Single cell analysis reveals transient expansion of marrow adipogenic lineage precursors as the mechanism for bone marrow recovery after radiation

Leilei Zhong, Lutian Yao[1], Zhen Miao[2], Mingyao Li[2], Jaimo Ahn[1], and Ling Qin[1]
[1]Department of Orthopaedic Surgery, [2]Biostatistics, Epidemiology and Informatics,
Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA.

Introduction

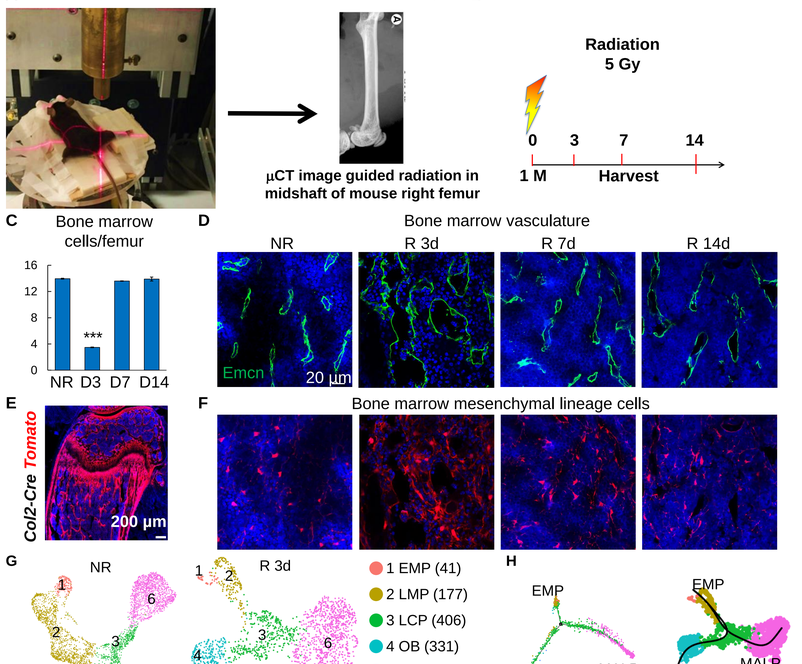
Radiotherapy treats malignant tumors effectively but also damages surrounding tissues, such as bone marrow. High-dose radiation often leads to bone marrow suppression and damage of vasculature. Bone marrow cells, including mesenchymal, hematopoietic, and endothelial cells, attempt to regenerate after radiation injury but the repair mechanism is still largely unknown. Decades of studies have demonstrated that mesenchymal lineage cells provide supportive environment for hematopoiesis and angiogenesis. Using single cell RNA-sequencing (scRNA-seq) technique, we recently discovered a novel subpopulation of cells that express most adipogenic markers but with no lipid accumulation. Based on their location in the differentiation route, we named them marrow adipogenic lineage precursors (MALPs). By examining scRNA-seq dataset of bone marrow mesenchymal lineage cells from irradiated mice, we discovered that MALPs play a critical role in assisting bone marrow regeneration after radiation.

Methods

Animals- All animal work performed was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. *Col2/Td* and *Adipoq/Td* mice were generated by breeding *Rosa-tdTomato* mice with *Col2-Cre* or *Adipoq-Cre* mice. *Adipoq/Td/DTR* mice was generated by breeding *DTR* mice with *Adipoq/Td* mice. Diphtheria toxin (DT, 50ug/Kg) or veh were injected i.p. twice a week for cell ablation assay. A clinically relevant radiation dose of 5 Gy was delivered to the midshaft of right femurs (15 mm in diameter) of 1-month-old mice from a focal irradiator (SARRP, Xstrahl) at a rate of 1.65 Gy/min with the aid of built-in μCT and X-ray. *Sorting bone marrow Td+ cell for scRNA-seq-* Endosteal bone marrow cells were isolated using an enzymatic digestion method as we described previously[1] and resuspended into FACS buffer for sorting Td+ cells. A total of 2 batches of single cell libraries were constructed from non-radiated (2 batches, n=5), radiated (1 batch, n=3) male *Col2/Td* mice. Libraries were generated by Chromium controller (10X Genomics) and sequencing was performed on an Illumina HiSeq platform. Unsupervised clustering was conducted by Seurat and trajectory analysis was conducted by Monocle. *Whole mount immunofluorescence-* Freshly dissected bones were processed for cryosections and fluorescent imaging. Statistics- All analyses were conducted using t-tests.

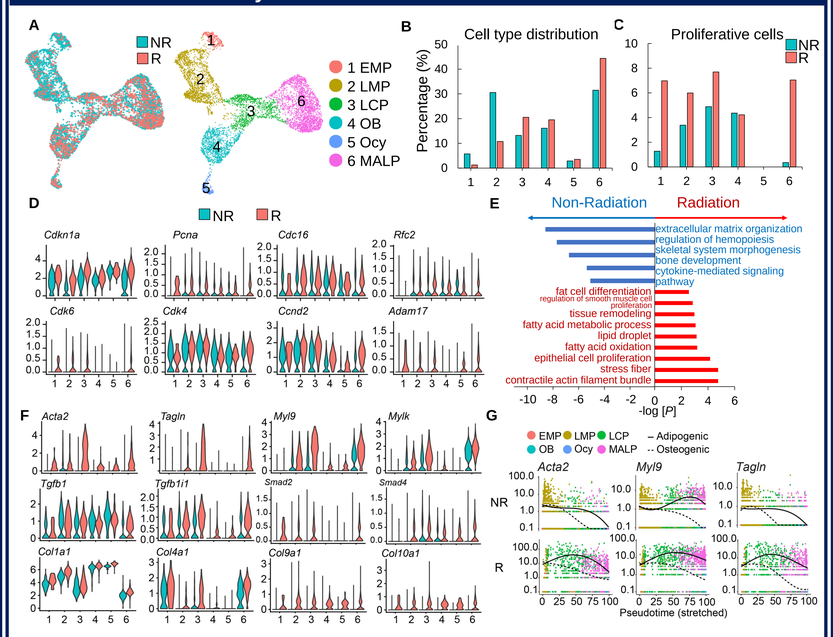
Results

Radiation quickly expands bone marrow mesenchymal lineage cells and Single cell RNAseq analysis of mesenchymal lineage population after radiation



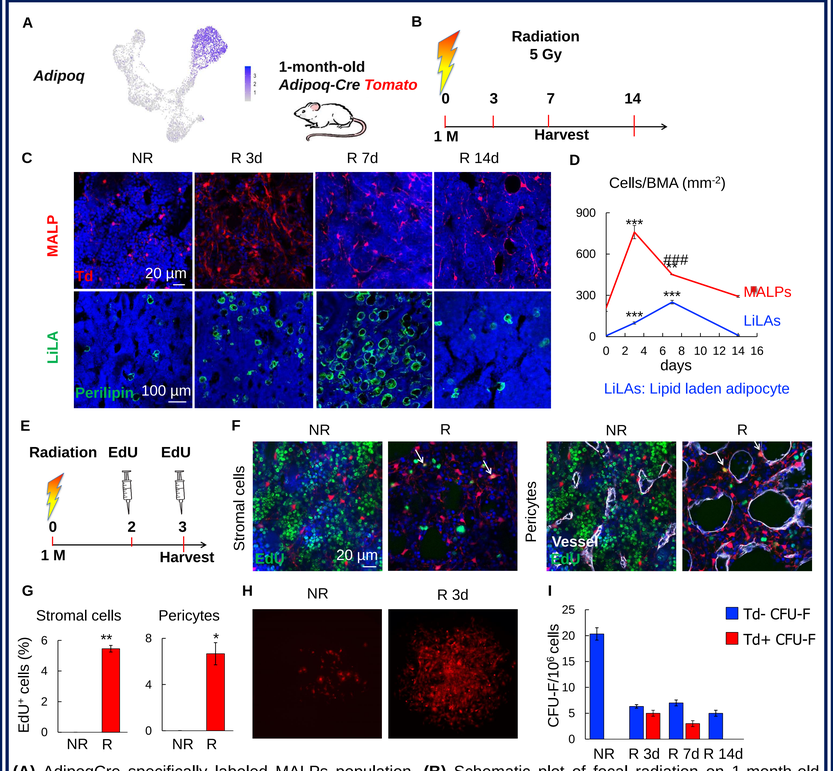
(A) Photo of mouse undergo focal radiation on femur on Small animal radiation research platform. **(B)** Schematic plot of focal radiation on 1-month-old mice femurs. **(C)** 5 Gy of focal SARRP radiation to the distal femurs of 1-month-old mice drastically reduced bone marrow cellularity at day 3. Starting from day 7, bone marrow cellularity was mostly returned to normal. **(D)** Radiation damaged endothelial cells and remarkably altered vessel structure, resulting in increased vessel diameter and area and reduced vessel density at day 3. The vessel microstructure could also recover from day 7 post radiation. **(E)** In *Col2-Cre/Tomato* mice, Td labeled all the mesenchymal lineage cells. **(F)** Td^+ cells inside the bone marrow of Col2/Td mice strikingly increased at 3 days after radiation, then gradually decreased over the time. **(G)** Large scale scRNA-seq on Td^+ cells sorted from bone marrow of 1-month-old nonradiated or day 3 post radiated Col2/Td mice. Unsupervised clustering of mesenchymal cells yielded a similar set of cell clusters for both conditions. **(H)** Pseudotime trajectory analyses of radiation dataset. **(I)** Violin plots of cluster-specific makers in radiation dataset.

ScRNA-seq analysis predicts cell cycle entry and myofibroblast conversion of MALPs.



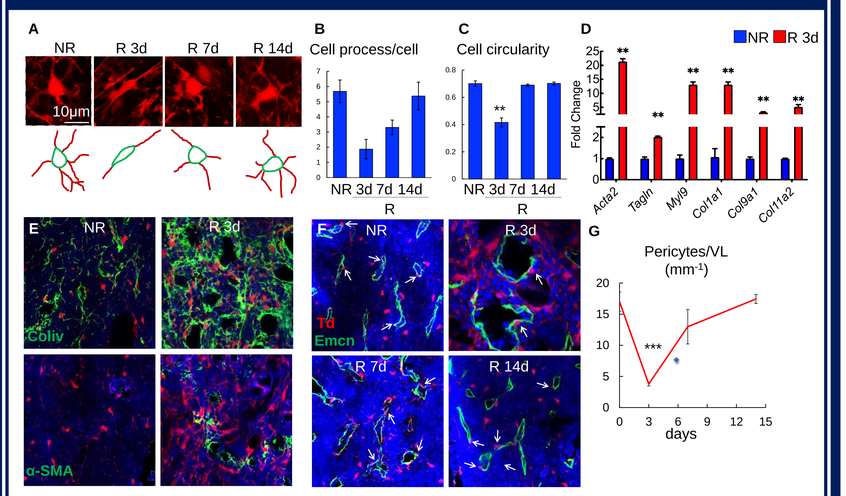
(A) Integration of radiation dataset with non-irradiated 1 month-old datasets generated a UMAP plot with same cell clusters as above. **(B)** EMPs and LMPs were drastically shrunk in the irradiated sample while MALPs were expanded. **(C)** Cell cycle analysis revealed that the percentage of proliferative cells in progenitors and MALPs are all increased in the irradiated dataset. **(D)** Violin plots showed that several prominent cell cycle-promoting genes are upregulated particularly in MALPs from the irradiated dataset. **(E)** GO term and KEGG pathway analysis shown enriched pathways in non-radiation or radiation dataset of MALPs. **(F)** Violin plots showed that myofibroblast marker genes and extracellular matrix protein genes were upregulated particularly in MALPs from the irradiated dataset. **(G)** Pseudotime analysis revealed that myofibroblast markers are expressed more in adipogenic route after radiation.

Radiation transiently expands MALP pool via stimulating its proliferation.



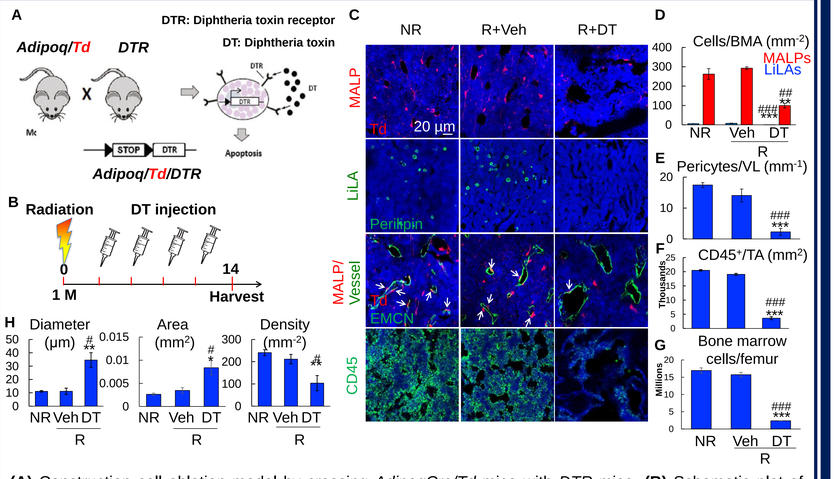
(A) AdipoqCre specifically labeled MALPs population. **(B)** Schematic plot of focal radiation on 1-month-old *AdipoqCre/Td* mice femurs. **(C)** Td+ cells in *AdipoqCre/Td* mice were remarkably increased in bone marrow at day 3 after radiation, followed by a graduate decrease at later time points. Meanwhile, LiLAs peaked around day 7 and then declined. **(D)** Quantification of C. **(E)** Schematic plot of EdU proliferation assay of MALPs after radiation. **(F)** EdU staining in bone marrow of *AdipoqCre/Td* mice. In normal bone marrow, Td+ stromal cells and pericytes, did not have any EdU incorporation. After radiation day 3, EdU signal was largely diminished in bone marrow, while a significant portion of Td+ stromal cells and pericytes were EdU+, indicating that radiation insult stimulates their proliferation, which likely contributes to the expansion of MALPs after radiation. **(G)** Quantification of EdU+ cells in Td+ stromal cells or pericytes. **(H)** Immunofluorescence images of Td+ cell clusters (do not form CFU) in normal condition and Td+ CFU at 3 days after radiation. **(I)** 5 Gy radiation drastically reduced CFU-F frequency in bone marrow. Strikingly, while bone marrow cells from non-irradiated mice only formed Td- CFU-F colonies, those from radiated mice at days 3 and 7 formed Td+ CFU-F colonies. They peaked at day 3 and declined afterwards.

MALPs lose cell processes and acquire myofibroblast properties after radiation



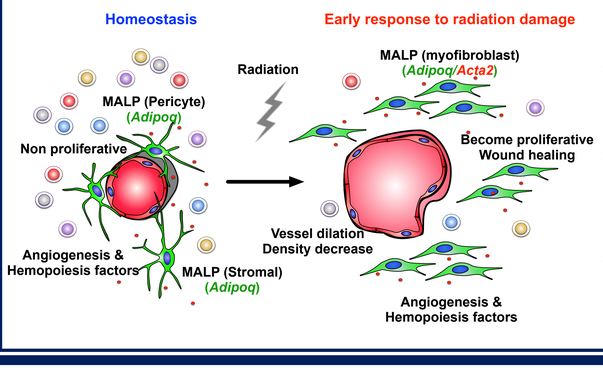
(A) MALPs possess a unique shape with a central cell body and multiple cell processes, thus forming a 3D network structure throughout the bone marrow in normal condition. Radiation greatly reduced cell process number and cell circularity of MALPs, resulting in a shape more closely to myofibroblasts. These changes gradually disappeared over the time. **(B, C)** Quantification of cell process number and cell circularity. **(D)** qRT-PCR of sorted bone marrow Td+ cells from Adipoq/Td mice confirmed that myofibroblast markers, as well as ECM proteins are increased at day 3 after radiation. **(E)** Immunofluorescence images of Coliv and αSMA staining. **(F)** After radiation, contrary to the increase of Td+ stromal cells, Td+ pericytes covering the vessels significantly decreased. Considering radiation could reduce the cell processes of MALP,which might cause the detachment of pericytes from vessels, thus explaining the paradox that radiation increases total MALPs but decrease the pericyte portion of MALPs. **(G)** Quantification of Td+ pericytes number along vessels.

MALPs mediate the recovery of marrow vasculature after radiation damage



(A) Construction cell ablation model by crossing *AdipoqCre/Td* mice with *DTR* mice. **(B)** Schematic plot of studying MALPs function in restoring bone marrow compartment after radiation. 1-month-old *AdipoqCre/Td/DTR* mice were subjected to veh or DT injection right after radiation and harvest at day 14 post radiation. **(C)** 14 days post radiation, in R+veh group, MALPs and perilipin+ LiLAs, vasculature and CD45+ haemopoietic cells in bone marrow already recovered to normal, while diphtheria toxin (DT) injections effectively eliminated Td+ cells, including MALPs and perilipin+ LiLAs in bone and prohibited vessels and CD45+ cells to recover. **(D)** Quantification of MALPs and LiLAs. **(E)** Quantification of pericytes number along the vessels. **(F)** Quantification of number of CD45+ cells per tissue area. TA: Tissue area. **(H)** Quantification of vessel diameter, area and density.

Conclusions



- *ScRNA-seq analysis predicts cell cycle entry and myofibroblast conversion of MALPs after radiation.*
- *Radiation transiently expands MALP pool via stimulating its proliferation.*
- *Radiation converts MALPs into myofibroblasts which involve in wound healing.*
- *MALPs are indispensable for bone marrow repair after radiation.*