Single Cell Transcriptome Analysis of Aging Effect on Bone Marrow Mesenchymal Progenitors

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Introduction

Bone marrow mesenchymal lineage cells are a heterogeneous cell population involved in bone homeostasis and diseases such as osteoporosis. While it is long postulated that they originate from mesenchymal stem cells (MSCs), the true identity of MSCs and their in vivo bifurcated differentiation routes into osteoblasts and adipocytes remain poorly understood. Previously we and others reported that Td labels the entire bone marrow mesenchymal lineage cells in *Col2-Cre Rosa-tdTomato* (*Col2/Td*) mice.[1] Here, we applied large scale single cell RNA-sequencing (scRNA-seq) on sorted bone marrow Td$^+$ cells from *Col2/Td* mice at various ages to identify the subpopulations of mesenchymal progenitors and to examine the aging effects on them.

Methods

Animals

All animal work performed was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. *Col2/Td* was generated by breeding *Rosa-tdTomato* mice with *Col2-Cre* mice. α*SMA-CreER Rosa-tdTomato* (α*SMAER/Td*) was generated as described previously[2]. Mice received Tamoxifen (Tam) injections (75 mg/kg) for 2 days at 1 mo of age and bones were harvested 3 mo later.

Sorting bone marrow Td$^+$ cell for scRNA-seq-

Endosteal bone marrow cells were isolated using an enzymatic digestion method as we described previously[3] and resuspended into FACS buffer for sorting Td$^+$ cells. A total of 4 batches of single cell libraries were constructed from 1-(2 batches, n = 5), 3-(1 batch, n = 3), and 16-mo-old (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

In all age groups, sorted Td$^+$ bone marrow cells from *Col2/Td* mice contained all the CFU-F forming cells as unsorted cells and almost all CFU-Fs were Td$^+$, suggesting that Td$^+$ cells include all bone marrow mesenchymal progenitors. Large scale scRNA-seq of those Td$^+$ cells yielded 5102, 1693, and 7066 bone marrow mesenchymal cells from 1-, 3-, and 16-mo-old mice, respectively. Seurat revealed a similar cell clustering pattern among 3 age groups (Fig. 1A). Examination of lineage-specific markers identified clusters with gene signatures of osteoblast, osteocyte, and adipocyte (Fig. 1B). In each group, pseudotemporal trajectory analysis always placed cluster 1 cells at one end while osteoblast/osteocytes and adipocytes, at the opposite ends (Fig. 1C), suggesting that cluster 1 cells are the ancestor of other mesenchymal cells and that they undergo bi-differentiation routes into osteoblasts and adipocytes. In 1 and 3 mo datasets, cluster 1-4 had gradually increased osteogenic gene expression. Thus, clusters 2 and 3 were named late MSC and mesenchymal bi-potent progenitor (MBP), respectively. Cluster 6 is lineage committed progenitor (LCP) due to its distribution around the branch point. Cell cycle analysis revealed that MSCs are quiescent among progenitors and adipocytes and osteocytes are non-proliferative (Fig. 1D). Lacking the late MSC cluster, 16 mo dataset had marked reduced

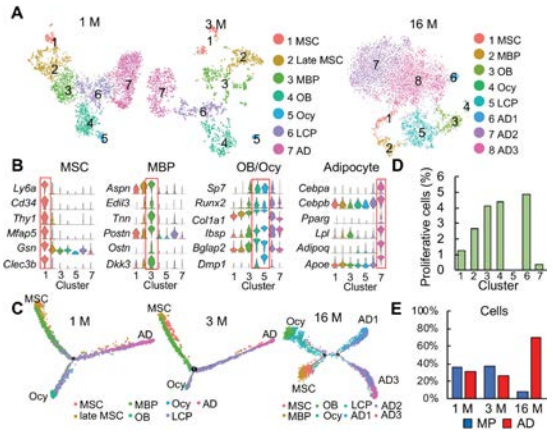

Figure 1. Single cell transcriptomics analysis of bone marrow mesenchymal lineage cells of 1-, 3-, and 16-mo old mice. **(A)** tSNE plots of 3 age datasets. **(B)** Violin plots of cluster-specific makers in 1 mo dataset. The patterns are same in the other 2 datasets. **(C)** Monocle trajectory of mesenchymal lineage cells. **(D)** The percentage of proliferative cells (S/G2/M phase) among each cluster are quantified. **(E)** The percentages of mesenchymal progenitors (MSCs, late MSCs, and MBPs) and adipocytes (ADs) are quantified

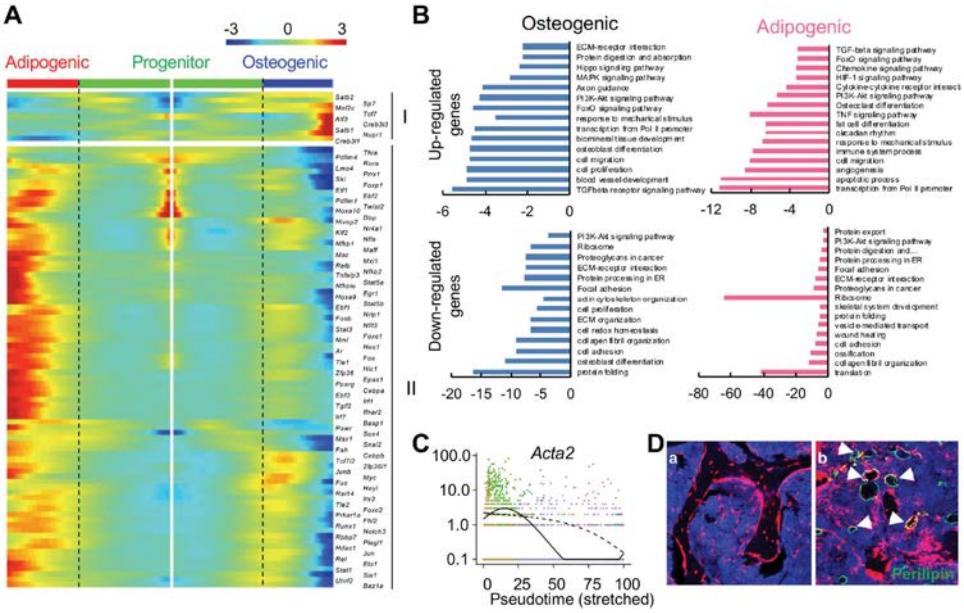

Figure 2. The bifurcated osteo- and adipo-lineage differentiation routes of in vivo bone marrow MSCs. **(A)** Pseudotemporal depiction of differentially expressed transcription factors. **(B)** GO term and KEGG pathway analyses of genes during osteogenic and adipogenic differentiation. **(C)** *Acta2* (α*SMA*) expression peaks at MBP. **(D)** In 4-mo-old α*SMAER/Td* mice, Td labels osteoblasts, osteocytes (a) and many adipocytes (b, arrows).

mesenchymal progenitors and increased adipocytes compared to young ones (Fig. 1E). Positioning individual cells along a linear pseudotimeline with MSCs as the root revealed transcription factors (TFs) differentially expressed after the branch point of osteogenic and adipogenic lineages (Fig. 2A). Consistent with its longer pseudotime, adipogenic differentiation required much more unique TFs than osteogenic differentiation, including *Pparg* and *Cebp*α. Analyzing differentiated expressed genes (DEGs) revealed that up-regulated genes are distinct for each lineage whereas there is considerable overlap between down-regulated genes. Pathway analyses of DEGs found unique and common features of osteogenic and adipogenic differentiation processes (Fig. 2B). Our seq data indicated α*SMA* as a MBP marker (Fig. 2C). This was confirmed in αSMAER/Td mice, in which Td labeled many osteoblasts, osteocytes, and bone marrow Perilipin$^+$ adipocytes (Fig. 2D). Combining all datasets generated similar clusters and pseudotime trajectory as individual ones (Fig. 3A). While MSCs and MBPs in 1- and 3-mo datasets were more centered at the starting point of pseudotime, those cells in 16 mo dataset shifted toward differentiated status, particularly the adipocyte end (Fig. 3A). Furthermore, adipocyte markers in 16-mo dataset were expressed at higher levels in mesenchymal subpopulations (MSC and MBP) than in young ones (Fig. 3B), suggesting an adipocytic drift of progenitors during aging.

Discussion

Our large scale scRNA-seq revealed the in vivo identity of MSC population, which expresses several common adult stem cell markers (Ly6a, CD34, and Thy1) but not most previously reported MSC markers, including LepR. Indeed, in our analysis, LepR is an adipocyte marker with an age-related increase in MSCs (Fig. 3C). The same pool of MSCs followed by the same hierarchy differentiation pattern is responsible for bone formation by mesenchymal lineage cells at adolescent, adult, and aging stages. During aging, MSCs are not only reduced in numbers but drifted toward more adipocyte status, which might further account for the loss of progenitor activity.

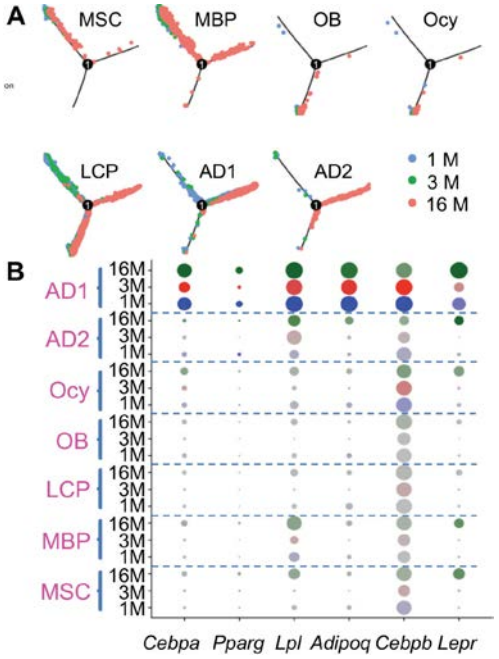

Figure 3. Aging causes an adipocytic drift of mesenchymal progenitors. **(A)** Monocle trajectory of mesenchymal lineage cells of integrated database. Cells are labeled based on age groups. **(B)** Dotplot of *Cebpa, Pparg, Lpl, Adipoq* and *Lepr* in Seurat clusters across different age groups. The circle size is proportional to the percentage of cells expressing the gene and the transparency of circle is reversely correlated with the gene expression level.

Significance

Understanding of the mechanism of in vivo bone marrow mesenchymal progenitor subpopulations and discovery of their adipocytic drift during aging will shed light in treating age-related osteoporosis.

References

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3. Zhu, J *et al.* *Methods Mol Biol.* 2015;1226, 19-29.