Heterogeneity of Cellular Hypoxia in Murine Bone Marrow

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INTRODUCTION: Two critical events early in fracture healing determine the course of repair: 1) lack of oxygen caused by blood vessel rupture and 2) mechanical instability. Cellular hypoxia-induced signaling and mechanotransduction are therefore critical to bone healing, but how crosstalk between these pathways impacts fracture repair is unknown. Our goal is to define how mechanical loading alters the identity and distribution of hypoxic cells in early fracture repair. Toward this end, we here validated a methodological pipeline to precisely sort for hypoxic and non-hypoxic cells, using murine bone marrow as a model. Oxygen concentration can be expressed as a percentage of the partial pressure of atmospheric O_2. At sea level, atmospheric pressure is 760 mm Hg; thus the partial pressure of oxygen (pO_2) in the atmosphere is 160 mm Hg. Prior measurements characterize bone marrow as "hypoxic" (i.e., 7.5 – 45 mmHg, or 1% – 6% pO_2) [1]. However, intracellular pO_2 can differ from that of the bulk environment, depending on local pO_2 and the oxygen consumption rate in a given cell. Further, methods to specifically sort and further characterize cells by their intracellular O_2 levels are limited. Nitroimidazoles such as EF5 are selectively reduced by nitroreductase enzymes under hypoxic conditions, resulting in the formation of EF5 adducts that can be visualized with fluorophore-coupled antibody [2]. Here, we establish EF5 staining as a precise method to delineate cells by levels of intracellular oxygen.

METHODS: We performed two experiments to assess cellular hypoxia in femoral bone marrow. In **experiment 1**, we performed flow cytometry of isolated bone marrow cells and cryohistological immunostaining *in situ*. Briefly, 12–14 week-old C57BL/6J female mice (Jackson Laboratory) were injected with 10 mM EF5 (Merck Millipore) and euthanized after 4 hours (n= 4). Femora were either directly cryo-embedded or flushed to isolate bone marrow cells. As a positive control for *in situ* EF5-labeling of marrow cells in low oxygen conditions, we injected one mouse with EF5 and euthanized after 30 min. The body was then kept at 37°C in entirely anoxic conditions for 45 min (termed euthanized animal control, EAC). As a negative control for *in situ* EF5-labeling of cells with known high pO_2, we performed EF5 staining of the spleen (nominal pO_2 of 8-10%). To control for any nonspecific background staining, we also included bone marrow cells from non-EF5 treated animals ($EF5^-$). For flow cytometry, cells were fixed with 4% paraformaldehyde directly after collection, and stained overnight with Cy5-conjugated anti-EF5 antibody (clone: ELK3-51; Merck Millipore). To distinguish between cells of the erythroid lineage (erythrocytes, pro-erythrocytes) and nucleated hematopoietic cells, we stained for Ter119 and CD45 (Biolegend), respectively. A BD FACSCanto II was used for flow cytometry and data was analyzed using FlowJo software. For immunofluorescence staining, sections were fixed in 4% paraformaldehyde, blocked with 5% goat serum, and stained 2h with Cy3 conjugated anti-EF5 antibody (clone: ELK3-51; Merck Millipore). All procedures were conducted in accordance with UPenn IACUC regulations (protocol no: 806482). In **experiment 2**, we isolated and cultured bone marrow cells under defined oxygen concentrations and evaluated intracellular EF5 by flow cytometry (n=4). Bone marrow cells were isolated from femora of C57BL/6J female mice and transferred into culture medium (MEM + 5% FCS supplemented with HEPES and bicarbonate as buffer system and 100 µM EF5 compound). Cells were cultured without CO_2 in aluminum chambers for 2h and the gas phase in the chambers was changed to 10%, 2%, 0.5%, 0.1% pO_2 using a series of gas exchanges with oxygen-free nitrogen (n= 4). Afterwards, cells were fixed and stained as described before while an aliquot of cells was used for RNA isolation and qPCR.

RESULTS: In **experiment 1**, we were able to detect differences in cellular hypoxia by identifying EF5+ and EF5- cell populations as well as differences in the EF5 fluorescence intensity (Fig. 1A). In addition, anoxia (EAC) in the bone marrow resulted in a complete shift of cells with 92% EF5 positive cells (compared to 52%) verifying sufficient EF5 distribution in the bone marrow (Fig. 1A). Moreover, no EF5 positive cells were found in the spleen under normal conditions, although anoxia resulted in 100% EF5 positivity in spleen cells (Fig. 1A). Frequency quantification revealed that 51.25±5.39 % were positive for EF5 while 48.75±5.39 % of bone marrow cells were not positive for EF5 (Fig. 1B). Immunofluorescence staining of the whole bone marrow identified regions with high and low EF5 signal intensity, indicating heterogenous intracellular hypoxia (Fig. 1C). Distinctive analysis of bone marrow cell subpopulations such as erythroid cells (Ter119) and hematopoietic cells (CD45) indicated that erythrocytes and their progenitors are not positive for EF5 while a large cell fraction of nucleated CD45+ hematopoietic cells is positive for EF5. In **experiment 2**, murine bone marrow cells were cultivated under oxygen concentrations resulting in the presence of a EF5+ and EF5- cell fraction at 10% pO_2 and almost exclusively EF5+ cells at 0.1% oxygen (Fig. 1E). RNA expression analysis indicated an increasing expression of *Slc2a1* (HIF target gene) and *Ccn1* (YAP/TAZ target gene) under decreasing oxygen availability. This suggests that even if bone marrow cells are kept under the same hypoxic conditions, there is a heterogeneity of in intracellular O_2 concentration.

DISCUSSION: Our results indicate a substantial heterogeneity in intracellular hypoxia (equivalent to EF5 staining intensity) among bone marrow cells *in situ* but also when cultivated *in vitro* under defined oxygen concentrations. This underlines that intracellular pO_2 can differ from that of the bulk environment, depending on local pO_2, the oxygen consumption rate but also potential other unknown factors. Using this tool, we now aim to define how mechanical cues and hypoxia direct the course of bone fracture repair.

SIGNIFICANCE/CLINICAL RELEVANCE: Here we describe a unique methodology to pre-select hypoxic and non-hypoxic cells for sophisticated single-cell based analysis which will enable us to determine how *in vivo* mechanical cues direct intracellular hypoxia and mechano-signaling during bone fracture repair in future studies.

REFERENCES: [1] Spencer et al. 2014 *Nature*; [2] Koch, C. 2002 *Methods Enzymology*

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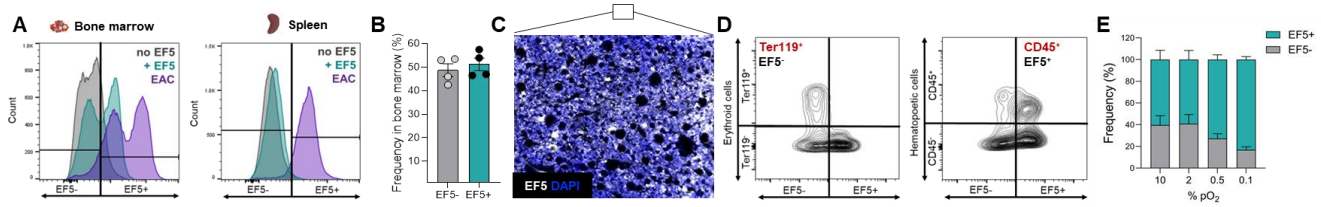

Fig. 1: EF5 staining of murine bone marrow cells – results from experiment 1 (A–D) and experiment 2 (E). (**A**) Exemplary histograms – flow cytometric analysis of EF5 intensity in isolated bone marrow and spleen cells from mice treated without or with EF5 (no EF5 – untreated mice; +EF5 – mice injected with EF5 compound 4h prior to dissection; EAC – euthanized animal control/anoxia). (**B**) Quantification of EF5+ and EF5- cell frequencies in the bone marrow (n=4). (**C**) Exemplary image of EF5 immunofluorescence staining in whole fresh frozen bone marrow samples. (**D**) Contour plots for combined staining of Ter119 (erythroid cells) or nucleated CD45+ hematopoietic cells with EF5. (**E**) Quantified cell frequencies for *in vitro* experiment – cultivation of bone marrow cells at different pO_2 (n=4 – two independent experiments).