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## Marrow adipogenic lineage precursor: a new cellular component of marrow adipose tissue

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### Abstract

Bone marrow mesenchymal stromal cells are a highly heterogeneous cell population containing mesenchymal stem cells as well as other cell types. With the advance of single cell transcriptome analysis, several recent reports identified a prominent subpopulation of mesenchymal stromal cells that specifically express adipocyte markers but do not contain lipid droplets. We name this cell type marrow adipogenic lineage precursor, MALP, and consider it as a major cellular component of marrow adipose tissue. Here, we review the discovery of MALPs and summarize their unique features and regulatory roles in bone. We further discuss how these findings advance our understanding of bone remodeling, mesenchymal niche regulation of hematopoiesis, and marrow vasculature maintenance.

### Keywords

marrow adipogenic lineage precursor; single cell RNA-sequencing; mesenchymal stem cells; bone remodeling; hematopoiesis; angiogenesis

### Introduction

The primary functions of bone are to provide mechanical support, serve as a reservoir for calcium and phosphorus, and to produce blood cells. To achieve these functions, two lineages of bone-residing cells are essential: mesenchymal and hematopoietic. Mesenchymal cells originate from mesenchymal stem cells (MSCs). First identified by Friedenstein et al. in 1970s<sup>1</sup>, bone marrow MSCs have been extensively studied because of their critical roles in skeletal development, maintenance, and injury repair, as well as their

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clinical applications in regenerative medicine<sup>2</sup>. However, their true identity has remained ambiguous or even controversial. Inside the bone, MSCs give rise to marrow stromal cells, marrow adipocytes, and bone forming osteoblasts/osteocytes that synthesize mineralized bone matrix. Meanwhile, osteoclasts are derived from hematopoietic stem and progenitor cells (HSPCs) and are responsible for resorbing existing bone matrix. The dynamic balance between bone formation and resorption, namely bone remodeling, shapes bone structures and maintains mineral metabolism. Shifting this balance toward more bone resorption leads to osteoporosis, a highly prevalent skeletal disorder associated with bone fragility and fracture<sup>3</sup>.

Mesenchymal cells in the bone marrow, except for adipocytes, are referred to as marrow stromal cells, a heterogeneous population with unclear cellular composition. To be specific in this review, we reserve the term MSC only for the most primitive form of mesenchymal progenitor cells (MPCs) with self-renewal ability, but not for descendant non-renewing MPCs that have multipotentiality as well but are more abundant. Additionally, we reserve the term adipocyte for cells containing large cytoplasmic lipid droplets. Although prior research has generated an extensive list of markers for MSCs/MPCs, not a single marker or a set of markers has been consistently agreed upon and some of them are even mutually exclusive. This clearly illustrates the heterogeneity of marrow stromal cells and demands further investigation.

The recent emergence of single-cell RNA-sequencing (scRNA-seq) has provided an unprecedented opportunity to investigate cellular heterogeneity in tissues. A seminal study in 2009 reported the gene expression profile of a single mouse blastomere<sup>4</sup>. Initial platforms were able to analyze a few hundred cells per sample. Available from 2017, the 10x Genomics platform allows for the analysis of tens to hundreds of thousands of cells at an affordable price<sup>5</sup>. Briefly, this technology uses microfluidic partitioning to capture single cells and prepare barcoded, next-generation sequencing cDNA libraries. As a result, all cDNAs from a single cell have the same barcode, allowing the sequencing reads to be mapped back to their cell of origin. Subsequent computational analysis can group cells with similar gene expression profiles together without prior knowledge of any cell type markers. At a large scale, scRNA-seq has the power to interrogate rare cell types, elucidate transitional states, delineate relationships among subpopulations, and predict the course of differentiation or reprogramming<sup>6</sup>. By applying this powerful technique to bone, our group discovered a new adipogenic cell population in bone marrow that we named marrow adipogenic lineage precursor (MALP)<sup>7</sup>. In this review article, we present the most updated research on MALPs, compare this cell population with previously proposed bone marrow mesenchymal populations and adipoprogenitors in peripheral fat tissues, and explain how this new finding could change the perspectives on marrow adipose tissue (MAT), bone remodeling, and bone marrow environment regulation.

## Identification of MALPs as a new bone marrow mesenchymal cell population

We initially sought to determine the mesenchymal cell hierarchy in bone marrow through applying scRNA-seq. To do this, we isolated Tomato (Td)+ cells from enzymatically digested bone marrow of *Col2a1-Cre Td* mice at 1 (young), 3 (adult), and 16 (aging) months of age. Studies have shown that *Col2a1-Cre* labels all mesenchymal lineage cells in bone, including all colony forming unit-fibroblast (CFU-F) forming cells, osteoblasts, osteocytes, adipocytes, and all chondrocytes<sup>7-9</sup>. Computational analysis identified the following mesenchymal lineage subpopulations among all age group datasets (Fig. 1A): early mesenchymal progenitor (EMP), late mesenchymal progenitor (LMP), lineage committed progenitor (LCP), marrow adipogenic lineage precursor (MALP), osteoblast (OB), osteocyte (Ocy), and chondrocyte (CH, presumably from the growth plate). Pseudotemporal cell trajectory analyses positioned EMPs at one end of a Y shape trajectory, with osteoblasts/osteocytes and MALPs at the opposite, divergent ends. EMP expresses several common stem cell markers (*Ly6a/Sca1*, *Cd34*, and *Thy1*) and thus, resembles previously proposed PDGFR $\alpha$ /Sca1 (Pa.S) MSCs<sup>10</sup>. Sca1 is a common marker for mouse tissue-resident stem and progenitor cells, including hematopoietic stem cells (HSCs)<sup>11</sup>. Our datasets show that *Pdgfra* expression labels all bone marrow mesenchymal cells and thus the combination of *Pdgfra* and *Sca1* expression (Pa.S) identifies MSCs without HSCs. Prior studies show that PDGFR $\alpha$ +Sca1- cells also generate CFU-Fs, an indicator of *in vivo* MPCs, albeit at a relatively lower frequency than Pa.S cells<sup>10, 12</sup>. Due to their large number compared to Pa.S cells, PDGFR $\alpha$ +Sca1- cells contain most bone marrow CFU-F forming cells. Since Sca1 expression only labels EMPs, we reason that PDGFR $\alpha$ +Sca1- cells correspond to LMPs and LCPs, which are likely to be abundant, non-renewing progenitors. Genetic lineage tracing and functional studies are required to validate this proposed progenitor cell hierarchy.

Amongst all marrow stromal cells from young animals, MALPs are the only population displaying high enrichment of several *bona fide* adipocyte markers, including *Cebpa*, *Pparg*, *Adipoq*, and *Lpl*<sup>7</sup>. MALPs also specifically express *Zfp423*, a marker recently proposed to label a more mature form of pre-adipocyte (CD45-CD31-Sca1-Zfp423+) in the bone marrow<sup>13</sup>. On the other hand, MALPs do not express markers associated with lipid metabolism, such as *Plin1* (encoding lipid droplet coating protein) and *Fabp4* (encoding a fatty acid binding protein), that are necessary for mature adipocyte function. These data suggest that MALPs represent the immediate precursors of marrow adipocytes. Compared to young mice, aged mice had much fewer MPCs and many more MALPs<sup>7</sup>, consistent with the general concept that aging is accompanied by a reduced mesenchymal progenitor pool and increased marrow adiposity<sup>14</sup>.

In the past two years, five other groups have also analyzed the heterogeneity of mouse bone marrow mesenchymal lineage cells. The Scadden group sorted non-hematopoietic cells from the long bones of young mice for scRNAseq analysis. They identified several clusters, including leptin receptor-expressing MSCs (Lepr-MSCs), osteolineage cells, chondrocytes, fibroblasts (fibro-1-5), endothelial cells, and pericytes<sup>15</sup>. Interestingly, the annotated Lepr-MSCs cluster also expresses *Adipoq*, thus resembling our MALPs. Integrated analyses of

the dataset from the Scadden group with our 1 month dataset (Fig. 1B) reveals that MALP cluster overlap completely with the *Lepr*-MSC cluster, while the EMP cluster merges well with fibro-1,2 clusters (Fig. 1C-E). These results suggest that *Lepr*-MSCs correspond to adipocyte-lineage cells, i.e. MALPs, and may not represent MSCs.

The Aifantis group profiled gene expression in various bone marrow HSC niches, including cells labelled by *Lepr-Cre* (mesenchymal stromal niche), *Cdh5-Cre* (vascular niche), and *Col2.3-Cre* (osteoblast niche)<sup>16</sup>. *Lepr*<sup>+</sup> and *Col2.3*<sup>+</sup> cells contained 4 (P1-P4) and 3 (O1-O3) subpopulations, respectively. Strikingly, P1-3 merged very well with MALP, forming a single large cluster (Fig. 1F-H). In addition, O2, O3, and P4 merged with EMP, osteoblasts and LCP, respectively. Thus, similar to the Scadden dataset, the Aifantis dataset also contains a cell population similar to MALP. In addition, all three studies noted that this cell population is enriched with key HSC niche factors SDF1 and SCF, encoded by *Cxcl12* and *Kitl*, respectively.

The Welner group used scRNA-seq to analyze non-hematopoietic and non-endothelial cells in bone<sup>17</sup>. A total of 7 clusters were identified: pre-adipocyte (P1), adipocyte progenitor (P2), MSC (P3), osteoblast/chondrocyte progenitor (P4), pre-osteoblast/chondrocyte (P5), pro-osteoblast (P6), and pro-chondrocyte (P7). Most cells, P1-4, highly and specifically express *LepR*, *Adipoq*, and *Cxcl12*. The Hass group analyzed total bone marrow cells, followed by progressive depletion of abundant cell types or enrichment of rare populations<sup>18</sup>. They identified the following mesenchymal lineage clusters: chondrocyte, osteoblast, Ng2<sup>+</sup> MSC, fibroblast-like populations, adipo-lineage CAR (Adipo-CAR) cells, and osteo-lineage CAR (Osteo-CAR) cells. CAR cells refer to *Cxcl12*-abundant reticular cells that marks bone marrow MPCs<sup>19</sup>. The authors noted that among all bone marrow cells, Adipo-CAR and Osteo-CAR cells produce the highest number and levels of cytokines and growth factors, thus naming these cells “professional cytokine producing cells”. Interestingly, the two CAR cell clusters are also enriched for *Adipoq* expression. Lastly, the Ono group sequenced GFP<sup>high</sup> cells isolated from the bone marrow of *Cxcl12*-CreER Td *Cxcl12*-GFP mice at P28 after Tamoxifen induction at P21<sup>20</sup>. Three mesenchymal lineage cell clusters were identified and only one of them (cluster 0) is Td<sup>+</sup>, which is quiescent and enriched for *Adipoq*, *Cxcl12*, and *Kitl*. Most recently, two more scRNA-seq studies, one on Gli1-CreER labeled mouse bone marrow cells (by Dr. Fanxin Long’s group), and the other on human CD271<sup>+</sup> bone marrow-derived mononuclear cells (by Dr. Hongwen Deng’s group), identified a large *Adipoq*<sup>+</sup> cell cluster in their datasets (personal communication). Taken together, despite different cell labelling and sorting strategies, all scRNA-seq studies published to date identified one or more mesenchymal cell clusters with a gene signature similar to MALPs,.

## Characteristics of MALPs

Recent studies demonstrate that the bone marrow stroma of young mice does contain abundant *Adipoq*<sup>+</sup> cells while having few adipocytes<sup>7, 21, 22</sup>. MALPs first appear in marrow perinatally between P0 and P1<sup>21</sup>, 2-3 weeks before the emergence of adipocytes. Lineage tracing revealed that MALPs give rise to adipocytes, but not osteoblasts and osteocytes, suggesting that MALPs are fate-determined cells<sup>7, 23</sup>. In young mice, MALPs

(Adipoq+Plin1-) far exceed adipocytes (Adipoq+Plin1+) in number by 400-1000 folds, depending on location (metaphyseal vs diaphyseal marrow). They constitute 0.2-0.6% (unpublished data, <sup>21</sup>) of total bone marrow cells, and thus are a major component of mesenchymal stromal cells. In addition to long bones, MALPs can also be found in calvariae and vertebrae, suggesting a universal distribution in the bone marrow.

Computational cell cycle analysis predicts that MALPs are non-cycling cells <sup>7</sup>. Consistently, the Aifantis group predicted that P1 cells, analogous to MALPs, have the lowest proliferative status among all cell clusters <sup>16</sup>. In our study, *Adipoq-Cre* labeled cells did not incorporate EdU in vivo nor form CFU-F colonies <sup>7</sup>. By contrast, Zhou et al. show that a subset of *Adipoq-CreER* labeled cells incorporate BrdU can form CFU-Fs and expand over time after discontinuation of Tam injections <sup>22</sup>, suggesting a proliferative capacity. A possible explanation for these apparently discrepancy could be due to tamoxifen treatment, which promotes adipocyte turnover in certain fat depots <sup>24</sup>. Tamoxifen may thus induce some level of proliferation and turnover in MPCs and/or MALPs. Using alternative lineage tracing method, such as tetracycline-on transcription system, might resolve this question.

Distinguished from adipocytes, MALPs do not possess lipid content. They exist as pericytes and stromal cells with a central cell body and multiple long dendritic processes. This cell shape is reminiscent of an osteocytic canaliculi network <sup>25</sup>. Through their cell processes, MALPs form an extensive 3D network throughout the marrow cavity, making numerous connections among themselves and with other marrow cells, blood vessels, and bone surface. Taken together, MALPs represent committed adipocyte precursor cells in bone marrow. They are evolutionarily beyond multi-potent progenitor stages and positioned as adipogenic precursors in the mesenchymal cell hierarchy.

## The relationship between MALPs and LepR+/CAR cells

In addition to adipocyte markers, MALPs highly express some previously defined bone marrow MPC markers, including *Lepr* and *Cxcl12*. While this confirms prior observation that LepR+ cells overlap well with CAR cells <sup>12</sup>, it also raises two important questions: are LepR+/CAR cells a heterogenic population? And to what extent do MALPs overlap with LepR+/CAR cells?

First reported by the Nagasawa group using *Cxcl12-GFP* mice, CAR cells were found to be scattered throughout the bone marrow with long processes that create a network <sup>26, 27</sup>, a similar morphology we described for MALPs. Subsequent studies indicate that CAR cells are progenitors for osteoblasts and adipocytes, making *Cxcl12* a MPC marker <sup>19</sup>. In *Cxcl12-CreER* Td reporter mice, Td labels a majority of LepR+/CAR cells in the diaphyseal marrow and only a small portion of LepR+/CAR cells in the metaphyseal marrow <sup>20</sup>. Lineage tracing found that Td gradually labels trabecular osteoblasts from 0% at 9 weeks to a plateau of ~35% at 6 months of age (induction at 8 weeks of age). The adipocyte labeling pattern was not examined in this study.

*Lepr* was identified by the Morrison group as another MPC marker <sup>12</sup>. In *Lepr-Cre Td* mice, Td labels adipocytes quickly (~70% and 90% at 2 and 6 months of age, respectively)

and osteoblasts slowly (~5%, 20%, 40%, and 75% at 2, 6, 10, and 14 months of age, respectively). Accordingly, it was concluded that LepR<sup>+</sup> cells are the major source of bone and adipocytes in the adult bone marrow.

Bone remodeling peaks in young mice and declines in adult and aging mice. The rapid bone formation in adolescent mice requires a constant supply of osteoblasts from MPCs. The slow labeling pattern of osteoblasts by LepR<sup>+</sup>/CAR cells indicates that they do not comprise all MPCs in young mice. Meanwhile, the quick labeling pattern of adipocytes suggests that many of those cells are primed to become adipocytes. Indeed, both CAR cells and LepR<sup>+</sup> cells are noticed to express a high level of adipocyte markers, such as *Adipoq*, *Pparg*, *Cebpb* etc, compared to the rest of bone marrow, or more specifically, PaS cells<sup>12, 19</sup>. In culture, *Lepr* and *Cxcl12* expression is greatly elevated during the adipogenic differentiation of bone marrow MPCs, qualifying them as adipocyte markers<sup>7</sup>.

In our datasets, *Cxcl12* and *Lepr* are also expressed in progenitor clusters, albeit at a much lower level than in MALPs. Thus, we believe that a major portion of LepR<sup>+</sup>/CAR cells are MALPs with adipo-lineage fate and a small portion of LepR<sup>+</sup>/CAR cells are MPCs with both adipo- and osteo-lineage fates. Most studies on LepR<sup>+</sup> cells are based on a constitutive *Cre* model. Future studies characterizing inducible mouse lines (*Lepr-CreER* and *Cxcl12-CreER*) will provide more detailed insight on the relationship between MALPs and LepR<sup>+</sup>/CAR cells in mesenchymal cell hierarchy.

## MALPs as adipose lineage cells are unique to bone

Research on adipose tissue traditionally focuses on white adipose tissue (WAT) that stores excess energy as triglycerides, and brown adipose tissue (BAT) that regulates thermogenesis by producing energy in the form of heat. Marrow adipose tissue (MAT) has attracted relatively little attention until it recently emerged as a functionally distinct type of adipose tissue. Based on lipid content, the amount of MAT is significant in human, accounting for ~13% of total adipose tissue in lean and healthy individuals<sup>28</sup>. Adipocytes in MAT are morphologically similar to adipocytes in WAT with a characteristic unilocular lipid droplet. MAT does not express uncoupling protein-1 (UCP1), a key brown fat-specific component, either during development or after adrenergic stimulation, indicating that MAT is transcriptionally different from BAT<sup>29</sup>. The resemblance between MAT and WAT raises an intriguing question as to whether these two types of adipose cells share the same differentiation route from MSCs to adipocytes.

Adipocytes in WAT and BAT are derived from adipocyte progenitor cells (APCs) residing in the stromal vascular fraction (SVF). Those APCs are defined by their expression of mesenchymal markers, such as *Pdgfra*, *Cd34*, and *Sca1*, and the lack of expression of hematopoietic lineage markers<sup>30</sup>. To date, several scRNA-seq studies profiled gene expression in SVF cells from mouse and human peripheral adipose tissues at a single cell resolution<sup>31-36</sup>. While most adipocytes were discarded at the centrifugation step when harvesting SVF, a few of them were still detected in some studies<sup>34, 36</sup>. In peripheral fat depots, only adipocytes (*Plin1*<sup>+</sup>), but not APCs, express *Adipoq*. Interestingly, APCs in the newly developing subcutaneous WAT of fetal mice express *Plin1* and *Adipoq*<sup>37</sup>, likely

representing transitional cells undergoing adipocyte differentiation. However, in peripheral fat depots of adult mice, *Adipoq* is exclusively expressed in mature Plin1+ adipocytes<sup>38</sup>. MALPs thus appear to represent a distinctive adult adipogenic cell type or state that express a subset of mature adipocyte genes and are poised to become mature adipocytes. It remains unclear if mature adipocytes in marrow can lose their lipids and revert or de-differentiate into MALPs. In this regard, it is interesting to note that mature adipocytes in certain fat depots and in the skin have the capacity to de-differentiate into fibroblast cells<sup>39-41</sup>.

Analyzing mouse subcutaneous inguinal WAT at P12 identified 3 APC subpopulations (Fig. 2A)<sup>34</sup>. Among these, dipeptidyl peptidase 4 (DPP4)+ cells are multipotent early progenitors, capable of giving rise to Icam1+ committed preadipocytes and Cd142+ cells. While DPP4+ cells reside in the reticular interstitium, Icam1+ cells are intercalated among adipocytes and poised to undergo adipocyte differentiation with minimal stimulation. Interestingly, Cd142+ cells resemble adipogenesis-regulatory cells, or Aregs, an APC subpopulation discovered by the Deplancke group<sup>33</sup>.

To compare MALPs to APC subpopulations, we integrated this WAT dataset with bone marrow dataset (Fig. 2B). Interestingly, the EMP cluster from bone marrow merged well with the DPP4+ cell cluster from WAT; LMP, LCP, and osteoblast clusters formed a new cluster with Icam1+ preadipocytes but these cells did not overlap very well; MALPs remained as a separate cluster (Fig. 2C, D). Several implications can be drawn from this clustering pattern. First, bone marrow MSCs and WAT MSCs share a high degree of similarity. Second, they adopt different differentiation strategies to produce adipocytes. Compared to WAT MSCs, bone marrow MSCs go through more complicated differentiation steps to become adipocytes, probably reflecting their additional role of generating osteoblasts/osteocytes. Third, MALPs have no counterpart in WAT, and thus, unique to bone. Historically, MAT refers to adipocytes only. The discovery of MALPs greatly expands the content of MAT to include both MALPs and adipocytes.

## A master regulator of bone marrow environment

According to the scRNA-seq datasets, the most prominent feature of MALPs is that they express the greatest number of secreted factors at the highest levels among bone mesenchymal lineage cells. These factors are known for their actions in osteogenesis, osteoclastogenesis, hematopoiesis, and angiogenesis (Fig. 3), implying a master regulatory role of MALPs on nearby bone marrow cells.

### Bone formation

The most conspicuous phenotype observed after MALP ablation in young mice is rapid and massive bone formation throughout the marrow cavity, especially in the diaphyseal marrow that is normally devoid of any trabeculae<sup>7, 42, 43</sup>. In these studies, MALPs were ablated through toxin gene expression driven by *Adipoq-Cre*, which also targets peripheral fat depots. However, subcutaneous fat transplantation did not reverse the bone phenotype<sup>7, 42</sup>, suggesting that MALPs exert local effects to suppress osteogenesis. Mechanistic studies revealed that the expression of two bone morphogenetic protein (BMP) antagonists, *Grem1* and *Chrdl1*<sup>44</sup>, are diminished in the bone marrow after MALP ablation<sup>42</sup>. Consistent

with this finding, *Grem1* and *Chrdl1* are highly and specifically expressed in MALPs (Fig. 3). Additionally, MALPs produce a large amount of IGFbps, which antagonize the IGF signaling pathway. BMPs and IGFs are two of the most robust osteogenic signals for MPCs<sup>45, 46</sup>. One more line of evidence supporting the osteogenic inhibitory effect of MALPs comes from a prior study of mice with *Pparg* deletion using *Adipoq-Cre*. Since *Pparg* is a master transcriptional regulator of MSC adipogenesis<sup>47</sup>, these mice have no bone marrow adipocytes but they have a remarkable increase of trabecular bone mass<sup>48</sup>.

Cultured mouse MPCs are normally obtained from diaphyseal bone marrow. However, the paradox is that those cells do not become osteoblasts nor form bone *in vivo*. We believe that the ubiquitous distribution of MALPs in the bone marrow blocks nearby MPCs from entering the osteogenic differentiation route and thus prevents unwanted bone formation. Future studies on MALP-specific deletion of osteogenesis inhibitors are required to validate this hypothesis.

### Bone resorption

Osteoclast formation relies on two cytokines: macrophage colony-stimulating factor (M-CSF, encoded by *Csf1*) and receptor activator of NF- $\kappa$ B ligand (RANKL, encoded by *Tnfrsf11*)<sup>49</sup>. Previous *in vitro* studies found that adipocytes derived from bone marrow MPCs support osteoclast formation in culture<sup>50, 51</sup> and that adipogenic differentiation is accompanied by an increase of RANKL<sup>52</sup>. Moreover, MPCs from aged mice support osteoclast formation better in the coculture experiment than those from young mice. Strikingly, our scRNA-seq datasets showed that MALPs highly and specifically express not only *Rankl* and *Csf1*, but also other osteoclast regulatory factors, such as *Il7*, *Il34*, *Ccl2*, *Vcam1*, and *C3*<sup>23</sup>. Moreover, MALPs have much more interactions with osteoclast precursors, monocytes/macrophages, than other mesenchymal subpopulations<sup>23</sup>. RANKL deletion in MALPs using *Adipoq-Cre* causes a remarkable gain of trabecular bone mass at a level comparable to or even higher than RANKL depletion in osteocytes<sup>23</sup>, a cell type previously demonstrated to be the main stimulator of osteoclastogenesis<sup>53-55</sup>. MALP-derived RANKL also plays a critical role in pathological bone loss, such as lipopolysaccharide (LPS)- and ovariectomy-induced bone resorption<sup>23</sup>.

Bone remodeling initiates with bone resorption via osteoclasts followed by bone formation via osteoblasts. Many past studies point to osteocytes, particularly apoptotic osteocytes induced by different stimuli, as the initiator of osteoblast activity<sup>56</sup>. Considering their characteristic cell shape and marrow location, it is conceivable that MALPs function as a sensor of changed marrow environment and subsequently orchestrate bone remodeling at the specific trabecular bone site. Whether MALPs detect the environmental signal via their cell processes deserves further investigation.

### Hematopoiesis

Bone is the primary site for hematopoiesis in adult mammals. Indeed, hematopoietic cells comprise of >98% total bone marrow cells. While at much lower numbers, mesenchymal and endothelial cells provide niches to support HSPC renewal, quiescence, and multi-lineage differentiation<sup>57</sup>. It is well-recognized that *LepR+ / CAR* cells are the major producer of



SDF1/Cxcl12 and SCF/Kitl in the bone marrow<sup>19</sup>. As chemokines, Cxcl12 is required for the retention and maintenance of HSPCs<sup>58</sup> and Kitl is critical in the survival, migration, and differentiation of HSPCs<sup>59</sup>. LepR+/CAR cells control many aspects of hematopoiesis, particularly the maintenance of HSPCs under steady state and in regenerative conditions<sup>19, 60-64</sup>. As discussed above, LepR+/CAR cells are a heterogeneous cell population containing MPCs and MALPs. It would be interesting to tease out the role of each subpopulation in hematopoiesis.

scRNA-seq datasets indicate that MALPs produce hematopoietic regulatory factors, such as Cxcl12, Kitl, Il7, Angiopoietin 1 (encoded by *Angpt1*), Adiponectin (encoded by *Adipoq*) at the highest levels among all mesenchymal subpopulations. Deletion of *Kitl* using Adipoq-Cre significantly decreases the frequencies and numbers of HSPCs, including Lineage-Sca1+c-Kit+ (LSK) cells, long-term HSCs, common myeloid progenitors (CMPs), megakaryocyte-erythrocyte progenitors (MEPs), and granulocyte-monocyte progenitors (GMPs) in long bones of adult mice<sup>65</sup>. These mice also have altered peripheral blood components and develop macrocytic anemia at the steady state. Deletion of *Kitl* using *Adipoq-CreER* only affects HSPCs in caudal vertebrates but not in long bones<sup>22</sup>, which might reflect the lower recombination efficiency of *Adipoq-CreER* compared to *Adipoq-Cre*. However, these mice have severely impaired bone marrow hematopoietic regeneration after irradiation or 5-FU treatment, suggesting a role of MALP-derived *Kitl* in injury repair.

Il-7 is a cytokine important for B cell development<sup>66</sup>. Interestingly, *Adipoq-Cre Il7*CKO mice have reduced pro B, pre B, immature B and mature B cells in bone marrow with more severe effects in precursors than mature cells<sup>21</sup>, suggesting that B cell development relies on MALP-derived Il7 production. Angpt1 and its receptor Tie2 play dual actions in the maintenance of HSPCs and vasculature<sup>67, 68</sup>. Deletion of Angpt1 from LepR+ cells in mouse bone marrow accelerates hematopoietic and vascular recovery after irradiation while increasing vascular leakiness<sup>69</sup>, implicating that MALPs could regulate hematopoiesis and angiogenesis through secreting Angpt1. As a major product of MALPs, Adiponectin promotes HSPCs proliferation and retaining them in a functionally immature state<sup>70</sup>. Future research on MALP-specific deletion of this adipokine could strengthen the important HSC niche role of MALP.

## Angiogenesis

Bone is a highly vascularized tissue receiving 10-15% of total cardiac output<sup>71</sup>. In addition to serving as a transport conduit system, blood vessels play multifaceted roles in bone development, homeostasis, and regeneration via a spatiotemporally angiogenic-osteogenic coupling mechanism<sup>72</sup>. The extensive network of blood vessels in bone marrow include arteries, veins, and capillaries but the vast majority are Emcn<sup>+</sup> sinusoidal capillaries<sup>73</sup>. Prior work described LepR+/CAR cells as perivascular stromal cells<sup>27, 61</sup>. Using whole-mount and 3D confocal imaging technique, we found that MALPs exist as both stromal cells and pericytes<sup>7</sup>. Anatomically, pericytes are defined as cells encapsulated under the basal membrane of microvessels where they form a single layer around and in close contact with endothelial cells<sup>74</sup>. In a 3D view, MALP stromal cells and pericytes are morphologically similar, possessing a stellate shape with many long dendritic processes extending into the

marrow, contacting each other, and surrounding endothelial walls<sup>7</sup>. PDGFR $\beta$  is a general pericyte marker<sup>75</sup> and basement membrane are made of Laminin and type IV collagen<sup>76</sup>. Genes encoding these proteins are highly expressed in MALPs<sup>7</sup>. Pericytes regulate the formation, permeability, and stabilization of blood microvessels<sup>75</sup>. We found that MALP ablation severely damages marrow vasculature, leading to vessel swelling and a reduction in vessel number<sup>7</sup>.

Angiogenesis is mainly controlled by two families of growth factors, VEGF<sup>77</sup> and Angiopoietin<sup>78</sup>. Strikingly, MALPs express *Vegfa*, *Vegfc*, *Angpt1*, and *Angpt4* at the highest level within all mesenchymal subpopulations. While VEGF is generally considered as a master lymphangiogenic factor, a recent report found that it is highly expressed in bone marrow LepR+ cells<sup>79</sup>. Moreover, LepR+ cells-derived VEGF contributes to marrow vascular development and vascular and HSPC regeneration after irradiation<sup>79</sup>. Interestingly, MALPs also highly express *Esm1*, encoding Endothelial cell-specific molecule 1 that modulates VEGF signaling in endothelial cells<sup>80</sup>. Future studies are needed to delete these factors specifically in MALPs and understand their roles in the development and maintenance of marrow vasculature.

## Unanswered questions and perspectives

The discovery of MALPs and their profound actions on neighboring cells (Fig. 4) provides new perspectives on some fundamental concepts in the bone biology and has broad impact on stem cell, adipose, and vascular biology. It also invokes many interesting questions that demand future research for clarification.

First, what is the identity of bone marrow MSCs? Theoretically, a single MSC should have the ability to form the entire mesenchymal tissue in bone, just like a single HSC being able to reconstitute the entire hematopoietic system. While scRNA-seq analysis nominate PaS cells as the most likely MSC population in bone, lineage tracing experiments using cluster specific inducible Cre models are required to demonstrate that they descend into non-renewing MPCs, adipocytes, and osteoblasts/osteocytes.

Second, why are MALPs unique to bone? This might reflect distinct tissue organization and functions of different adipose tissues. Peripheral fat pads are primarily made of adipocytes, which are supplied by APCs. On the contrary, MAT is only a small part of bone. Mesenchymal cells, including MALPs, are interspersed among hematopoietic and endothelial cells. Thus, for whatever reasons, MALPs are evolutionally selected to take on additional regulatory roles. For that, its adipogenic differentiation must be de-coupled from lipid accumulation. Comparing the transcriptome profiles of MALPs and marrow adipocyte will provide interesting clues to how adipocyte formation is paused at MALP stage.

Third, what is the function of marrow adipocytes? Are they mere a side product of MALPs or do they have unique functions? Currently available Cre drivers target both MALPs and marrow adipocytes. Past studies on marrow adipocytes cannot exclude the involvement of MALPs and thus should be re-evaluated. Since MALPs outnumber marrow adipocytes, it

is imperative to construct a new adipocyte-specific *Cre* line for studying the function of marrow adipocytes.

Lastly, do MALPs contribute to injury repair? To date most MALP studies are limited to bone development and homeostasis. LepR+/CAR cells play critical roles in marrow regeneration after radiotherapy and chemotherapy. Our current knowledge of MALPs raises a question whether the regenerative ability of LepR+/CAR cells comes from its MALP portion or MPC portion. Moreover, although MALPs do not exist at the periosteum, it will be still interesting to study whether they participate into fracture repair.

No doubt, studying MALPs open new venues of developing therapies for osteoporosis and other bone-related disorders. Discovering new cellular mechanisms to control bone turnover and regeneration will enable fine-tuning of existing therapies or design of novel therapeutics. With the advance of gene-editing technology and novel cell-specific delivery approaches<sup>81</sup>, in the future it would be possible to spatiotemporally regulate MALP behavior as a therapy for skeletal and blood diseases. The future of MALP research looks both promising and exciting.

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## Abbreviation:

<b>Adipo-CAR:</b>	adipo-lineage CXCL12-abundant reticular
<b>Adipoq:</b>	Adiponectin
<b>Angpt1:</b>	Angiotensin 1
<b>APC:</b>	adipocyte progenitor cell
<b>BAT:</b>	brown adipose tissue
<b>BMP:</b>	bone morphogenetic protein
<b>CFU-F:</b>	colony forming unit-fibroblast
<b>CH:</b>	chondrocyte
<b>CMP:</b>	common myeloid progenitor
<b>Dpp4:</b>	dipeptidyl peptidase 4
<b>EMP:</b>	early mesenchymal progenitor
<b>GMP:</b>	granulocyte-monocyte progenitor
<b>HSC:</b>	hematopoietic stem cell
<b>HSPC:</b>	hematopoietic stem and progenitor cell

<b>LCP:</b>	lineage committed progenitor
<b>LMP:</b>	late mesenchymal progenitor
<b>LPS:</b>	lipopolysaccharide
<b>LSK:</b>	Lineage-Sca1+c-Kit+
<b>MALP:</b>	marrow adipogenic lineage precursor
<b>MAT:</b>	marrow adipose tissue
<b>M-CSF:</b>	macrophage colony-stimulating factor
<b>MEP:</b>	megakaryocyte-erythrocyte progenitor
<b>MPC:</b>	mesenchymal progenitor cell
<b>MSC:</b>	mesenchymal stem cell
<b>OB:</b>	osteoblast
<b>Ocy:</b>	osteocyte
<b>Osteo-CAR:</b>	osteo-lineage CXCL12-abundant reticular
<b>PaS:</b>	PDGFR $\alpha$ /Sca1
<b>RANKL:</b>	receptor activator of NF- $\kappa$ B ligand
<b>scRNA-seq:</b>	single-cell RNA-sequencing
<b>SVF:</b>	stromal vascular fraction
<b>Td:</b>	Tomato
<b>UCP1:</b>	uncoupling protein 1
<b>WAT:</b>	white adipose tissue

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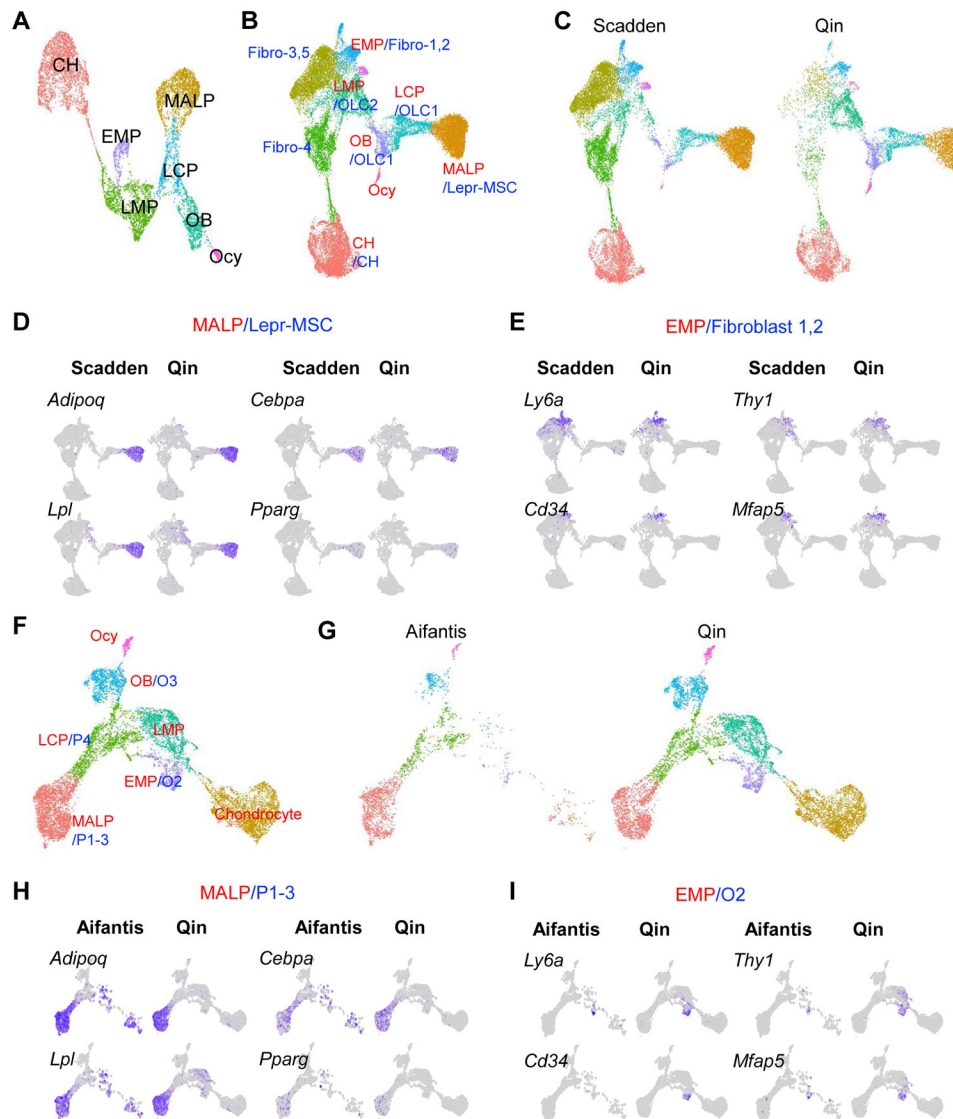


### Practice Points

- Bone marrow adipose tissue consists of MALPs and mature adipocytes.
- MALPs represent a unique adipocyte-lineage cell or state in bone that are not found in peripheral fat depots.
- MALPs are a major constituent of the marrow stroma and play critical roles in regulating bone turnover, hematopoiesis and angiogenesis by elaborating cytokines and growth factors that target adjacent cells.
- MALP represents a new cellular target for treating bone disorders, such as osteoporosis.

### Research Agenda

- Genetic lineage tracing experiments are needed to ascertain the existence and functional properties of multipotent MSCs in bone marrow.
- Transcriptome analysis of MALPs and marrow adipocytes will provide insights into the pathways that regulate the persistence of MALPs in bone.
- Generating a mature adipocyte-specific *Cre* will tease out the distinctive functions of MALPs and marrow adipocytes.
- Understanding the role of MALPs in bone injury is imperative for targeting these cells for novel therapies.



**Figure 1. scRNA-seq datasets of bone marrow mesenchymal lineage cells contain a large MALP subpopulation.**

A. UMAP plot of bone marrow mesenchymal lineage cells from Qin group's 1 month dataset.

B. UMAP plot of integrated dataset containing cells from Qin (GSE145477) and Scadden (GSE128423) scRNA-seq datasets. Newly clustered subpopulations are named by their corresponding cluster names from Qin dataset (red) followed by corresponding cluster names from Scadden dataset (blue). Fibroblast clusters 1,2 (fibro-1,2) from Scadden dataset were previously annotated as MSC-like cells. Fibroblast clusters 3-5 (fibro-3,4,5) from Scadden dataset were previously annotated as tenocyte lineage cells, which are mostly absent in Qin dataset. OLC: osteolineage cell.

C. Split UMAP view of integrated dataset by the original datasets.

D. UMAP plots of MALP/Lepr-MSc cluster markers.

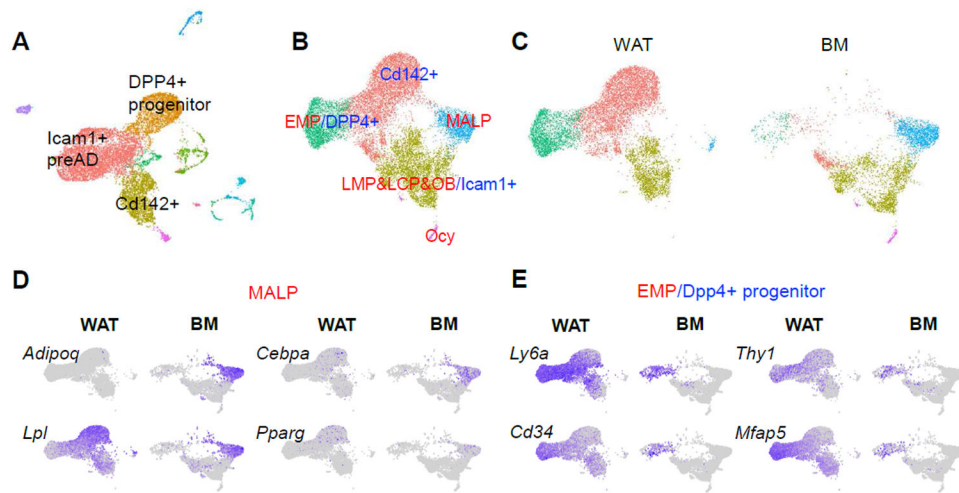
E. UMAP plots of EMP/Fibroblast 1 cluster markers.

F. UMAP plot of integrated dataset containing cells from Qin (GSE145477) and Aifantis (GSE108892) scRNA-seq datasets. Newly clustered subpopulations are named by their corresponding cluster names from Qin dataset (red) followed by corresponding cluster names from Aifantis dataset (blue).

G. Split UMAP view of integrated dataset by the original datasets.

H. UMAP plots of MALP/P1-3 cluster markers.

I. UMAP plots of EMP/O2 cluster markers.



**Figure 2. WAT scRNA-seq dataset does not contain a MALP subpopulation.**

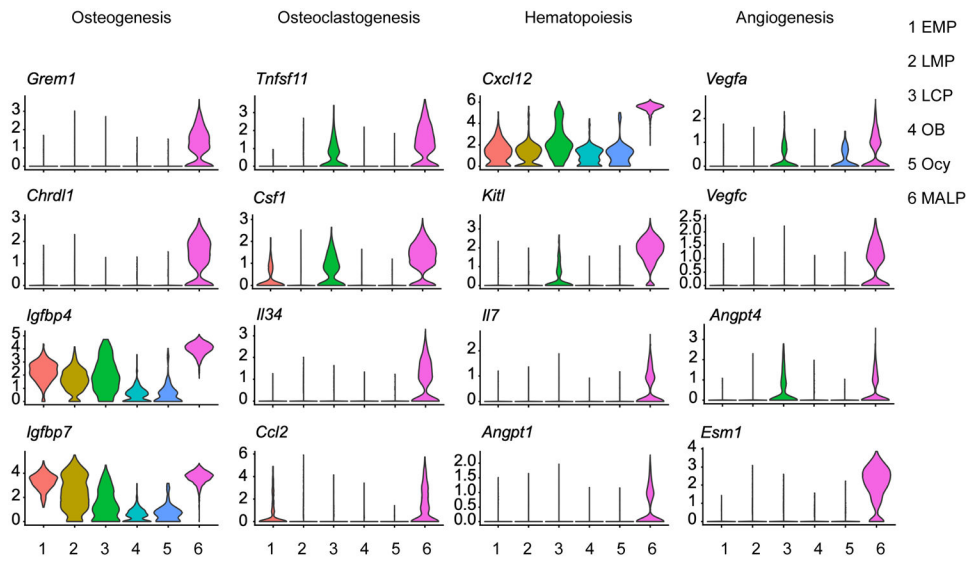
A. UMAP plot of WAT from the Seale group. PreAD: preadipocytes.

B. UMAP plot of integrated dataset containing cells from WAT (GSE128889) and bone marrow mesenchymal cells (GSE145477). Newly clustered subpopulations are named by their corresponding cluster names from bone marrow dataset (red) followed by corresponding cluster names from WAT dataset (blue).

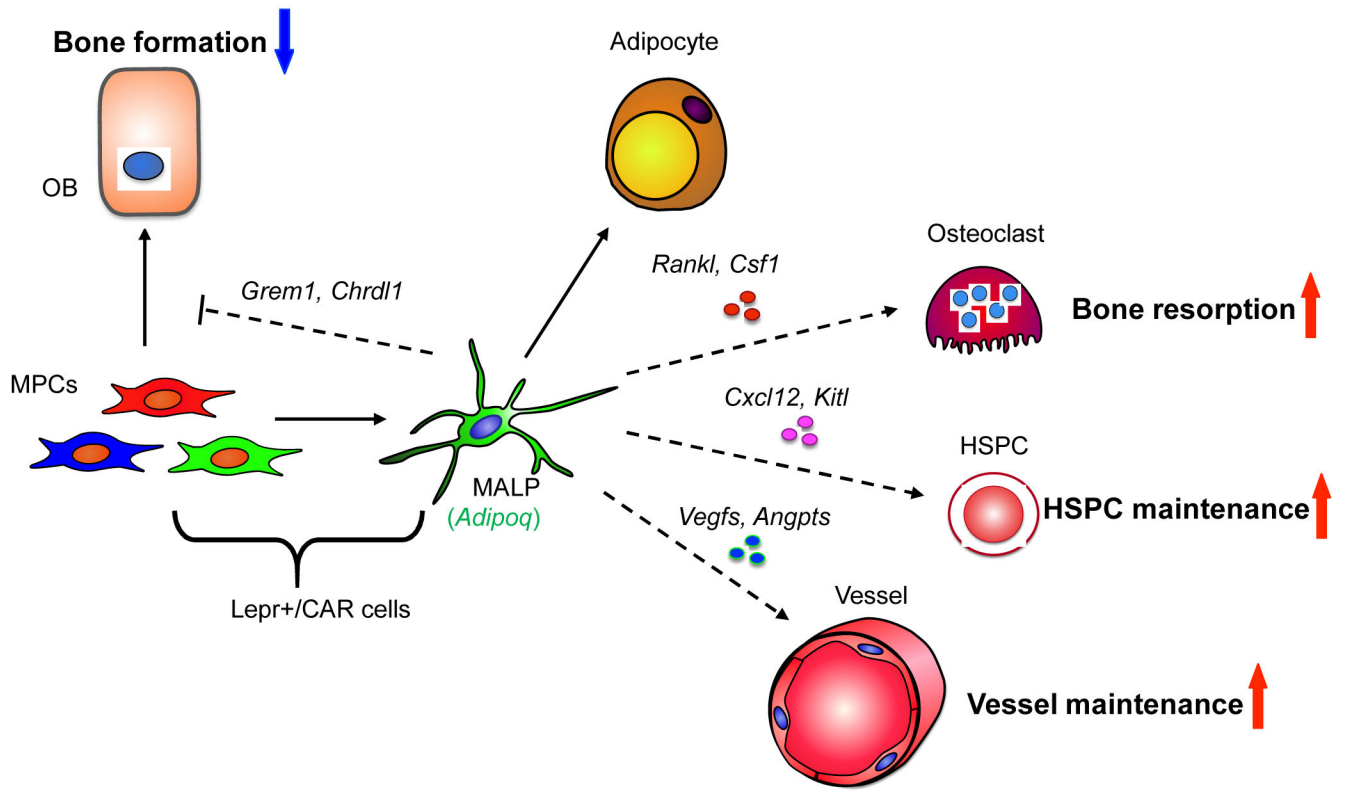
C. Split UMAP view of integrated dataset by the original datasets.

D. UMAP plots of MALP cluster markers.

E. UMAP plots of EMP/Dpp4+ progenitor cluster markers.



**Figure 3.** Violin plot of marker genes of osteogenesis, osteoclastogenesis, hematopoiesis and angiogenesis of bone marrow mesenchymal lineage cells.



**Figure 4.** A schematic diagram depicts the master regulatory role of MALPs on bone marrow cells.