Targeting CD71^{+} Oxygen-Releasing Cells Induces Fracture Gap Hypoxia and Enhances Vascularized Bone Healing

Annemarie Lang[1], Simin Belali[2], Joseph M. Collins[1], Madhura Nijsure[1], Sergei A. Vinogradov[2], Cameron J. Koch[3], Joel D. Boerckel[1]
[1]Departments of Orthopaedic Surgery and Bioengineering, University of Pennsylvania, Philadelphia, PA; [2]Department of Biochemistry and Biophysics, and Department of Chemistry, University of Pennsylvania, Philadelphia, PA; [3]Department of Radiation Oncology, University of Pennsylvania, Philadelphia, PA
Email: annemarie.lang@pennmedicine.upenn.edu

Disclosures: A.L. (N), S.B. (N), J.M.C. (N), M.N. (N), S.A.V. (N), C.J.K. (N), J.D.B. (N)

INTRODUCTION: 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 [1]. However, the oxygen levels in the bone marrow and fracture gap are understudied due to methodological limitations. Oxygen levels direct cell survival, proliferation, and differentiation, with optimal oxygen levels depending on cell type and context. Different tissues exhibit different average oxygen levels, which are determined by the balance between the supply and consumption. The spleen, for example, stores erythrocytes and exhibits high oxygen levels (~8% pO_2), while the bone marrow environment is relatively hypoxic (1-6% pO_2 [2]). Erythrocytes are major regulators of metabolism and survival, delivering oxygen to cells and tissues. Billions of new erythrocytes are produced every day by erythropoiesis in the bone marrow to oxygenate the body. 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.

METHODS: 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 [3]. Second, oxygen levels were measured by the phosphorescence quenching method using an established probe Oxyphor PtG4 [4].

Using these methods, we performed two experiments. In **Experiment 1**, we used EF5 labeling, followed by flow cytometry, and independently, phosphorescence quenching 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). Eye ointment, clindamycin, sustained-release buprenorphine (ZooPharm; s.c.; 1 mg/kg) and NaCl were applied during the preparation. The left femur was bluntly exposed, and the pins were placed though the connector bar of the external fixator laterally parallel to the femur. A 0.5 mm gap was created between the middle pins using a Gigli wire. For EF5 staining (3 days post-fracture – dpf): Mice were injected with 10 mM EF5 compound and euthanized after 2 hours. Cells were isolated from the fracture gap (Fig. 1A; GAP); the adjacent bone marrow of the same limb (Fig. 1A; iBM) and the bone marrow from the uninjured contralateral bone (Fig. 1A; cBM) and fixed with 4% paraformaldehyde before staining overnight with Cy5-conjugated anti-EF5 antibody (clone: ELK3-51). A BD FACSCanto II was used for flow cytometry and data were analyzed using FlowJo software. For in vivo measurements, Oxyphor PtG4 was injected in the tail vein 1 day prior to surgery to provide the compound evenly accessible throughout the fracture hematoma and gap after osteotomy. Osteotomy surgery was performed as described above. At 3, 7 and 14 dpf, mice were anesthetized with isoflurane and the osteotomized and contralateral bones were carefully dissected and exposed. Oxygen levels were measured at different locations of the bone using a fiber-optic phosphorimeter. Next, in **Experiment 2**, we injected either a monoclonal rat anti-mouse CD71 antibody (clone: 8D3; 100 µg) or a rat IgG2a isotype control in the fracture gap during surgery. EF5 staining and Oxyphor measurement were performed at 3 dpf as described above. MicroCT analysis (Scanco µCT 45) and histology/immunofluorescence (H&E; endomucin – Emcn = vessel; osterix – Osx = osteoblasts) were performed at 14 dpf. Statistical analysis was performed using ANOVA/Tukey's and Student's t-test, as appropriate. Finally, to determine which cells are responsive to CD71 antibody blockade, we performed single cell RNA-sequencing at 3 dpf (N = 3).

RESULTS: In **Experiment 1**, oxygen in the fracture gap was measured at 3, 5 and 7 dpf after osteotomy with stiff external fixation (Fig. 1A, B). 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 (iBM), or bone marrow of the un-injured contralateral bone marrow (cBM; Fig. 1B). 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. These measurements confirmed that the fracture hematoma is not hypoxic, but rather exhibits oxygen levels commensurate with those of the spleen (57.9±11.5 mmHg; equivalent to ~8% pO_2) (Fig. 1C). Oxygen tension in the contralateral bone marrow was 28.4±7 mmHg (equivalent to ~3% pO_2), consistent with previously reported values [2]. 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. An inevitable consequence of bone fracture is the rupture of blood vessels, resulting in bleeding, release of red blood cells (erythrocytes) and perhaps stress erythropoiesis (erythroid progenitors). We therefore 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. In **Experiment 2**, we treated fractures with CD71-blocking antibody (or Isotype IgG control), and quantified oxygen in the fracture gap at day 3 post-fracture (Fig. 1D). 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 (Fig. 1E) and significantly decreased the tissue oxygen tension (Fig. 1F). In addition, CD71 blockade significantly increased osteoprogenitor activation, angiogenesis, and bone formation in the callus and fracture gap at 14 dpf (Fig. 1G, H). Using scRNA-sequencing, we identified erythroid progenitors as predominately expressing *Tfrc* (CD71). Further, when the anti-CD71 antibody was administered during the onset of fracture, these erythroid progenitors were enriched for the hallmarks of hypoxia, consistent with contralateral bone marrow and in line with our previous data.

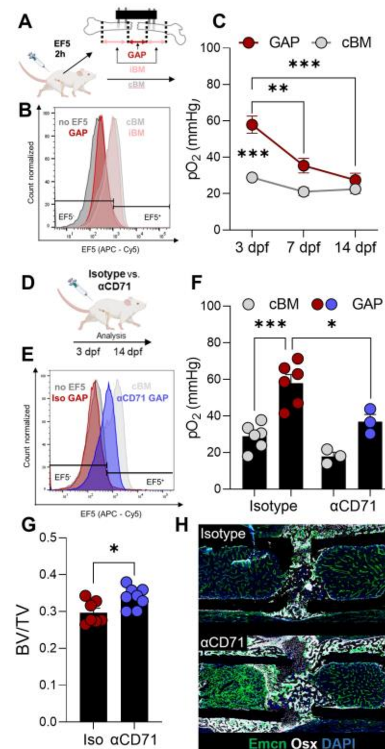

Fig. 1: Modulating oxygen in the fracture by blocking CD71^{+} cells. (A) Cells from osteotomized (GAP: fracture gap; iBM: ipsilateral bone marrow) and un-injured bones (cBM: contralateral bone marrow) were isolated and analyzed for EF5 frequency – (B) representative histograms (3 dpf) and (C) absolute pO_2 measurements (in mmHg). (D) Anti (α)-CD71 antibody was administered during surgery and cells were analyzed at 3 dpf – (E) representative histograms of EF5 frequency and (F) absolute pO_2 measurements (in mmHg). (G) MicroCT analysis and (H) immunofluorescence staining were performed at 14 dpf after treatment with isotype (Iso) or αCD71 antibody. Bar graphs show mean ± SEM and individual data points.

DISCUSSION: While the dogma in the field dictates that the initial phase of fracture repair is hypoxic, our data demonstrate that this is not the case. Rather, we show that the early fracture gap exhibits high oxygen tension and identify a population of CD71^{+} cells that modulate oxygen. Treatment of the fracture with CD71-blocking antibody induces both cell and tissue hypoxia and enhances fracture repair. These data establish that the initial phase of fracture repair is not hypoxic, identify a putative cellular mechanism, and establish a novel intervention that promotes hypoxia and improves bone repair.

SIGNIFICANCE/CLINICAL RELEVANCE: Taken together, our results overturn the long-held dogma regarding the role of oxygen in bone repair and introduce a new paradigm, advocating for hypoxia-promoting therapeutics for enhancement of bone healing.

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