Targeting oxygen distribution in the fracture gap to induce vascularized bone healing

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The initial phase of bone healing has long been thought to be characterized by severe lack of oxygen due to the rupture of blood vessels. However, the oxygen levels in the bone marrow and fracture gap are understudied due to methodological limitations. Erythrocytes are major regulators of metabolism and survival, delivering oxygen to cells and tissues. Stress erythropoiesis is an acute type of erythropoiesis that can be induced by nutrient deficiency or trauma. In this study, we aimed to understand how tissue oxygen levels change during the initial phase of bone fracture healing and how cells of the erythroid lineage modulate local oxygen levels to direct bone repair.

We established orthogonal methods for in vivo measurements of oxygen levels. First, we used EF5 staining. EF5 is selectively reduced by nitroreductase enzymes under hypoxic conditions, resulting in the formation of EF5 adducts that can be visualized with fluorophore-coupled antibody. Second, oxygen levels were measured by the phosphorescence quenching method using an established probe Oxyphor PtG4. Both methods were used to quantify oxygen levels in bone marrow and early bone fracture hematoma in vivo. C57BL/6J female mice aged 12–16 weeks underwent osteotomy surgery with stiff external fixation (MouseExFix, RISystem). All procedures were conducted in accordance with UPenn IACUC regulations (protocol no: 806482). Oxygen in the fracture gap was measured at 3, 5 and 7 dpf after osteotomy with stiff external fixation. The frequency of EF5-positive (i.e., hypoxic) cells at 3, 5 and 7 dpf in the gap was significantly lower than that in either the adjacent bone marrow of the ipsilateral limb, or bone marrow of the un-injured contralateral bone marrow. This indicates that cells in the early fracture gap are not hypoxic, as long believed. To verify this unexpected result, we performed non-invasive oxygen measurements in live mice using Oxyphor PtG4 at 3, 7 and 14 dpf and confirmed that the fracture hematoma is not hypoxic. Together, these two orthogonal methods showed that, in contrast to the prior assumptions, the early fracture gap features high initial oxygenation, which gradually decreases and approaches bone marrow pO_2 levels by 14 dpf.

Next, we sought to determine why the fracture gap exhibits such high oxygen levels. We hypothesized that local erythropoiesis plays a role in fracture gap oxygenation and subsequent fracture repair. CD71 (Transferrin receptor 1) is a marker for erythrocyte precursors, which is required for iron import into erythroid cells. We treated fractures with CD71-blocking antibody (clone: 8D3; 100 µg or a rat IgG2a isotype control), and quantified oxygen in the fracture gap at day 3 post-fracture. We found that injection of CD71-blocking antibody in the fracture gap at the time of surgery increased the fraction of EF5-positive (i.e., hypoxic) cells and significantly decreased the tissue oxygen tension. In addition, CD71 blockade significantly increased osteoprogenitor activation, angiogenesis, and bone formation in the callus and fracture gap at 14 dpf. Using scRNA-sequencing, we identified erythroid progenitors as predominately expressing *Tfrc* (CD71) which were enriched for hypoxia-related gene expression upon anti-CD71 antibody treatment.

Taken together, our results provide new insights on the role of oxygen in bone repair, identify a putative cellular mechanism for the elevated local oxygen levels and establish a novel intervention that promotes hypoxia and improves bone repair.