Deciphering the in vivo Heterogeneity of Mouse Bone Marrow Mesenchymal Progenitors at Single Cell Resolution

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Introduction

In bone, it is commonly believed that mesenchymal progenitors (MPs), including their most primitive form mesenchymal stem cells (MSCs), constantly generate bone surface osteoblasts and marrow adipocytes via sequential, unidirectional, and branched differentiation steps. Almost all types of osteoporosis are associated with diminished bone formation and increased marrow adiposity. Stem cell heterogeneity has been well-recognized in mammalian tissue stem cells. However, owing to a lack of proper *in vivo* investigative tools, in the past we deliberately ignored this feature of mesenchymal progenitors by simply referring to them as MSCs or MPs, and searched for a single marker to label them. Recently, we discovered that in *Col2-Cre Rosa-tdTomato* (*Col2/Td*) mice, Td signal labels all endosteal (metaphyseal) mesenchymal lineage cells but no other cell types[1], thus providing a perfect system to comprehensively analyze the subpopulations of mesenchymal lineage cells from MSCs to mature cells. In this project, we applied the most advanced large scale single-cell sequencing (scRNA-seq) technique to this animal model and solved the in vivo heterogeneity of bone marrow MPs.

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 *Adiponectin-Cre Rosa-tdTomato* (*Adipoq/Td*) mice were obtained by crossing *Col2-Cre* or *Adipoq-Cre* mice with *Rosa-tdTomato* mice.

Endosteal bone marrow Td+ cell isolation

The outer surfaces of long bones were scraped and digested to remove the periosteum. After cutting off the epiphyses and flushing out the central bone marrow, metaphyseal bone fragments were longitudinally cut into two halves and digested by proteases to collect endosteal bone marrow cells [2]. Those cells were then sorted for the top 1% Td^+ cells.

Large scale scRNA-seq

20,000 sorted cells were loaded in the chromium controller (10X Genomics) to generate barcoded, single-cell libraries of 10,000 cells for sequencing via Ilumina HiSeq 2500. After stringent quality controls, 8,456 cells were analyzed. Unsupervised clustering was conducted by Seurat to generate a t-SNE plot of the overall cell populations. Trajectory analysis was conducted using Monocle.

Whole mount immunofluorescence

Freshly dissected bones were fixed in 4% PFA, decalcified in 10% EDTA, and immersed into 20% sucrose and 2% polyvinylpyrrolidone. After frozen embedding, bones were cryosectioned at 50 μm in thickness and stained with Lepr1, Endomucin, Perilipin, and CD45 antibodies for immunofluorescent imaging analysis.

Statistics

All analyses were conducted using t-tests or two-way ANOVA with a bonferroni's post-test for multiple comparisons using Prism (GraphPad).

Results

We harvested endosteal bone marrow from 1-month-old *Col2/Td* mice and sorted for the top 1% Td^+ cells (Figure 1A). Those cells contained all CFU-F forming cells found in unsorted parental cells (Figure 1B) and all their CFU-Fs were Td^+ (Figure 1C). Our harvest method also collects Td^+ osteoblasts [3]. Therefore, the top 1% Td^+ cells contain enriched and all-inclusive mesenchymal lineage cells ranging from MSCs to mature cells in bone. We subjected those cells to large scale scRNA-seq. After excluding hematopoietic and endothelial cells, we identified 2,489 mesenchymal lineage cells expressing ~2500 sequenced genes per cell. Clustering analysis divided those cells into 8 clusters with known markers in some of them: early MSCs (Sca1), late MSCs, chondrocytes (Col2a1), mesenchymal bi-potent progenitors (MBPs), osteoblasts (Osteocalcin), osteocytes (SOST), adipoprogenitor (AP)1, and AP2 (adiponectin) (Figure 2A). Interestingly, plots suggest that these cells exist in a continuum, rather than in discrete

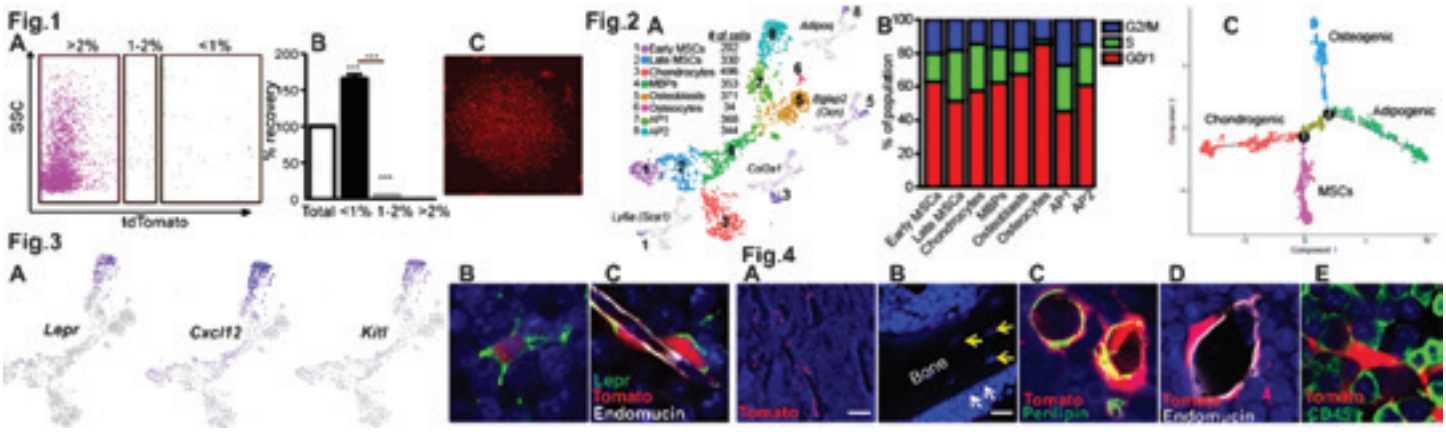

Figure 1. (A) FACS sorting of bone marrow cells from Col2/Td mice into 3 groups: top 1% (< 1%), 1-2%, and > 2% Td+ expressing cells; **(B)** CFU-F assay of unsorted and sorted groups. N = 3-4/group; **(C)** All CFU-F colonies from top 1% group are Td+. **Figure 2. (A)** Clustering of bone marrow mesenchymal lineage cells reveals the identity of MSCs and their multi-differentiation routes. The gene expression tSNE plots of some cluster markers are shown on the side. Cells with a high expression of indicated gene are labeled in blue; **(B)** Cell cycle state of each cluster; **(C)** Clustered cells were subjected to dimensional reduction by Monocle to generate a trajectory plot with 4 directions. **Figure 3. (A)** tSNE plots of CAR cell markers and LepR show their high expression (blue) in AP clusters, especially AP2; **(B)**, **(C)** Fluorescence images of Col2/Td tibia revealed that LepR+ cells are mostly Td+ cells in **(B)** bone marrow; **(C)** and at perivascular niche. **Figure 4**. Fluorescence images of Adipoq/Td tibia revealed abundant adipogenitors in bone marrow. **(A)** Metaphyseal bone marrow; **(B)** Trabecular bone. Yellow arrows point to osteocytes and white arrows point to bone surface. Scale bar: 100 μm; **(C)** Td+ marrow adipocytes; **(D)** Td+ pericytes; (E) Td+ bone marrow cells are CD45- stromal cells.

states. Cell cycle analysis based on the expression levels of > 90 cell cycle-related genes revealed that early MSCs are quiescent, osteocytes are the least proliferative cells and that AP1 and late MSCs are the most proliferative cells (Figure 2B). Monocle trajectory analysis generated three differentiation directions with the first branch separating chondrocytes from MBPs and the second branch bi-pronged into osteoblasts and APs (Figure 2C). Positioning individual cells along a linear pseudotimeline with MSC as the root revealed known and novel genes differentially expressed during MSC tri-differentiation processes (data not shown). Cells in AP clusters, particularly those in AP2, highly and specifically express LepR, Cxcl12 and Kitl (Figure 3A), resembling the previously identified CAR/LepR$^+$ MSC population [4]. Fluorescence imaging revealed that 40.2% of Td$^+$ cells in bone marrow are LepR$^+$ cells and almost all LepR$^+$ stromal (Figure 3B) and pericytes cells (Figure 3C) were Td$^+$. To validate AP clusters, we generated *Adipoq/Td* mice (Figure 4). At 1 month of age, these mice had abundant Td$^+$ cells in metaphyseal bone marrow (Figure 4A) but not on bone surfaces as osteoblasts or inside bone as osteocytes (Figure 4B). Td$^+$ cells included all adipocytes (Figure 4C), many pericytes (Figure 4D) and stromal cells (Figure 4E) and contributed to a large portion of CFU-Fs, demonstrating that AP clusters are fate-determined progenitors.

Discussion

Our work demonstrated the power of large scale scRNA-seq at identifying and characterizing subpopulations of mesenchymal lineage cells. To our knowledge, this is the first study that computationally delineates the entire in vivo differentiation process of MSCs and put true MSCs and their descendants in an ordered fashion. One unexpected finding is that we identified and validated abundant APs in 1-month-old mouse bone marrow when mature adipocytes are rarely detected in the metaphyseal area. Based on our sequencing data, APs are a major source of cytokines and chemokines. Together with their abundance and high proliferation ability, our data indicate that they play an essential role in regulating bone metabolism.

Significance

Our study established the *in vivo* heterogeneity of bone marrow MPs by identifying their novel subpopulations and delineating their relative positions along the MSC tri-differentiation axes.

References

1. **Chandra A, *et al***. *J Bone Miner Res*, 2017. 32:360-372.
2. **Zhu J, *et al.*** *Methods Mol Biol*, 2015:19-29.
3. **Siclari, VA, *et al.*** *Bone*, 2013. 53:575-86.
4. **Zhou, BO, *et al.*** *Cell Stem Cell*, 2014. 15:154-68.