bone production is significant.

METHODS:

Mice: All experiments were approved by IACUC. We engineered *Clec3b*^{CreERT2} knock-in mice using CRISPR-Cas9. We bred them with conditional reporter mice to generate *Clec3b*^{CreERT2}*; R26*^{tdTom}*; Bglap*^{eGFP} mice, wherein FAPs and their descendants were labeled with tdTomato fluorescence after tamoxifen treatment, and osteoblasts were GFP+. Similarly, to deplete FAPs, we generated *Clec3b*^{CreERT2}*; R26*^{DTA} mice, which allowed us to kill FAPs with tamoxifen injections. Finally, we blocked WNT-signaling in FAPs by deleting β-catenin in *Clec3b*^{CreERT2}*; Ctnnb1*$^{fl/fl}$ mice.

Fracture Model: We induced stabilized long bone fracture by performing a mid-diaphysis osteotomy on the right femur and aligning the bones by inserting a stainless-steel pin into the intramedullary cavity.

Heterotopic Ossification: To induce muscle mineralization, we performed a single injection of recombinant BMP2-Matrigel mixture (2.5μg in 50ml) into the right hamstring muscles.

Histology: Bone, muscle and fracture callus specimens were fixed in cold PFA, decalcified for up to 2 weeks, sectioned and fluorescently imaged with an automated slide scanner and confocal microscope.

RESULTS:

We confirmed that cells labeled with tdTomato in *Clec3b*^{CreERT2}*; R26*^{tdTom}*; Bglap*^{eGFP} mice are FAPs, based on their localization to muscle tissue, absence on bone-lining surfaces, and protein marker expression profile (SCA1^{+}CD45^{-} CD31^{-} TER119^{-}). FAPs did not become osteoblasts during postnatal skeletal growth and homeostasis. However, they began proliferating immediately after fracture, and differentiated to fibroblasts, chondrocytes, osteoblasts (indicated by Bglap.GFP expression) and bone marrow stromal cells (indicated by Cxcl12.GFP expression) in the callus over 3 weeks (Figure 1). Our time course histology analyses showed a highly significant enrichment for tdTomato+ cells in the vascular portion of the early fracture callus ($p<0.001$, paired t-test, 6- and 10-days post-surgery), whereas few tdTomato+ cells were found in the avascular cartilaginous callus. These results suggest that FAPs migrate from muscle to the fracture callus in coordination with blood vessels. When we depleted FAPs with repeated tamoxifen injections (every 3 days for 3 weeks post-surgery), we measured a significant reduction in callus bone volume with μCT analysis ($p < 0.05$), indicating the significance of FAPs in fracture repair (Figure 2).

We found that BMP2-injections resulted in robust bone formation in the hindlimb muscles. These ectopic bones were heavily populated by FAP-derived osteoblasts, osteocytes and adipocytes, indicated by tdTomato-fluorescence. Blocking WNT-signaling, a pathway essential for bone formation, through deletion of β-catenin in FAPs resulted in reduced heterotopic ossification (Figure 2).

DISCUSSION AND CONCLUSION:

Our results indicate that FAPs are osteogenically inactive under normal conditions. However, following fractures in a nearby bone, they acquire the ability to convert to multiple cell-types, including activated fibroblasts, osteoblasts, osteocytes, chondrocytes, and bone marrow stromal cells. Further, their absence reduces fracture callus mineralization. Importantly, FAPs also make important contributions to heterotopic ossification of muscle. These results indicate that FAPs serve as a common point of cellular origin for both regenerative and pathologic mineralization processes. Understanding how FAPs are osteogenically mobilized and make cell-fate decisions can help promote fracture repair and reduce heterotopic ossification. Such interventions would significantly benefit patients suffering from high-energy-trauma, and similar skeletal injuries involving heterotopic ossification.

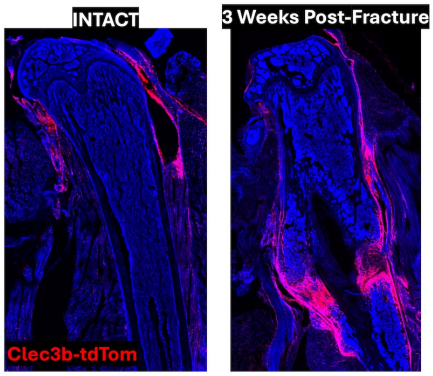

Figure 1. A mouse model of diaphyseal stabilized fracture induces FAP cell activation. (A) FAP cells are osteogenically quiescent and are localized in the outer periosteum, connective tissue, and are not found in the bone marrow postnatally. **(B)** Representative histology showing the migration of FAP cells into the fracture callus.

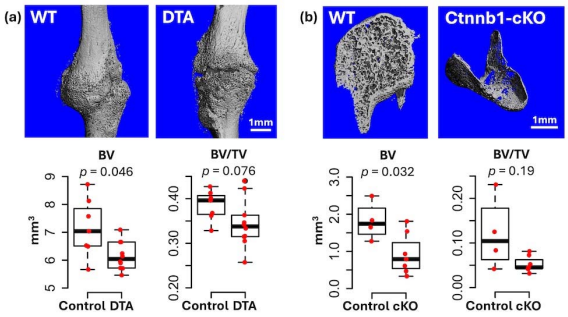

Figure 2. FAP cell-depletion reduced callus bone volume and conditional deletion of the Wnt-signaling associated transcription factor β-catenin in Clec3b+ cells reduce heterotopic ossification. (a) μCT data showing FAP cell-depletion reduced callus bone volume. **(b)**μCT data showing decreased BV and mineralization of heterotopic ossification with conditional deletion of the Wnt-signaling associated transcription factor β-catenin.