Leptin-receptor lineage cells are abundant in fracture nonunion fibrotic tissue and contribute to both osteogenic and fibrotic processes

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INTRODUCTION: Fracture nonunion is a common complication following a fracture, affecting an estimated 5-10% of individuals who have sustained fractures[1]. In cases of fracture nonunion, the gap between fractured bone fragments becomes filled with fibrous tissue rather than the expected regrowth of normal bone tissue[2]. This outcome is problematic because the fibrous tissue lacks the necessary mechanical strength to adequately stabilize the bone fragments[3]. Consequently, this lack of stability results in persistent pain and the inability of patients to effectively utilize the injured limb[1]. Currently, there exists a knowledge gap concerning the cellular origins of the fibrous tissue that forms between fractured bone fragments. In our study, we have demonstrated the prevalence of cells expressing the leptin receptor (LEPR+ cells) within this fibrous tissue. Notably, our research has also revealed that these LEPR+ cells within the fibrotic tissue express two key markers: ACTA2, commonly associated with myofibroblasts, and osteocalcin (OCN), a well-recognized marker of osteoblasts. This intriguing discovery implies that the LEPR+ cells present in the interfragmentary fibrous tissue may possess a dual role, potentially contributing both to osteogenic (bone-forming) and pro-fibrotic processes.

METHODS: All experiments were approved by local IACUC. *Animals:* Lepr-cre (Stock 008320), Rosa26-CAG-loxp-stop-loxp-tdTomato (Stock 007909), Acta2-DsRed (Stock 031160), Ocn-GFP (Stock 017469) were purchased from Jackson Laboratories.

Model of fracture non-union: Non-union model was adapted from Garcia, P et al 2007. Briefly, the femur was exposed through a lateral approach. Two osteotomies were made with a diamond saw to create a 1.8 mm diaphyseal defect at the mid-shaft of the femur. A needle with both ends flattened was then inserted into the intramedullary canal and the fracture gap to stabilize the bone. A clip of 8 mm in length was implanted ventro-dorsally into the femur to provide extra rotational stabilization and to prevent the collapse of the gap between proximal and distal fragments. To induce non-union, the periosteum <2 mm from the gap was stripped by surgical scalpel. Fracture without periosteum removal was used as control.

Micro-CT: Scans (µCT 45, Scanco Medical, Switzerland) were performed at 6 µm voxel size, 90 kVp, 145 mA, and 0.36 rotation step (180 angular range) per view. Volume of interest was defined as a 4 mm-long cylindrical region whose is center was the center of the fracture line. Radiological bone morphological parameters, particularly bone volume fraction (bone volume/total volume; BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th), and trabecular separation (Tb.S) were assessed.

Statistical Analysis: Statistical analysis was performed using Student's t-test. $p<0.05$ was considered as significant.

RESULTS: At the 10-week mark post-surgery, we observed a persistent gap in the femurs of mice subjected to a fracture non-union model (Figure 1a). Further examination using micro-CT imaging of the femurs from the non-union model confirmed the lack of bony bridges forming between the proximal and distal fragments (Figure 1b). Conversely, the micro-CT scans of femurs from the healed fracture model revealed complete bridging between these proximal and distal fragments (Figure 1b). The nonunion group exhibited a significantly lower BV/TV when compared to the healed fracture group (Figure 1c). Histological analysis further confirmed the existence of fibrotic tissue occupying the space between the proximal and distal segments, without any bony bridges (Figure2a-b). Immunofluorescence imaging of non-union femur from *LepR-Cre;ZsGreen;Acta2-DsRed* showed significantly higher percentage of $LEPR^+ACTA2^+$ cells in the fibrous tissue than the healed fracture counterpart (Figure 3a,c). Immunofluorescence imaging of non-union femur from *LepR-Cre;tdTomato;Ocn-GFP* showed significantly higher percentage of $LEPR^+OCN^+$ cells in the fibrous tissue than the healed fracture counterpart (Figure 3b,d).

DISCUSSION: Our data suggests that $LEPR^+$ cells are abundantly present in the fibrotic tissue in fracture nonunion. $LEPR^+$ cells in the fibrotic tissue express both ACTA2, a commonly used marker of myofibroblast, and OCN, a commonly used marker of osteoblast. Further studies are currently being performed in to explore the osteoblastic differentiation potential of $LEPR^+$ $ACTA2^+$ OCN^+ cells.

SIGNIFICANCE/CLINICAL RELEVANCE: The LEPR+ cells present in the interfragmentary fibrous tissue may possess a dual role, potentially contributing both to osteogenic (bone-forming) and pro-fibrotic processes.

REFERENCES: 1, Zura R et al., 2016, *JAMA Surgery*, 151(11):e162775. 2, Panteli M et al., *J Cell and Mol Med*, 2022, 26:601-623. 3, Suhardi V et al, ORS NIRA Presentation 2023, Paper #250.

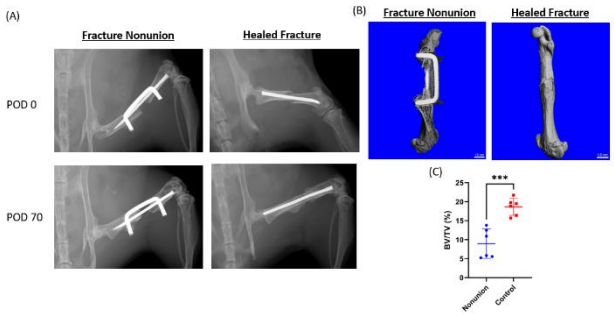

Figure 1. Plain radiograph (A) and representative 3D-reconstructed microCT (B) of mice that underwent fracture nonunion and healed fracture model. (B) BV/TV of the peri-fracture area of mice that underwent nonunion vs healed fracture (control) model. Data is represented as mean +/- s.d. ***p<0.001.

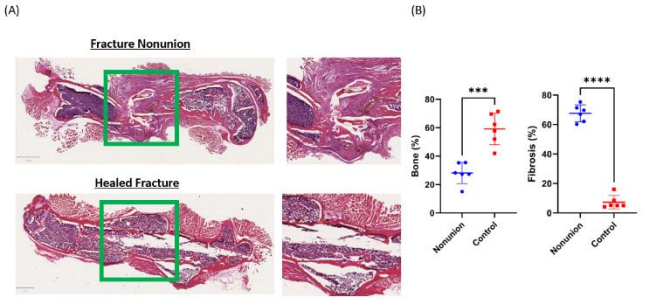

Figure 2. Histology of mice that underwent nonunion or healed fracture model ten weeks after surgery**.** (A) Representative H&E image of surgical femur of mice that underwent fracture nonunion or healed fracture model. (B) Percent fibrotic area and bone area around the fracture line. Data is represented as mean +/- s.d. *** p<0.001, ****P<0.0001.

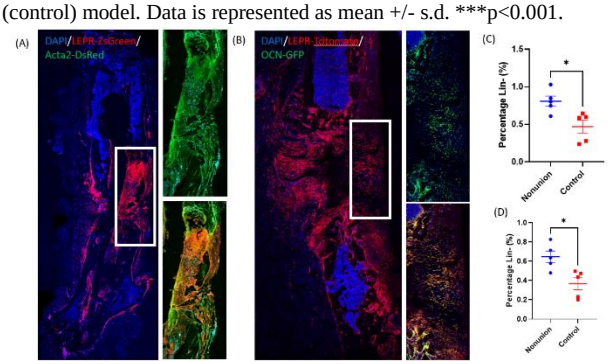

Figure 3. Immunofluorescent imaging of mice that underwent nonunion or healed fracture model ten weeks after surgery**.** (A) Representative immunofluorescent image of surgical femur of *LepR-Cre;ZsGreen;Acta2-DsRed* mice that underwent fracture nonunion or healed fracture model. Red= LEPR-Zsgreen, Green= ACTA2-DsRed, Blue= cell nucleus. (B) Representative immunofluorescent image of surgical femur of *LepR-Cre;tdTomato;Ocn-GFP* mice that underwent fracture nonunion or healed fracture model. Red= LEPR-tdTomato, Green= OCN-GFP, Blue= cell nucleus. (C-D) There were significantly more $Lin^-LEPR^+ACTA2^+$ cells (C) and $Lin^-LEPR^+BGLAP^+$ cells (D) in the fracture nonunion group than healed fracture (control) group. Data are the mean ± s.d. *p<0.05