Hypertrophic Chondrocytes Serve as a Reservoir for Unique Marrow Associated Skeletal Stem and Progenitor Cells, Osteoblasts, and Adipocytes During Skeletal Development

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ABSTRACT

Hypertrophic chondrocytes give rise to osteoblasts during skeletal development, however the process by which these non-mitotic cells make this transition is not well understood. Prior studies have also suggested that skeletal stem and progenitor cells (SSPCs) localized to the surrounding periosteum serve as the primary source of marrow associated SSPCs, osteoblasts, osteocytes, and adipocytes during skeletal development. To further understand the cell transition process by which hypertrophic chondrocytes contribute to osteoblasts or other marrow associated cells, we utilized hypertrophic chondrocyte genetic reporter mouse models combined with single cell RNA-sequencing, immunofluorescence staining, and bulk RNA-sequencing approaches. Our data demonstrate that hypertrophic chondrocytes undergo a process of dedifferentiation to generate unique marrow associated SSPCs, previously characterized as a CXCL12-abundant reticular (CAR) cells, that not only establish a molecular niche for hematopoiesis, but also serve as a primary source of osteoblasts during skeletal development and contribute to the adipogenic lineage with age.

INTRODUCTION

Most skeletal elements arise from a cartilage template via the process of endochondral ossification (Olsen et al., 2000). During skeletal development, mesenchymal stem and progenitor cells condense and differentiate to form chondrocytes or cartilage cells (Olsen et al., 2000). These chondrocytes undergo rapid proliferation while depositing an extracellular matrix (ECM) rich in type II collagen (COL2A1), forming the initial immature cartilage rudiments. As cartilage growth proceeds, the chondrocytes localized nearest the center of each rudiment exit the cell cycle and undergo a process of maturation and hypertrophic differentiation (Olsen et al., 2000). Hypertrophic chondrocytes, which are identified by their specific expression of type X collagen (COL10A1), are ultimately responsible for secreting additional proteins such as Vascular Endothelial Growth Factor alpha (VEGFα), Platelet Derived Growth Factor beta (PDGFβ), and Indian Hedgehog (IHH) among other factors to promote both vascular invasion and osteoblast differentiation of the surrounding perichondrial progenitors (Olsen et al., 2000). As vessels from the perichondrium invade the hypertrophic cartilage to establish the marrow cavity, newly formed osteoblasts and their progenitors are delivered by these vessels to the marrow space (Olsen et al., 2000). There, osteoblasts secrete a type I collagen (COL1A1) rich matrix that ultimately mineralizes to replace the cartilage with bone, while the hypertrophic cartilage is remodeled and reduced with age (Olsen et al., 2000). Hypertrophic chondrocytes are essential for both establishment of the marrow cavity and bone formation, however their precise fate and direct or indirect contribution to bone formation has been questioned (Tsang et al., 2015).

Recent genetic evidence suggests that hypertrophic chondrocytes undergo a process of transdifferentiation to directly generate osteoblasts and osteocytes that contribute to the formation of trabecular and endocortical bone (Park et al., 2015; Yang et al., 2014a, 2014b; Zhou et al., 2014b). To determine the fate of growth plate chondrocytes during endochondral bone formation, investigators have utilized mouse lines combining constitutive and inducible CRE recombinases under the control of the *Col2a1, Acan,* or *Col10a1* promotors/enhancers with a recombination sensitive reporter. Utilization of the constitutive and inducible *Col2a1Cre/Col2a1Cre*^{ERT2} mouse lines have complicated our understanding of this lineage by the fact that in addition to their expression in immature chondrocytes, *Col2a1* and these genetic tools have been noted to be expressed within and/or label directly perichondrial and marrow associated cell populations known to give rise

to osteoblast lineage cells (Hilton et al., 2007; Long et al., 2001). Some of the more convincing data generated utilizing a *Col2a1Cre*^{ERT2} mouse line and recombination sensitive reporter comes from Yang et al., 2014a, which established an approach for low dose tamoxifen delivery to drive recombination in clones or clusters of immature chondrocytes that could be tracked over time. When the chase period was short (1-5 days post tamoxifen), reporter expression was largely restricted to immature chondrocyte clusters or extensions of these clusters into early hypertrophic chondrocytes. However, when the chase period was extended (10 days post tamoxifen), reporter expression could be observed throughout individual columns of proliferating to hypertrophic chondrocytes that also extended into *Col1a1*-expressing osteoblasts localized only to the regions of metaphyseal bone directly underneath the labeled chondrocytes; a result that is consistent with chondrocytes transitioning to an osteoblastic fate (Yang et al., 2014a). Similar pulse-chase experiments were performed utilizing the *AcanCre*^{ERT2}*; R26-tdTomato* reporter model at both embryonic and adult time points utilizing doses of tamoxifen that elicit high rates of recombination and are more restricted to chondrocytes within the growth plate. These data suggested that following short (1-3 days post tamoxifen) or long (2 weeks post tamoxifen) chase periods, non-chondrocytic cells associated with the primary spongiosa and trabecular bone were reporter positive with some cells labeling with the *Col1a1(2.3kb)-eGFP*.(Zhou et al., 2014b) The most convincing data was generated utilizing a combination of constitutive and inducible *Col10a1Cre/Col10a1Cre*^{ERT2} mouse lines and recombination sensitive reporters; models that are much more restricted in their expression to hypertrophic chondrocytes of the growth plate (Yang et al., 2014b). This study, as well as others solely utilizing a constitutive *Col10a1Cre* model, determined that hypertrophic chondrocytes can relatively quickly give rise to osteoblastic cells expressing OSTERIX (OSX), COL1A1, and eventually even OSTEOCALCIN (OCN) and/or SCLEROSTIN (SOST) expressing osteocytes within the trabecular and endocortical bone matrix (Park et al., 2015; Yang et al., 2014a, 2014b; Zhou et al., 2014b). Interestingly, Yang et al., 2014a utilized a *Col10a1Cre; R26-eYFP* reporter model and noted the presence of some YFP^{+}, *Perilipin*-expressing adipocytes and YFP^{+}, PDGFRβ^{+} pericytes within the bone marrow, suggesting the potential for alternate fates of hypertrophic chondrocytes beyond the osteoblast lineage. Utilizing similar techniques, Tan et al., 2020 also demonstrated the potential for mature adipocytes to arise from *Col10a1*-expressing hypertrophic chondrocytes.

Since the fates of mature osteoblasts, adipocytes, and pericytes are unlikely to arise from independent hypertrophic chondrocyte transdifferentiation events, we reasoned that the hypertrophic chondrocytes may undergo a dedifferentiation process to generate skeletal stem and progenitor cell (SSPC) population(s) that localize to the bone marrow and maintain the capacity of redifferentiating into more mature cell fates. To address this issue, we utilized hypertrophic chondrocyte genetic reporter mouse models combined with single cell RNA-sequencing, immunofluorescent staining validation assays, and bulk RNA-sequencing approaches of clonal cell populations. Our data indicate that hypertrophic chondrocytes indeed undergo a process of dedifferentiation to generate unique bone marrow associated SSPCs that likely serve as a primary source of osteogenic cells during skeletal development and also contribute to the adipogenic lineage, but not the pericyte lineage. Further, these studies indicate that the hypertrophic chondrocyte derived SSPCs are strikingly similar at the transcriptomic level to marrow associated SSPCs derived from other sources, with some notable exceptions that may be responsible for their rapid contribution to the osteoblast lineage and bone formation during skeletal development.

RESULTS

Hypertrophic chondrocytes give rise to osteoblasts and other marrow associated cells

To validate the osteogenic fate of hypertrophic chondrocytes, we first utilized the *Col10a1Cre*^{ERT2} mouse line crossed with a *Rosa26-tdTomato* reporter (*Col10a1Cre*^{ERT2}*; R26-tdTomato*). These mice were injected with a single dose of tamoxifen (TM) at postnatal day 6 (P6) and harvested at 12-hours, 24-hours, and 2-weeks following TM (Figure 1a-d). At 12-hours following TM administration, specific expression of the tdTOMATO reporter was observed within hypertrophic chondrocytes and no cells within the marrow or surrounding tissues (Figure 1a). At 24-hours following TM administration, the tdTOMATO reporter could now be observed in a limited number of marrow-associated cells directly below the cartilage growth plate, as well as within hypertrophic chondrocytes (Figure 1b). At 2-weeks following TM administration, the tdTOMATO reporter had cleared from the hypertrophic chondrocytes and was now exclusively present in a number marrow associated cells; some of those being at a significant distance from the growth plate (Figure 1c). In this distal region of the marrow, we also observed trabecular bone lining cells that co-labeled for tdTOMATO and OCN (Figure 1d; white arrows), suggesting that these cells had transitioned from hypertrophic chondrocytes to osteoblasts. Interestingly, other tdTOMATO positive marrow associated cells were not directly in contact with bone matrix and did not co-label with OCN or other osteogenic markers (Figure 1d; orange arrow), suggesting the presence of another cell type or cell stage within the bone marrow that may be derived from hypertrophic chondrocytes.

To get a better understanding of the proportionality of these hypertrophic chondrocyte derived marrow and osteogenic cells, we performed similar analyses on constitutively active *Col10a1Cre; R26-tdTomato* mice. While tdTOMATO reporter expression was observed within hypertrophic chondrocytes and osteogenic cells associated with trabecular and endocortical bone of mice at P0 (Figure 1e-g) and 2-months (2M) of age (Figure 1h-j), CRE recombinase could only be detected within hypertrophic chondrocytes (Figure S1). The vast majority of the trabecular and endocortical bone was lined with tdTOMATO reporter positive cells, and 69.4% ± 14.8% of osteocytes were reporter positive (Figure 1k,l – white arrows). We also observed that 83.3% ± 9.7% of all OCN-positive osteoblasts were tdTOMATO reporter positive, confirming that hypertrophic chondrocytes can give rise to the majority of intramedullary osteoblasts during bone development (Figure 1k,l – green arrows). These data were also consistent with our inducible *Col10a1Cre*^{ERT2}*; R26-tdTomato* data and prior

reports (Yang et al., 2014a, 2014b; Zhou et al., 2014b). Of note, a number of tdTOMATO negative and OCN positive cells were also identified lining portions of the trabecular and endocortical bone, indicating that some of these osteoblasts originate from an alternate source such as the perichondrium/periosteum (Figure 1k). Finally, utilizing this model we were able to confirm the existence of tdTOMATO reporter expressing cells within the marrow that were not associated with bone matrix; however, did appear to be either loosely associated with the marrow (white arrow) or directly associated with vessel-like structures (yellow arrow) (Figure 1j).

Single cell transcriptomics identifies several cell populations, including osteoblasts, that are derived from hypertrophic chondrocytes.

Following the observation that tdTOMATO reporter positive cells were both marrow- and bone-associated (Figure 1), we hypothesized that the hypertrophic chondrocytes may give rise to multiple cell fates. We therefore took an unbiased approach by performing single cell RNA-sequencing (scRNA-seq) on cells derived from 2M *Col10a1Cre; R26-tdTomato* mice. To isolate and specifically enrich hypertrophic chondrocyte derived marrow associated cells, we performed centrifugation of the marrow followed by a gentle flush of femurs and tibias and subjected these marrow plugs to a brief collagenase digestion. Following the digest, red blood cells were lysed and the remaining cell populations were subjected to fluorescence activated cell sorting (FACS). We selected tdTOMATO reporter positive cells that demonstrated high levels of unambiguous tdTOMATO fluorescence for further analysis.

Using the 10x Genomics platform, we performed scRNA-seq and further analyzed 1066 tdTOMATO reporter positive sorted cells following quality control processing and elimination of any cells lacking *tdTomato* transcripts. We sequenced a median of 2820 genes per cell and a mean of 261,899 reads per cell. We reached a sequencing saturation of 92.3%, giving us confidence in the sequencing depth and the conclusions drawn from our downstream analyses. Since neither *Cre* nor *Col10a1* expression could be detected in any of these cells, they quite clearly represented descendants of *Col10a1Cre*-expressing hypertrophic chondrocytes. Finally, we performed a Seurat-based unsupervised clustering of gene expression profiles from the tdTOMATO positive cells, which ultimately yielded 5 distinct cell clusters (Figure 2a; see Methods for details).

Differential gene expression analyses further demonstrated that cells associated with clusters 1 and 2 expressed several known osteogenic genes (Figure 2b-h). Many cells in cluster 1 expressed genes such as *Mepe*, *Dmp1*, and *Fam20c* (Figure 2b-d,h), known for their roles in osteocytes and/or the bone mineralization process (Robling and Bonewald, 2020; Sapir-Koren and Livshits, 2014). Some cells in cluster 2 also expressed *Dmp1* and *Fam20c*; however, their expression was detected at much lower levels. Most cells in cluster 2 expressed other osteoblast associated genes, such as *Bglap* and *Col1a1* (Figure 2e-f,h), critical components of the organic bone matrix. In addition to the few genes highlighted here, additional osteoblast associated genes were also observed in cells within clusters 1 and 2 (Figure S2a-g). Expression analyses further determined that the master transcriptional regulator of osteoblast differentiation and known osteoprogenitor cell marker, S*p7* (encoding the protein OSTERIX) (Maes et al., 2010; Mizoguchi et al., 2014), not only exhibited expression within osteoblast clusters 1 and 2, but also within a limited number of cells associated with clusters 3, 4, and 5 (Figure2g-h). These data suggest that the majority of marrow associated cells derived from the hypertrophic chondrocytes localized to clusters 3, 4, and 5 are not osteoblasts or osteoprogenitors, but rather may be more primitive cells within the osteoblast lineage or distinct cell types altogether.

Hypertrophic chondrocytes give rise to marrow associated SSPCs.

We next utilized differential gene expression analyses to identify genes that were uniquely and broadly expressed across the cells of clusters 3, 4, 5, with limited expression in the more differentiated osteoblasts of clusters 1 and 2. This analysis identified a number of genes utilized for identifying skeletal stem and progenitor cell (SSPC) populations. Specifically, *Lepr, Grem1, Pdgfra* (but not *Ly6a* – SCA1), *Cxcl12*, and *Pdgfrb* were highly expressed within cells across all three clusters (Figure 3a-f,k). Each gene and/or their regulatory elements genetically identify or can be utilized to label SSPCs with the potential of becoming osteoblasts *in vivo* (Crisan et al., 2008; Morikawa et al., 2009; Omatsu et al., 2010; Worthley et al., 2015; Zhou et al., 2014a). To confirm that hypertrophic chondrocytes are capable of giving rise to marrow associated SSPCs expressing *Lepr*, *Pdgfra,* and *Pdgfrb in vivo*, we performed IF co-labeling assays on bone sections from *Col10a1Cre; R26-tdTomato* mice at 2M of age and *Col10a1Cre^{ERT2}; R26-tdTomato* at 3W of age (TM delivered at P6). Co-labeling of cells with tdTOMATO fluorescence and antibodies for LEPR, PDGFRα, and PDGFRβ in both

genotypes further demonstrate and validate that hypertrophic chondrocytes indeed give rise to marrow associated SSPCs during skeletal development (Figure S3).

FACS can also be utilized to identify, isolate, and enrich populations of SSPCs from bone marrow and periosteal tissues in mice and/or humans based on the expression of specific cell surface proteins (Chan et al., 2015, 2018; Debnath et al., 2018; Zhou et al., 2014a). FACS assays utilizing antibodies against Integrin Subunit Alpha V (ITGAV) have been used for the identification, isolation, and enrichment of bone marrow stromal cell populations in mice, which are noted to be largely quiescent and capable of generating most of the colonies within a colony forming unit – fibroblastic (CFU-F) assay; a technique utilized to identify heterogeneous, non-hematopoietic, marrow stromal cells with progenitor cell characteristics (Boulais et al., 2018; Robey et al., 2014). While we identified *Itgav* expression sporadically throughout cells of all clusters, it is enriched in the SSPC clusters 3, 4, and 5 (Figure 3g,k). Recently, CD200, ITGAV, and CD105 antibodies have been used in conjunction with antibodies detecting the hematopoietic and endothelial lineages (CD45, CD31, and Ter119) for flow cytometric evaluation and identification of unique subsets of SSPCs (Debnath et al., 2018; Gulati et al., 2018). These studies indicate that lineage negative ($CD45^-$,$CD31^-$,$Ter119^-$) and $CD200^+$,$ITGAV^+$,$CD105^-$ cells represent the most primitive self-renewing and multipotent skeletal stem cell (SSC) population, while lineage negative and $ITGAV^+$,$CD105^+$ cells represent a more committed SSPC population (Debnath et al., 2018; Gulati et al., 2018). Our gene expression analyses observed broad expression of *Cd200* across all clusters including the osteoblasts; however, *Eng* (encoding CD105) was more restricted to SSPC clusters 3, 4, 5 and enriched specifically within cluster 4 (Figure 3h-i,k). When assessing the precise cellular overlap of these factors, we observed 128 of 953 SSPCs to be *Itgav+, Cd200+, Cd105-*. While this most primitive SSC population exists among our SSPCs analyzed via scRNA-seq, they were relatively evenly distributed among cells within clusters 3, 4, and 5 (Figure 3g-k). These data suggest that hypertrophic chondrocytes likely undergo a process of dedifferentiation to generate even the most primitive SSCs; however, these cells themselves may be heterogeneous due to the differential expression of genes that allow for their segregation into multiple distinct clusters within our Seurat analysis.

In an attempt to further delineate each of the SSPC clusters in our Seurat analysis, we analyzed the data to identify genes restricted in their expression to a single SSPC cluster. A limited number of genes were

ultimately found to be uniquely expressed within solitary SSPC clusters; however, much more commonly we observed genes to be enriched within specific clusters with limited expression within the other one or two SSPC clusters (Figure S4).

Some SSPCs and marrow stromal populations have been identified as important niche cells and regulators of hematopoietic stem cells (HSCs) and hematopoiesis (Chan et al., 2013; García-García et al., 2015; Seike et al., 2018). To determine whether SSPCs within clusters 3, 4, or 5 may uniquely support HSCs or hematopoiesis, we examined the differential gene expression of various secreted proteins known to regulate these processes and cellular factors important for HSC support. We observed high expression levels of *Ebf1*, *Ebf3, Kitl,* and *Angpt1* throughout all SSPC clusters 3, 4, and 5 (Figure S5a-d). Additionally, ITGAV$^+$,CD105$^+$,6C3$^+$ SSPCs (known as 6C3 stromal cells) and ITGAV$^+$,CD105$^-$,6C3$^+$ SSPCs (known as HEC cells) have been identified as differentiated derivatives of the ITGAV$^+$,CD200$^+$,CD105$^-$ SSC population, as well as unique populations of HSC supportive stromal cells that maintain HSC stemness (Gulati et al., 2018). Our differential gene expression analyses determined that *Enpep*, which encodes 6C3, is largely restricted to the SSPCs of cluster 3, with some co-expression of *Eng* (CD105) (Figure S5e,g and Figure 3i,k). Furthermore, genetic studies have also recently identified LepR$^+$ and PTN$^+$ (Pleiotrophin) marrow stromal cells as a unique stromal population required for HSC maintenance. Our gene expression analyses also indicated that the SSPCs of cluster 3 uniquely express *Ptn* as compared to all other *Lepr*-expressing SSCs. Collectively, these data suggest that SSPCs of cluster 3 may act uniquely in a HSC supportive role as PTN expressing 6C3 and HEC cells (Figure S6f,g) (Himburg et al., 2018).

SSPCs, and especially those supporting HSCs and hematopoiesis, are known to reside within specific niches often associated with blood vessels (Zhou et al., 2014a). Previously we noted that some of the hypertrophic chondrocyte derived tdTOMATO reporter cells appeared to associate with vascular structures. To assess this directly, we performed immunofluorescent staining for ENDOMUCIN (EMCN) on bone sections of *Col10a1Cre; R26-tdTomato* mice at 2M of age and identified a number of tdTOMATO reporter cells directly adjacent to EMCN expressing endothelial cells (Figure S6a). To ensure that these tdTOMATO cells were not pericytes or vascular smooth muscle cells (vSMCs) we assessed our differential gene expression data for genes that serve a common markers for these specific cell types, including *Acta2, Nes, Kcnj8, Rgs5, Cspg4,*

and *Mcam* (Chasseigneaux et al., 2018; Yamazaki and Mukouyama, 2018). We observed limited or no gene expression for these markers in SSPCs of clusters 3,4, or 5 (Figure S6b-g).

To finally parse the relatedness or relationship between cell clusters, we performed Monocle 3 pseudotime cell trajectory analysis. These data indicate a clear hierarchy of cellular differentiation and relatedness between all cell clusters in an unbranched or continuous trajectory. After establishing cluster 1, osteoblasts expressing *Dmp1, Mepe,* and *Fam20c,* as a terminal root of the trajectory analysis, Monocle 3 was able to establish a clear and unbranched pseudotime trajectory towards the most primitive SSPCs of clusters 4 and 5. Cells within cluster 3 are identified as an intermediate state between the more primitive SSPC clusters 4/5 and the osteoblast clusters 1/2 (Figure 3j). Since Monocle 3 performs principal component analysis and differential gene expression with its own algorithms, we also mapped genes that identified each cluster from Seurat on this trajectory for ease of cluster association along the pseudotime (Figure S7). Taken together these data indicate that cluster 3, 4, and 5 are hypertrophic chondrocyte derived SSPCs that are capable of becoming osteoblasts; however, may have unique niche localization (vessel associated) and/or function (support and maintenance of HSCs).

***Chondrocyte derived SSPCs are dually primed for both osteoblast and adipocyte differentiation*.**

Since most bone marrow associated SSPCs have the potential to become either osteoblasts or adipocytes during development, aging, regeneration, and/or disease, we further examined our differential gene expression data for indicators of this competency. While we observed limited expression of the osteoprogenitor transcription factor *Sp7* in the SSPC clusters 3, 4, and 5, we noticed much broader and robust expression of *Runx2,* the earliest master transcriptional regulator of osteoblast specification, throughout all clusters and especially concentrated in the SSPC clusters 3, 4, and 5 (Figure 4a). RUNX2 has been observed in LEPR^{+} cells and appears to prime cells for osteoblastic specification and differentiation (Yang et al., 2017). We have already demonstrated in Figure 1 the efficiency by which hypertrophic chondrocytes can contribute to the osteoblast lineage, and that these osteoblasts are part of a differentiation trajectory that can originate from hypertrophic chondrocyte derived SSPCs.

Recent data has also shown that some LEPR$^+$ SSPCs also express PPARγ (encoded by *Pparg*) and ADIPONECTIN (encoded by *Adipoq*) (Zhong et al., 2020). Similar to this report we observed *Pparg* and *Adipoq* expression within *Lepr*-expressing SSPCs of clusters 3, 4, and 5 (Figure 4b-c). While PPAR-gamma is necessary and sufficient to induce adipogenesis of SSPCs, ADIPONECTIN enhances osteogenesis but is primarily secreted by adipocytes (Tencerova and Kassem, 2016). Given the expression of these adipogenic regulators and marker genes, we asked if hypertrophic chondrocyte derived cells in clusters 3, 4, and 5 represent mature adipocytes or whether these cells could ultimately give rise to mature adipocytes *in vivo*. Our differential gene expression analysis did not indicate the presence of *Perilipin* (*Plin1*), a mature lipid laden adipocyte marker, in SSPC clusters 3, 4, or 5, suggesting that none of these cells isolated utilizing our procedures were representative of mature adipocytes (Figure 4d). Providing evidence for an ultimate adipogenic fate for some of these SSPCs, *Col10a1Cre; R26-tdTomato* mice at 2M and 13M of age displayed co-localization of the tdTOMATO reporter and PERILIPIN antibody staining (Figure 4e). By 13M of age, approximately 25% of adipocytes labeled with PERILIPIN were also tdTOMATO reporter positive (Figure 4f). None of these double labeled cells are either known to express *Col10a1,* nor could we detect CRE expression within any of these cells. Therefore, our results suggest that hypertrophic chondrocyte derived SSPCs are also a significant contributor to the adipogenic fate; however, these mature cells likely eluded our scRNA-seq analyses due to the technical nature for isolating and sorting lipid laden adipocytes and their limited availability in the bone marrow at 2M of age.

To assess SSPC contribution to the osteoblast and adipocyte lineages, we also performed reporter studies utilizing *LeprCre;R26-tdTomato* and *AdipoqCre;R26-tdTomato* mice. Utilizing bone sections of P0 *LeprCre;R26-tdTomato* reporter mice, we observed reporter positive signals in both marrow associated cells and some bone lining cells; however, OCN$^+$, tdTOMATO$^+$ osteoblasts were not apparent (Figure S8a). By 2M of age we observed bone lining cells, OCN-expressing osteoblasts, and osteocytes that were tdTOMATO reporter positive (Figure S8a – white arrows), similar to data previously reported (Zhou et al., 2014a). As expected, a limited number of PERILIPIN$^+$, tdTOMATO$^+$ adipocytes were also observed within the bone marrow of 2M old *LeprCre;R26-tdTomato* mice (Figure S8a). Similarly, P0 *AdipoqCre;R26-tdTomato* bone sections exhibited marrow associated and bone lining reporter positive cells that not yet contributed to OCN-

positive osteoblasts; however, by 2M of age tdTOMATO reporter positive and OCN-expressing osteoblasts, osteocytes, and adipocytes were apparent in *AdipoqCre;R26-tdTomato* bone sections (Figure S8b – white arrows). In contrast with these studies, *Col10a1Cre;R26-tdTomato* descendants demonstrate a relatively quick commitment to the osteoblast lineage that is observed at P0 and earlier, while *Col10a1Cre^{ERT2};R26-tdTomato* mice document that bone lining cells and mature OCN-expressing osteoblasts can be generated from hypertrophic chondrocytes within 1-day to 2-weeks. IF analyses of marrow associated cells from 2M old *Col10a1Cre;R26-tdTomato* mice assessing LEPR$^+$; tdTOMATO$^+$ and LEPR$^+$; tdTOMATO$^-$ marrow associated cells indicate that hypertrophic chondrocyte derived SSPCs only constitute 37.4% ± 6.2% of the total LepR$^+$ population (Figure S9), indicating that most of these LEPR$^+$ marrow associated SSPCs are likely derived from other origins such as the perichondrium/periosteum. Further it highlights the potential for functional differences between sub-populations of LEPR and ADIPOQ expressing SSPCs based on their particular origin and/or niche.

***Hypertrophic* chondrocyte derived SSPCs may be uniquely primed for developmental bone growth *in vivo*.**

Col10a1Cre-derived marrow associated SSPCs constitute only a portion of LEPR$^+$ SSPCs and *LeprCre*-derived marrow associated SSPCs contribute to nearly 100% of all CFU-F colonies, therefore we reasoned that *Col10a1Cre*-derived marrow associated SSPCs would contribute to only a fraction of total CFU-Fs and that this may provide a method for separating hypertrophic chondrocyte derived progenitors from those derived from alternative sources such as the perichondrium/periosteum (Zhou et al., 2014a). Following plating at clonal density and 10 days of growth, nearly 50% of CFU-F colonies derived from *Col10a1Cre;R26-tdTomato* mice exhibited relatively homogeneous tdTOMATO reporter expression while the other colonies were largely devoid of tdTOMATO positivity (Figure 5a-c). To achieve a better understanding of the similarities and differences between progenitors derived from these different sources at the transcriptomic level, we isolated 3 tdTOMATO$^+$ and 3 tdTOMATO$^-$ CFU-F colonies and performed individual RNA-isolations and submitted for bulk RNA-sequencing. Of the differentially expressed genes reaching significance ($p < 0.05$, LogFC > 1), the 3 tdTOMATO$^+$ CFU-Fs clustered separately from 3 tdTOMATO$^-$ CFU-Fs on the basis of only 363 differentially

expressed genes (227 enriched in tdTOMATO$^+$ CFU-Fs and 136 enriched in tdTOMATO$^-$ CFU-Fs) out of approximately 12,000 genes with an average expression level above biological thresholds (Figure 5d and Methods for threshold calculations). We observed *Lepr, Grem1, Pdgfra, Adipoq, Pparg, Itgav, Cd200,* and *Cxcl12,* all highly expressed above biological thresholds in all CFU-Fs, indicating a general maintenance of SSPC phenotype while in culture and a striking similarity between progenitors from disparate sources.

To more readily identify differences between tdTOMATO$^+$ and tdTOMATO$^-$ CFU-Fs, we performed gene ontology analysis for biological processes and found two interesting terms arise due to enriched expression of genes within the tdTOMATO$^+$ or hypertrophic chondrocyte derived CFU-Fs; cell population proliferation and regulation of vascular development (Figure 5e) (Mi et al., 2019; The Gene Ontology Consortium, 2021; The Gene Ontology Consortium et al., 2000). Regulation of vascular development was interesting, although not a surprising finding, since we know that terminal hypertrophic chondrocytes secrete factors to initiate vessel invasion from the periosteum and we had identified a number of marrow associated hypertrophic chondrocyte derived SSPCs localized near blood vessels. The fact that the tdTOMATO$^+$ or hypertrophic chondrocyte derived CFU-Fs were enriched in genes associated with cellular proliferation was unanticipated. To this end, we investigated EdU incorporation into all CFU-Fs from *Col10a1Cre;R26-tdTomato* mice at day 5 in culture that consisted of at least 5 cells. We observed a greater percentage of cells in tdTOMATO$^+$ CFU-Fs that had incorporated EdU as compared to tdTOMATO$^-$ CFU-Fs (Figure 5f - top). By day 10 in culture, tdTOMATO$^+$ CFU-Fs on average were larger and displayed more colonies with 200+ cells as compared to tdTOMATO$^-$ CFU-Fs (Figure 5f - bottom). To determine whether this proliferative advantage could be observed in SSPCs immediately harvested from *Col10a1Cre;R26-tdTomato* bone marrow, we performed cell cycle analysis on our scRNA-seq dataset and found that as a percentage of the population there was an equal or greater percentage of tdTOMATO$^+$ SSPCs (cluster 3, 4, and 5) in a proliferative state (S, G2, or M phase) as compared to osteoblasts (clusters 1 and 2) (Figure 5g). This unexpectedly high proliferative capacity for hypertrophic chondrocyte derived SSPCs may be reflective of the fact that hypertrophic chondrocytes eventually re-enter the cell cycle as they transition fates within the marrow space (Park et al., 2015; Yang et al., 2014a).

To determine whether tdTOMATO^{+} and tdTOMATO^{-} CFUs contained inherent differences in their capacities to undergo osteogenic and adipogenic differentiation *in vitro*, we performed CFU-osteoblastic (CFU-Ob) and CFU-adipogenic (CFU-Ad) assays. Interestingly, colonies composed of cells from either hypertrophic or non-hypertrophic origins were able to differentiate at similar capacities *in vitro* when measured by Alkaline Phosphatase, Von Kossa, and Oil Red O staining (Figure S10). While these data validate the functional ability of hypertrophic chondrocyte derived SSPCs to undergo osteogenic and adipogenic differentiation, this did not seem to be a defining feature or difference between non-hypertrophic chondrocyte derived SSPCs. Due to the overall similarities in gene expression profiles and differentiation capacity *in vitro*, these data suggest that SSPC maintenance and programming may be highly regulated by the bone marrow and/or vascular niche regardless of the contributing cellular source.

DISCUSSION

Recent and historic data have suggested that hypertrophic chondrocytes are capable of becoming osteoblasts that directly contribute to trabecular bone formation (Giovannone et al., 2019; Park et al., 2015; Tsang et al., 2015; Yang et al., 2014a, 2014b; Zhou et al., 2014b); however, the process by which hypertrophic chondrocytes generate osteoblasts remains unknown. Current genetic lineage tracing studies have suggested a transdifferentiation mechanism by which hypertrophic chondrocytes differentiate directly into an osteoprogenitor or mature osteoblast without transiting through an intermediate or more primitive state (Park et al., 2015). Here we have provided genetic and transcriptomic evidence, consistent with our hypothesis, that hypertrophic chondrocytes undergo a process of dedifferentiation to form unique SSPC populations ultimately giving rise to osteoblastic and adipogenic cell fates within the marrow compartment of postnatal and adult bones. A number of genetic and flow cytometric approaches have been used to identify and isolate multipotent SSPC populations within both the bone marrow and periosteal compartments. Many of these efforts have used alternative genetic models (*Gremlin1Cre, LeprCre, Col2a1Cre, Cxcl12Cre*) with lineage tracing and/or RNA-seq analyses or have utilized flow cytometric based selection methods with antibodies against PDGFRα, CD200, and ITGAV among others followed by differentiation assays (Boulais et al., 2018; Chan et al., 2015; Debnath et al., 2018; Farahani and Xaymardan, 2015; Gulati et al., 2018; Mizuhashi et al., 2019; Ono et al., 2014; Worthley et al., 2015; Zhou et al., 2014a). Generally, these studies have identified the presence of non-hematopoietic, non-endothelial multipotent mesenchymal cell populations with at least the capacity for osteogenic and/or adipogenic differentiation (Robey et al., 2014). It has also largely been assumed that these marrow associated SSPCs are derived from the perichondrial/periosteal SSPCs during skeletal development. Our study demonstrates for the first time that hypertrophic chondrocytes of the growth plate serve as a reservoir and alternative source of unique marrow associated SSPCs likely utilized for rapid bone growth during skeletal development (Figure 6).

Specifically our analyses of *Col10a1Cre; R26-tdTomato* bone marrow identified not only hypertrophic chondrocyte derived osteoprogenitors (*Sp7* expressing) (Park et al., 2015) and osteoblast populations, but also hypertrophic chondrocyte derived marrow and vessel associated cells expressing *Lepr, Pdgfra, Pdgfrb, Grem1, Cxcl12,* with several expressing *Itgav, Cd200, and Eng* (CD105) while lacking the expression of *Col10a1, Cre,*

and most osteogenic genes. Since *Runx2*, *Pparg,* and *Adipoq* are also expressed throughout many of these cells, which lacked *Plin1*, we postulate that these hypertrophic chondrocyte derived cells encompass populations of SSPCs with the capability of osteogenic and adipogenic differentiation. This indeed was confirmed via our *in vitro* differentiation assays. Prior work has demonstrated that *Runx2* expressing and LEPR$^+$ progenitors are primed for osteoblastic differentiation (Yang et al., 2017), while LEPR$^+$ progenitors expressing *Adipoq* and *Pparg* are associated with adipogenic differentiation (Zhong et al., 2020). Priming of the SSPCs may allow for their rapid differentiation when in the proper supportive osteogenic/adipogenic niche or outside a niche that supports SSPC maintenance.

In addition to the osteogenic and adipogenic differentiation capacity of these cells, some SSPC populations also possess their own regulatory role in serving as a niche cell for HSCs aiding in either the maintenance of HSC stemness or regulating their proliferation, differentiation, and/or migration capabilities during hematopoiesis. CXCL12-abundant reticular (CAR) cells express high levels of *Cxcl12* (SDF)*, Kitl* (SCF), *Pdgfra*, *Lepr, Ebf1, Ebf3,* among other factors*,* while lacking the expression of *Ly6a* (SCA1) and *Cspg4* (NG2) (Baryawno et al., 2019; Derecka et al., 2020; Himburg et al., 2012; Seike et al., 2018). CAR cells are critical regulators of HSCs and hematopoiesis with the capacity for osteogenic and adipogenic differentiation and are known to reside within sinusoidal vascular niches (Omatsu et al., 2010). Further, subsets of CAR cells express PTN and are important regulators of HSC stemness (Himburg et al., 2012). In addition to CAR cells, a population of lineage negative, CXCL12$^-$, PDGFRα^+, SCA1$^+$ SSPCs (PαS cells) have been identified as a quiescent population associated with vessels near the endosteum (Morikawa et al., 2009). These PαS cells are also capable of forming CFU-Fs, can differentiate into osteoblasts, chondrocytes, and adipocytes, while also being important for the migration and maintenance of HSCs (Morikawa et al., 2009; Nusspaumer et al., 2017). The hypertrophic chondrocyte derived SSPCs of clusters 3, 4, and 5 identified in our study show striking resemblances to CAR cells based on gene expression profiles, vascular association, and osteogenic/adipogenic differentiation capacities, while even subsets of these cells (cluster 3) were identified as *Ptn* expressing cells that may specifically support HSC stemness. Unlike PαS cells, hypertrophic chondrocyte derived SSPCs/CAR cells robustly express *Cxcl12* and lack *Ly6a* (SCA1) expression*.* Collectively, these data indicate for first time that growth plate hypertrophic chondrocytes may serve as a unique reservoir for CAR cell

populations, and not PαS cells, that are required for normal hematopoiesis, HSC maintenance, and bone formation *in vivo*.

Since SSPC populations, including CAR cells, were thought to originate from perichondrial/periosteal cell sources and since LEPR+ CAR cells constitute 100% of CFU-Fs, we attempted to compare SSPCs/CAR cells derived from hypertrophic chondrocytes to those of other origins by utilizing *Col10a1Cre; R26-tdTomato* bone marrow cells plated at clonal density (Zhou et al., 2014a). Nearly 50% of the CFU-Fs were originally derived from hypertrophic chondrocytes (tdTOMATO^{+}), while the other half of CFU-Fs (tdTOMATO^{-}) were likely derived from a perichondrial/periosteal origin. Assessments of bulk RNA-seq data and EdU incorporation assays from CFU-F cultures, as well as cell cycle analysis of our scRNA-seq data, indicate an enhanced proliferative capacity for hypertrophic chondrocyte derived CFU-Fs and SSPCs/CAR cells. This data may be reflective of the fact that Yang et al. also identified the presence of both hypertrophic chondrocyte derived *Col1a1^{+} and Col1a1^{-}* marrow associated cells that were BrdU+ proliferating cells, indicating that hypertrophic chondrocytes eventually re-enter the cell cycle during this cell fate transition (Park et al., 2015; Yang et al., 2014a). Interestingly, our scRNA-seq dataset identified similar proportions of cycling cells in SSPCs/CAR cells and osteoblasts. This is in stark contrast to the more quiescent phenotype observed in most primitive SSPC populations that have been highlighted across numerous studies (Tikhonova et al., 2019; Zhong et al., 2020). However, recent work utilizing similar scRNA-seq approaches assessed proliferation rates of chondrocyte and perichondrial derived SSPCs and osteoblast populations in the *Col2a1Cre;R26-tdTomato* bone marrow and also indicated similar proliferative capacities between later committed progenitors and osteoblasts, which was greater than the most primitive of SSPCs (Zhong et al., 2020). Zhong et al., also utilized the *AdipoqCre^{ERT2};R26-tdTomato* model to investigate colony forming and proliferative potential of broad populations of *Adipoq*-expressing cells and descendants. These tdTOMATO^{+} cells contributed very little to CFU-Fs and exhibited minimal proliferation *in vitro* (Zhong et al., 2020). Tikhonova et al. also utilized scRNA-seq and identified a subset of cells from the *LeprCre;R26-tdTomato* lineage that cluster into a high cycling group (Tikhonova et al., 2019), which may represent a more proliferative LEPR^{+} SSPC population derived from the cartilage growth plate. Finally, it is worth noting again that the *in vivo* contribution of *AdipoqCre; R26-tdTomato* and *LeprCre; R26-tdTomato* lineages to osteoblasts and/or osteocytes is something that occurs over

developmental time postnatally, while the *Col10a1Cre; R26-tdTomato* lineage contributes to the osteoblast and osteocyte fates rapidly; as early as the embryonic period of skeletal development. We speculate based on our lineage tracing and reporter analyses, single cell and bulk RNA-seq data, and proliferation assays that the hypertrophic chondrocyte derived SSPCs/CAR cells represent a smaller more proliferative, active, and osteogenic prone population of LEPR^{+}, ADIPOQ^{+} cells *in vivo* as compared to other populations that share common markers.

STAR METHODS

Key Resources Table

1° Antibody	Vendor	Catalog Number	Concentration	Antigen retrieval
ENDOMUCIN	Abcam	ab106100	1:100	0.3% Triton
PERILIPIN	Cell Signaling	9349S	1:100	None
OSTEOCALCIN	Millipore	ab10911	1:200	Proteinase K
OSTERIX	Abcam	ab22552	1:400	None
CRE	Cell Signaling	15036S	1:100	0.3% Triton
RFP	Abcam	ab62341	1:100	None
LEPR	R&D Systems	AF 497	1:50	0.3% Triton
PDGFRa	R&D Systems	AF1062	1:50	0.3% Triton
PDGFRb	Invitrogen	14-1402-81	1:50	0.3% Triton

2° Antibody	Catalog number
Goat anti-Mouse 488	A11001
Donkey anti-Goat 488	A11055
Goat anti-Rat 488	A11006
Goat anti-Rabbit 594	A11037
Goat anti-Rabbit 488	A11034
Goat anti-Rat	A21247
Goat anti-Rabbit (for *LepRCre;tdTomato* stained PERILIPIN only)	A11008

Mouse strains

The *Col10a1Cre* mouse line was a generous gift from Dr. Klaus von der Mark and has been previously described.(Park et al., 2015) The *Col10a1Cre*^{ERT2} mouse line was a generous gift from Dr. Kathryn Cheah (University of Hong Kong). The *Rosa26-tdTomato*, *LeprCre*, and *AdipoqCre* mouse lines were obtained from Jackson Laboratories (007909, 008320, and 028020, respectively). All animal work was approved by Duke University Institutional Animal Care and Use Committees (IACUC). Both sexes of mice were utilized for experiments.

Tissue Analysis

Harvest and sectioning: Animals were harvested at postnatal day 0, 2 - 4 months, and 13 months of age. Tissues were washed in 1x PBS and then fixed overnight with 4% paraformaldehyde (PFA - Sigma). P0 animals were treated overnight with 14% EDTA (Sigma), while 2M old animals were treated for 10 days. Animals used for PERILIPIN labeling were not decalcified. Tissues were then transferred to 30% sucrose

(Sigma) for 2 days. Samples were imbedded in Shandon Cryomatrix (ThermoFisher Scientific) and cryosectioned at 10um, and slides were stored at -20C until staining.

For ENDOMUCIN and LEPR staining: Samples were prepared for staining as published.(Kusumbe et al., 2015) 2M animals were harvested and fixed overnight in 4% PFA. Samples were decalcified overnight in 14% EDTA. Tissues were transferred to 20% sucrose and 2% polyvinylpyrrolidone (PVP - Sigma) overnight. Samples were embedded in 8% gelatin (Sigma), 2% PVP, and 20% sucrose and cryosectioned at 30-200um with slides stored at -20C until staining.

Staining: Slides were brought to room temperature and rehydrated in PBS. Antigen retrieval was performed as shown in Table 1. Blocking was performed in 1% goat serum. Slides were incubated with antibodies at concentrations in Table 1 overnight at 4C in a humidified dark box. Slides were washed and incubated with secondary antibodies (Invitrogen catalog number in Table 1 – 1:200) for 30-60 minutes at room temperature in the dark. Slides were imaged on a Leica DMI3000B microscope using a Leica DFC3000G camera with the latest Leica imaging suite, a Zeiss 710 or Zeiss 780 inverted confocal microscope running the latest ZEN software. Images were reconstructed and pseudocolored on Fiji and quantified using built-in quantification measures.

Single-cell RNA sequencing

10x Transcriptome library prep: Single cells were isolated from bisected tibia and femur of 2-month-old *Col10a1Cre; R26-tdTomato* mice centrifuged at 4C twice pulsed up to 5K xg. A 27G needle attached to a syringe was then used to flush through the centrifuged bones. Bones and marrow were placed in 3mg/mL of Collagenase D (Roche 11088866001) for 30 minutes in hypoxia. Red blood cells were lysed and strained through a 70um filter. Cells were stained with DAPI for 30 mins on ice. Cells were washed with PBS and placed in PBS + 2% FBS for fluorescent activated cell sorting (FACS). Gating was performed for single cells, DAPI, and TOMATO as shown in Supplemental Figure 2a-c (Figure generated using FlowJo version 10). Cells were transferred to the Duke Molecular Physiology Institute Molecular Genomics core where they were combined with a master mix that contained reverse transcription reagents. The gel beads carrying the Illumina TruSeq Read 1 sequencing primer, a 16bp 10x barcode, a 12bp unique molecular identifier (UMI) and a poly-

dT primer were loaded onto the chip, together with oil for the emulsion reaction. The Chromium Controller partitions the cells into nanoliter-scale gel beads in emulsion (GEMS) within which reverse-transcription occurs. All cDNAs within a GEM, i.e. from one cell, share a common barcode. After the RT reaction, the GEMs were broken and the full length cDNAs cleaned with both Silane Dynabeads and SPRI beads. After purification, the cDNAs were assayed on an Agilent 4200 TapeStation High Sensitivity D5000 ScreenTape for qualitative and quantitative analysis.

Enzymatic fragmentation and size selection were used to optimize the cDNA amplicon size. Illumina P5 and P7 sequences, a sample index, and TruSeq read 2 primer sequence were added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contained P5 and P7 primers used in Illumina bridge amplification. Sequence was generated using paired end sequencing (one end to generate cell specific, barcoded sequence and the other to generate sequence of the expressed poly-A tailed mRNA) on an Illumina NextSeq500 with 400M read per run.

Analysis: The primary analytical pipeline for the SC analysis followed the recommended protocols from 10X Genomics. Briefly, we demultiplexed raw base call (BCL) files generated by Illumina sequencers into FASTQ files, upon which alignment to the mouse reference transcriptome, filtering, barcode counting, and UMI counting were performed using the most current version of 10X's Cell Ranger software. We used the Chromium cell barcode to generate feature-barcode matrices encompassing all cells captured in each library. The secondary statistical analysis was performed using the last R package of Seurat 3.(Butler et al., 2018; Konopka, 2019; R Core Team, 2013; Wickham et al., 2020) In Seurat, data was first normalized and scaled after basic filtering for minimum gene and cell observance frequency cut-offs (200-4000 genes, 7% mitochondrial genes, genes expressed in > 5 cells). We then closely examined the data and performed further filtering based on a range of metrics in an attempt to identify and exclude possible multiplets (i.e. instances where more than one cell was present and sequenced in a single emulsified gel bead). We thresholded *tdTomato* expression at 2 or higher to exclude a minimal number of cells that were separated via FACS but were *tdTomato* negative by gene expression.

After quality control procedures were complete, we performed linear dimensional reduction calculating principal components using the most variably expressed genes in our dataset (2000 variable genes, npcs = 25). The

genes underlying the resulting principal components are examined in order to confirm they are not enriched in genes involved in cell division or other standard cellular processes (subsetting out percent.mito). Significant principal components for downstream analyses are determined through methods mirroring those implemented by Macosko et al, and these principal components were carried forward for two main purposes: to perform cell clustering and to enhance visualization.(Macosko et al., 2015) Cells were grouped into an optimal number of clusters for de novo cell type discovery using Seurat's FindNeighbors and FindClusters functions (resolution = 0.4) graph-based clustering approach with visualization of cells being achieved through the use of UMAP, which reduces the information captured in the selected significant principal components to two dimensions. Differential expression of relevant genes was visualized on UMAP plots to reveal specific individual cell types. Additional downstream analyses included examining the cellular distribution and differential gene expression of a priori genes of interest and closer examination of genes associated within the identified 5 cell clusters. Cell cycle analysis of the scRNA-seq dataset was preformed using a gene list and commands packaged in Seurat.(Tirosh et al., 2016; Wickham, 2019)

Colony Forming Unit Assays

The Colony Forming Unit – Fibroblastic (CFU-F) assay was performed as published, with the exception of placing cells in hypoxia based on a recent publication demonstrating reduction of contaminating cells.(Guo et al., 2021; Robey et al., 2014) In short, cells were isolated from bisected tibia and femur of 2M old *Col10a1Cre; R26-tdTomato* mice centrifuged at 4C twice pulsed up to 5K xG. Following red blood cell lysis (Roche), cells were plated at 750K cells per 15cm tissue culture plate in complete media (a-MEM, 20% FBS, 1% penicillin-streptomycin) and glutamax (Gibco) after straining through a 70um filter. After incubation in hypoxia (2% oxygen, 5% CO2, 37C) for 3 hours, plates were washed three times with Hanks Balanced Salt Solution (Gibco) and replaced with complete media and glutamax. Media was changed on day 3 and 6. Colonies were utilized for analysis using a Leica DMI3000B microscope using a Leica DFC3000G camera with the latest Leica imaging suite, bulk RNA sequencing, or EdU labeling. TOMATO fluorescence was used to identify positive and negative colonies.

For CFU-OB, osteogenic differentiation was initiated after day 10 colony growth by replacing media with aMEM supplemented 10% FBS, 1% penicillin-streptomycin, 50ug/mL L-ascorbic acid (Sigma-A4544), and 10mM B-glycerophosphate (Sigma-G9422). Alkaline phosphatase and Von Kossa (Ricca) staining were performed per general histological procedures. For CFU-AD, adipogenic diffeentiation was performed as published; replacing media with aMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 1.7uM Insulin (Sigma), 1uM Dexamethasone (Sigma), and 0.5mM IBMX (Sigma) at day 10 colony growth.(Yu et al., 2019) Oil red O (Sigma) staining was performed per general histological procedures.

Bulk RNA sequencing

Colonies were imaged using a Leica DMI3000B microscope using a Leica DFC3000G camera with the latest Leica imaging suite to identify TOMATO positive and negative colonies grown as in the CFU-F assay section to day 10. Colonies were prepared using Corning 10mm cloning cylinders and vacuum grease to provide single colony isolation. RLT buffer was pipetted directly into the cloning cylinder and then transferred to ethanol for processing using the RNeasy Micro kit (QIAGEN). RNA was provided to the Duke Center for Genomic and Computational Biology core for quality assessment and sequenced 50bp single-end read on an Illumina HiSeq4000.

RNA-seq data was processed using the TrimGalore (https://github.com/FelixKrueger/TrimGalore) toolkit which employs Cutadapt to trim low-quality bases and Illumina sequencing adapters from the 3' end of the reads.(Martin, 2011) Only reads that were 20nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCm38v73 version of the mouse genome and transcriptome using the STAR RNA-seq alignment tool.(Dobin et al., 2013; Kersey et al., 2012) Reads were kept for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled using the HTSeq tool.(Anders et al., 2015) Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential gene expression was carried out using the DESeq2 Bioconductor package with the R statistical programming environment.(Huber et al., 2015; Love et al., 2014; R Core Team, 2013) The false discovery rate was calculated to control for multiple hypothesis testing. Biological thresholds were determined by taking the log2 expression value plotted on a histogram. A bimodal distribution is observed for low expressed genes

followed by a larger Guassian distribution. The split point between these was used as a value cutoff for biological relevance.

Gene Ontology Analysis

Gene ontology analysis was performed using geneontology.org Analysis Type: PANTHER Overrepresentation test released 02-24-2021. Annotation version and release date: release date 01-01-2021 and version 10.5281/zenodo.4437524 for biological processes using *Mus musculus* as the reference. Bulk RNA sequencing data was organized into log fold changes >1 and p value <0.05 for input into the enrichment analysis. A negative log fold change in the dataset represents higher expression in TOMATO positive colonies while positive log fold change represents higher expression in TOMATO negative colonies. Test type: Fisher's exact. Correction: Calculate False Discovery Rate.(Mi et al., 2019; The Gene Ontology Consortium, 2021; The Gene Ontology Consortium et al., 2000)

EdU Incorporation Assay

Colonies were grown as in CFU-F assays (in a 6-well plate at 500K cells per well instead of 15cm plate) until day 5. EdU staining was performed following the protocol of Click-iT Plus EdU Cell Proliferation Kit (Invitrogen – C10637). In short, EdU was added to growth media to allow a half media change of the cells. The cells were incubated at in hypoxia at 37C for 4 hours. Cells were fixed, permeabilized, and treated with staining solution. Cells were imaged using a Zeiss Axio Observer Z1 microscope at 5x magnification tiled across 70% of each well and stitched together using the latest Zen Blue software. Number of cells per colony calculations were performed at day 10 of plates grown in parallel to the EdU day 5 plate.

Statistical analysis

Statistical analyses were performed using two-tailed, unpaired Student's t-test. A p-value <0.05 was considered significant.

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FIGURES AND FIGURE LEGENDS

Figure 1.

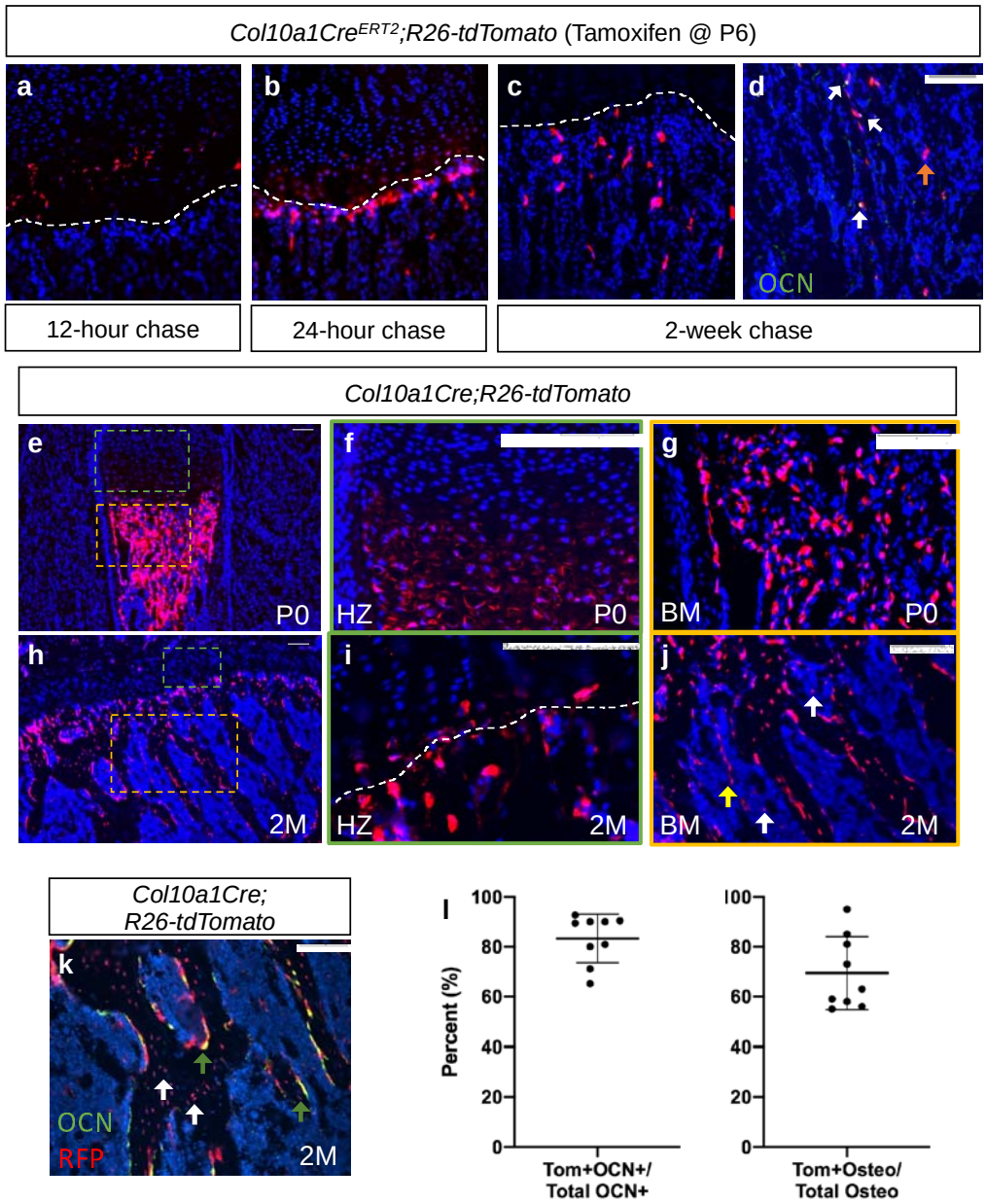


Figure 1. Hypertrophic chondrocytes are the primary source of osteoblasts/osteocytes in trabecular bone. Tibia sections of *Col10a1Cre^{ERT2};R26-tdTomato* mice injected with tamoxifen at post-natal day 6 and subsequently sectioned at (a) 12-hour chase, (b) 24-hour chase, and (c) 2-week chase. (d) OCN immunostaining of *Col10a1Cre^{ERT2};R26-tdTomato* bone sections following 2-week chase of TM. OCN^{+}, tdTOMATO^{+} descendants marked with white arrows. tdTOMTATO^{+} cells not associated with bone indicated by orange arrows. (e) Section of P0 *Col10a1Cre;R26-tdTomato* mouse tibia with higher magnifications regions of (f) hypertrophic zone (green box) and (g) bone marrow cavity (orange box). (h) Section of 2M old *Col10a1Cre;R26-tdTomato* tibia with higher magnifications of (i) hypertrophic zone (green box) and (j) bone marrow cavity (orange box). Non-bone lining TOMATO^{+} descendants marked with white arrows and potential vessel associated tdTOMATO^{+} descendants indicated by yellow arrow. Dotted line demarks the chondro-osseous junction. (k) OCN and RFP immunostaining of 2-month-old *Col10a1Cre;R26-tdTomato* tibia. OCN^{+}, tdTOMATO^{+} osteoblasts represented by green arrows and tdTOMATO^{+} osteocytes with white arrows. Quantifications shown in (l). Scale bars = 100um. N = 3 slides from 3 biologic replicates, SD ± 9.7% for OCN stain, SD ± 14.8% for osteocytes.

Figure 2.

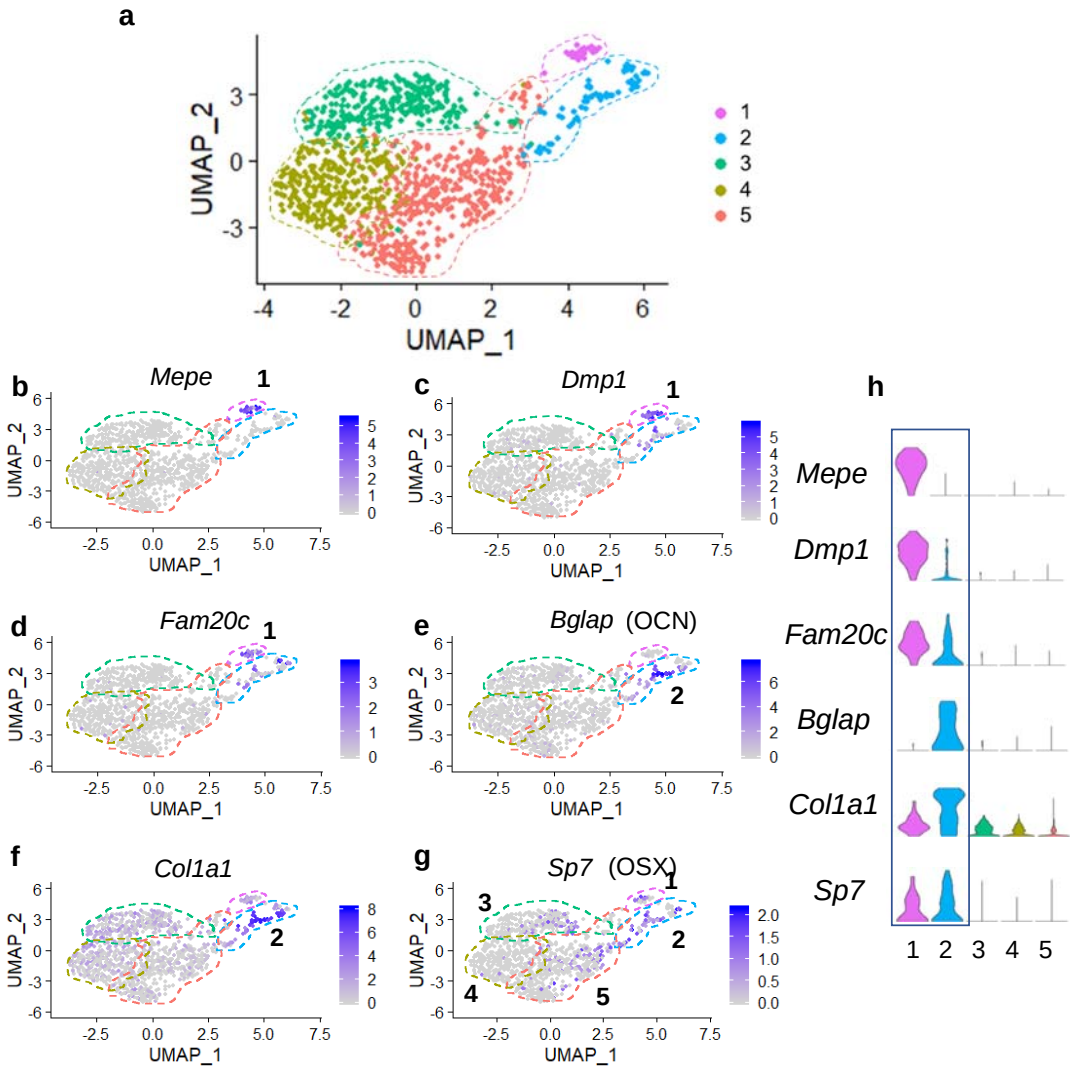


Figure 2. Single cell transcriptomics of hypertrophic chondrocyte descendants following FACS and 10x Genomics Sequencing. (a) UMAP shown in two-dimensional space produced using Seurat 3 package of R. (b-f) Feature plots of osteoblast specific genes identified in cluster 1 (b-d) and cluster 2 (e-f). (g) Feature plot of the osteoprogenitor associated gene, *Sp7*. (h) Violin plot representing the relative level of osteoblast associated gene expression (b-g).

Figure 3.

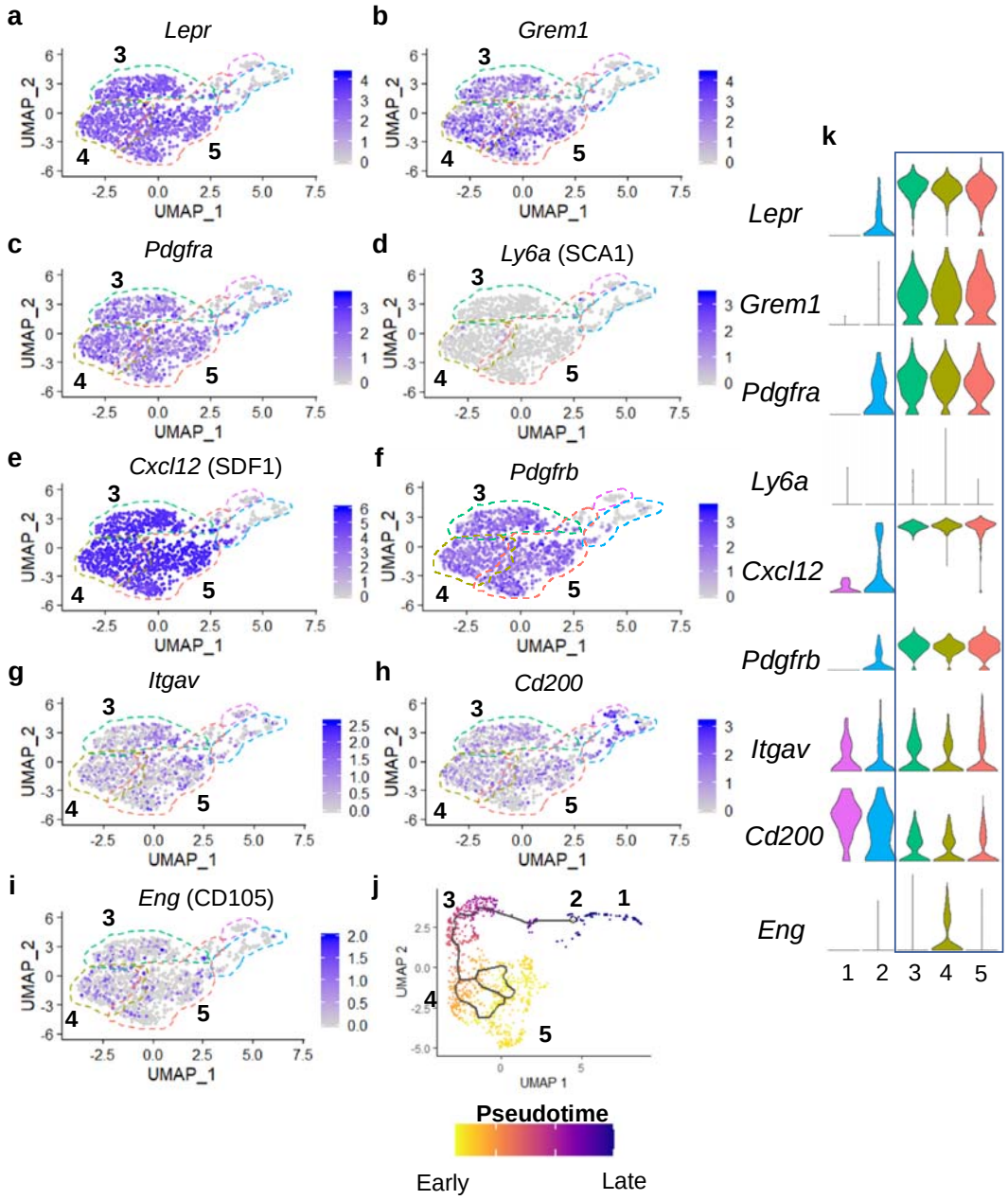


Figure 3. Many hypertrophic chondrocyte derived cells express genes associated with skeletal stem and progenitor cells (SSPCs). (a-f) Feature plots of genes previously identified as SSPC markers in genetic mouse models. (g-i) Feature plots of genes previously identified as SSPC markers for use in flow cytometry and FACS. (j) Monocle 3 trajectory analysis with clusters noted throughout pseudotime. (k) Violin plots of SSPC associated genes in (a-i).

Figure 4.

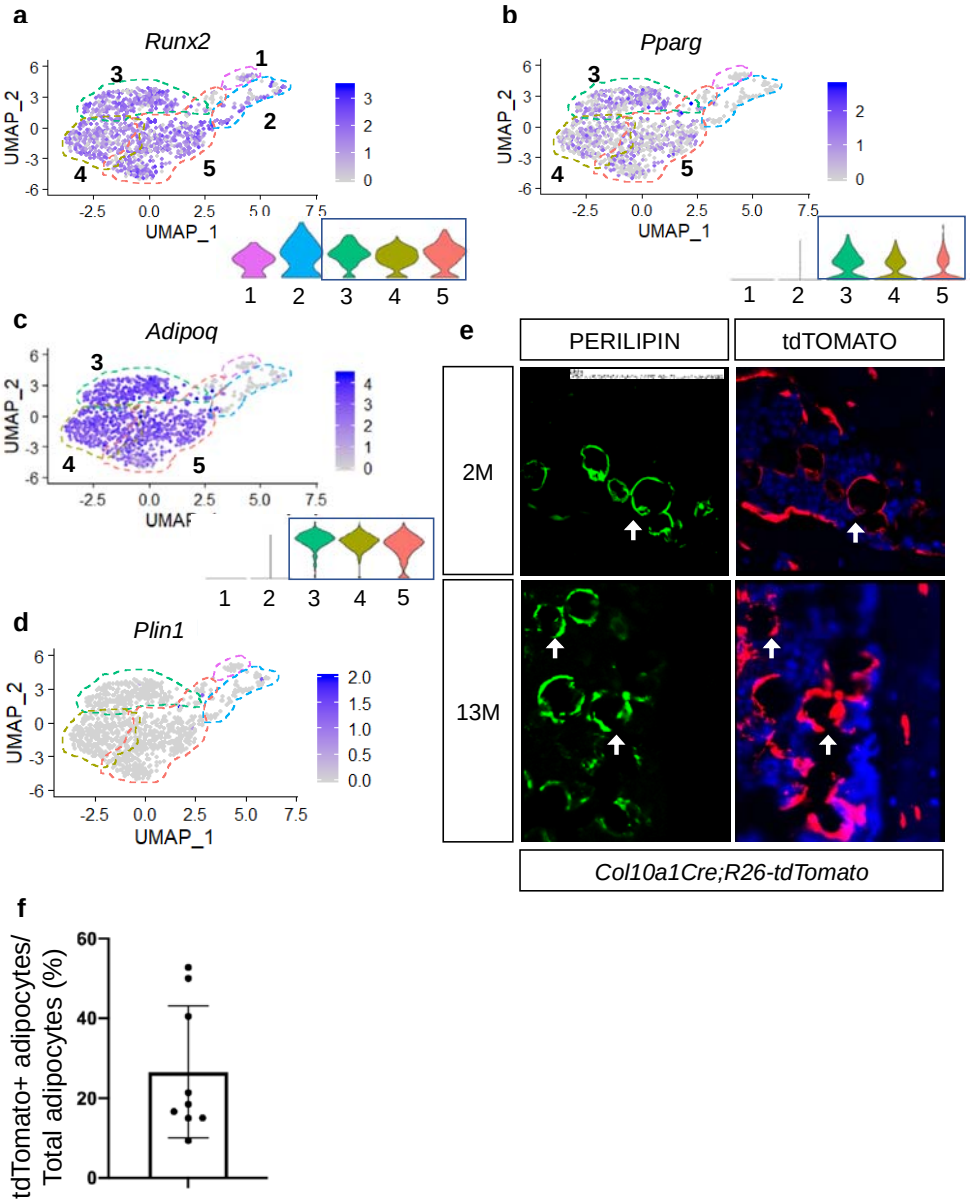
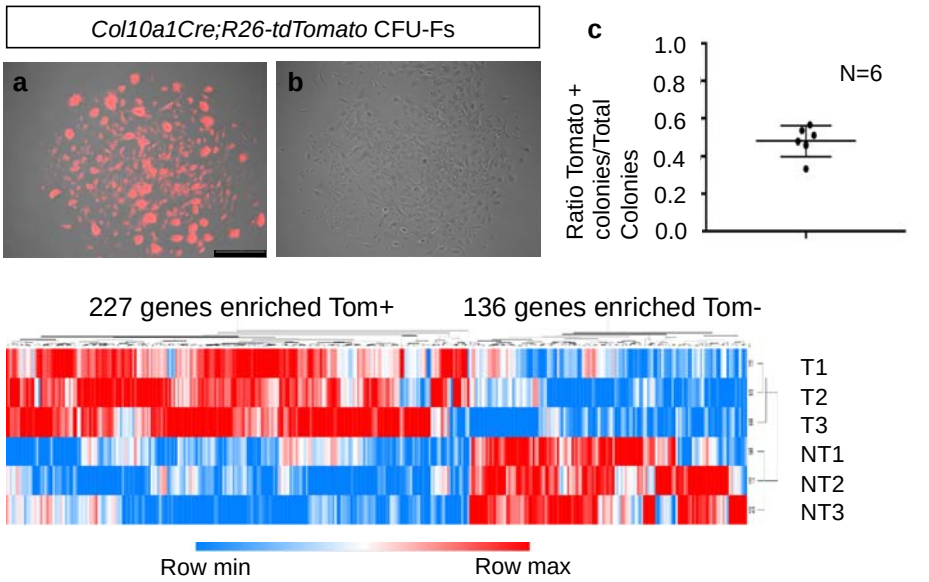


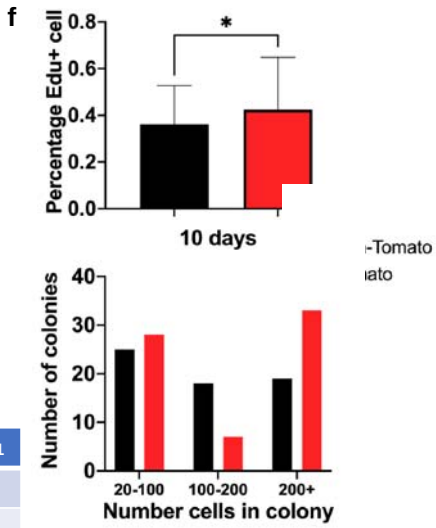
Figure 4. Hypertrophic chondrocyte derived SSPCs exhibit osteogenic and adipogenic differentiation capacities. (a) Feature plot and violin plot of the osteoblast specification gene, *Runx2*. (b-c) Feature plots and violin plots of the adipogenic specification gene, *Pparg, and adipogenic associated gene, Adipoq*. (d) Feature plot indicating a lack of expression of the mature lipid laden adipocyte gene, *Perilipin*. (e) Immunostaining for PERILIPIN in *Col10a1Cre;R26-tdTomato* mice at 2M and 13M of age. PERILIPIN^{+}, tdTOMATO^{+} adipocytes noted with white arrows. Scale bar = 100um (f) Quantification of PERILIPIN^{+}, tdTOMATO^{+} adipocytes at 13M of age in (e). N = 3 slides from 3 biologic replicates, SD ± 16.2%

Figure 5.

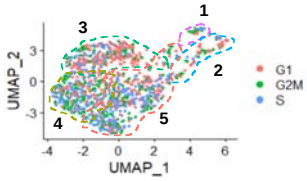


e

GO Term (T+)	Fold Enrichment	P-Value
Cell population proliferation	3.35	6.76E-06
Regulation of vasculature development	3.61	3.29E-04



g



	Cluster 5	Cluster 4	Cluster 3	Cluster 2	Cluster 1
G1/G0	37.04%	36.49%	58.56%	56.63%	43.75%
S/G2/M	62.96%	63.51%	41.44%	43.37%	56.25%

Figure 5. CFU-Fs derived from hypertrophic chondrocytes are similar to CFU-Fs derived from other cell sources, however contain SSPCs with enhanced proliferative capacities. (a-c) CFU-Fs derived from hypertrophic chondrocytes (tdTOMATO^{+}) (a) and those derived from other cell sources (tdTOMATO^{-})(b) are established and develop at similar frequencies (c). Scale bar = 500um (d) Heat-map from bulk RNA-seq data from 3 tdTOMATO^{+} CFU-Fs and 3 TOMATO^{-} CFU-Fs. (e) Gene ontology terms associated with tdTOMATO^{+} CFU-Fs. (f) EdU incorporation within tdTOMATO^{+} and tdTOMATO^{-} CFU-Fs (top) and differences in cell numbers between tdTOMATO^{+} and tdTOMATO^{-} CFU-Fs (bottom). N= 2 technical replicates of 3 biologic replicates, SD Tomato^{-} ± 16.5%, SD Tomato^{+} ± 22.3%, p-value = 0.044. (g) Cell cycle analysis of the SSPCs associated with clusters 1-5 using Seurat 3.

Figure 6.

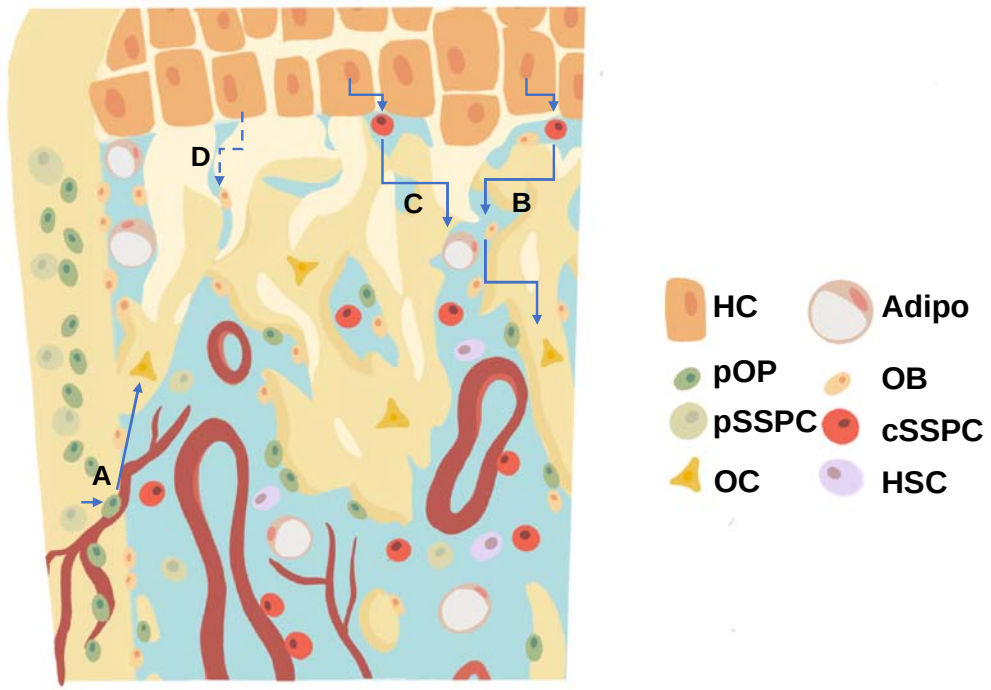


Figure 6. Revised model for the generation of trabecular/endocortical bone associated osteoblasts/osteocytes from multiple sources. (a) Perichondrial or periosteal SSPCs/osteoprogenitors (pSSPC/pOP) migrate into the marrow utilizing blood vessels and possess the ability to further differentiate into osteoblasts/osteocytes (OB/OC). (b-c) Hypertrophic chondrocytes (HC) dedifferentiate into chondrocyte derived SSPCs (cSSPC) with the capacity to generate osteoblasts/osteocytes (b) and/or adipocytes (Adipo) (c). (d) Data presented does not rule out the potential for the transdifferentiation of hypertrophic chondrocytes directly into osteoblasts/osteocytes. HSC= hematopoietic stem cells.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Supplemental Figure 1.

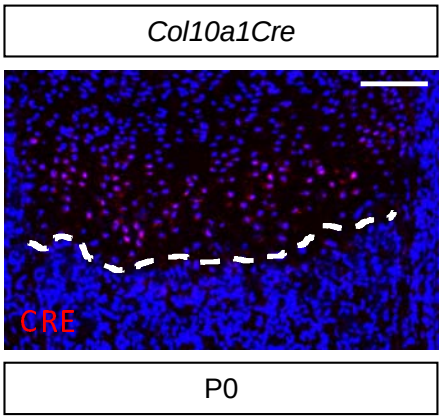


Figure S1. CRE expression is restricted to hypertrophic chondrocytes in *Col10a1Cre* mice. CRE immunostaining of P0 bone sections from *Col10a1Cre* mouse line. Chondro-osseous junction represented by white dotted line. Scale bar = 100um.

Supplemental Figure 2.

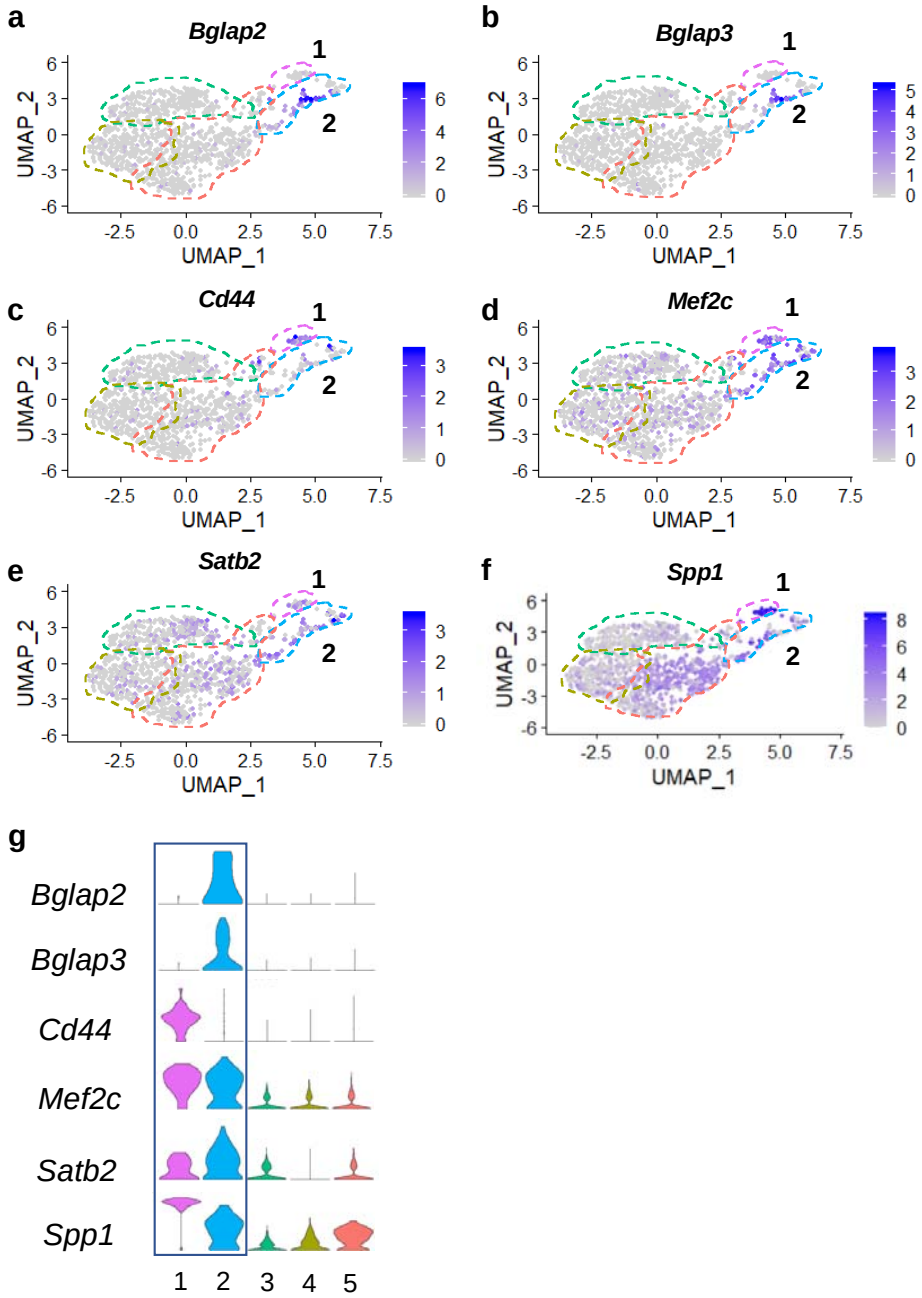


Figure S2. Additional osteoblast associated genes observed in clusters 1 and 2. (a-f) Feature plots of genes associated with osteoblasts were observed in clusters 1 and 2 with minimal expression in clusters 3-5. (g) Violin plots representing relative gene expression levels in (a-f) among the 5 identified clusters.

Supplemental Figure 3.

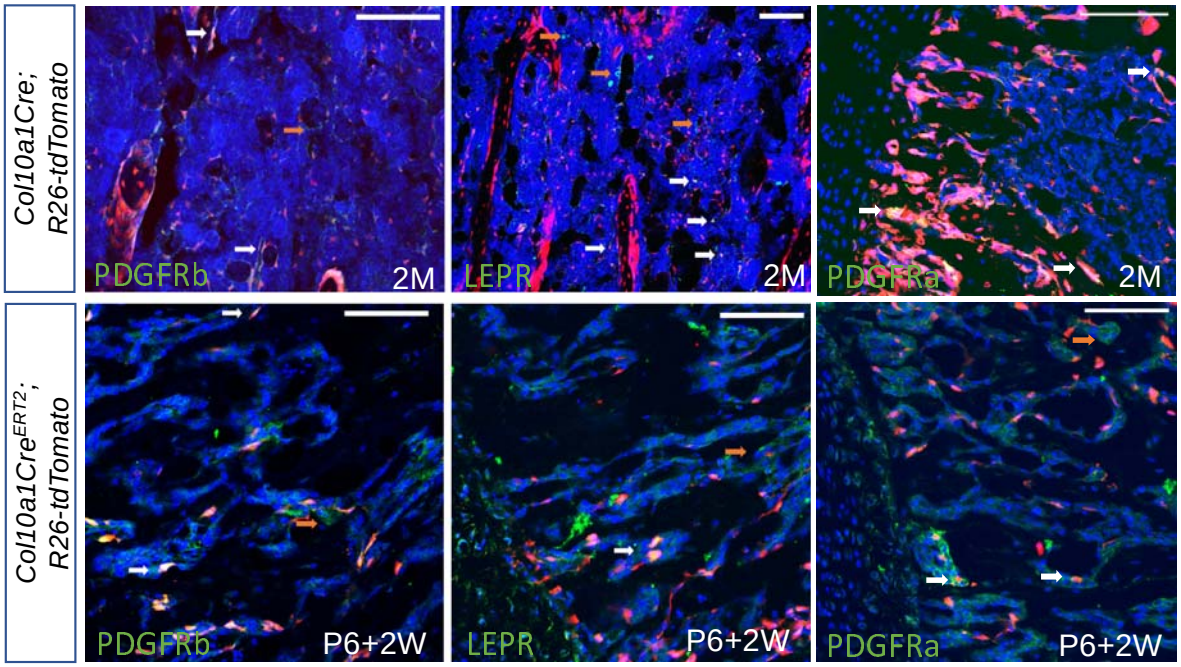


Figure S3. SSPC associated proteins are detected in hypertrophic chondrocyte derived marrow associated cells. Immunostaining of SSPC associated proteins in both 2M old *Col10a1Cre;Rosa26-tdTomato* bone sections (top) and bone sections from *Col10a1CreERT2;Rosa26-tdTomato* mice injected with tamoxifen at P6 and chased for 2 weeks (bottom). Representative co-labeled (LEPR, PDGFRa, or PDGFRb and tdTOMATO) cells are identified by white arrows. Representative cells positive for each respective antibody that are tdTOMATO^{-} are identified by orange arrows. Scale bars = 100um.

Supplemental Figure 4.

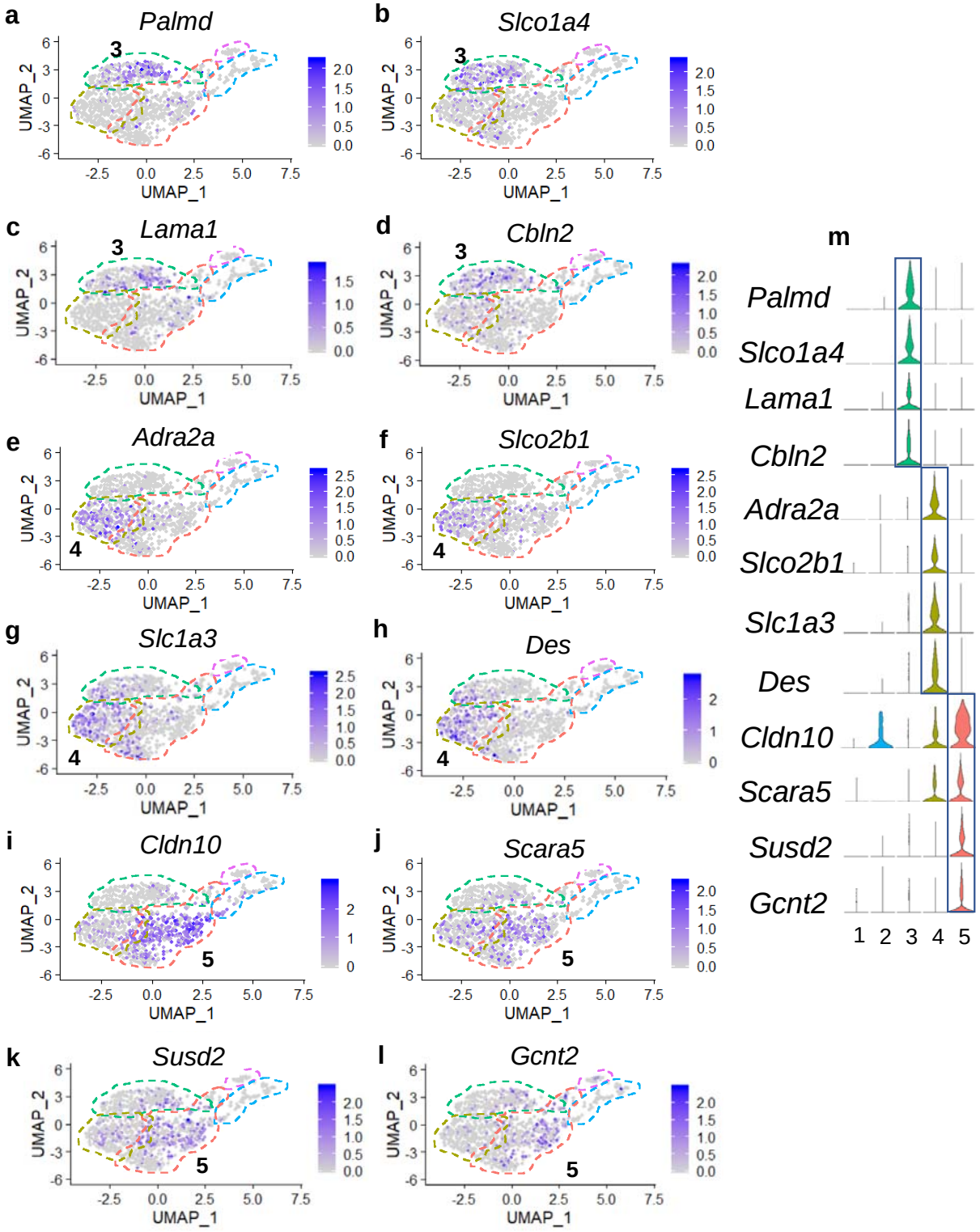


Figure S4. Differentially expressed genes mostly unique to clusters 3, 4, and 5. (a-j) Feature plots of genes identified by differential gene expression in cluster 3 (a-d), cluster 4 (e-h), and cluster 5 (i-l). (m) Violin plots of differentially expressed genes represented in (a-l).

Supplemental Figure 5.

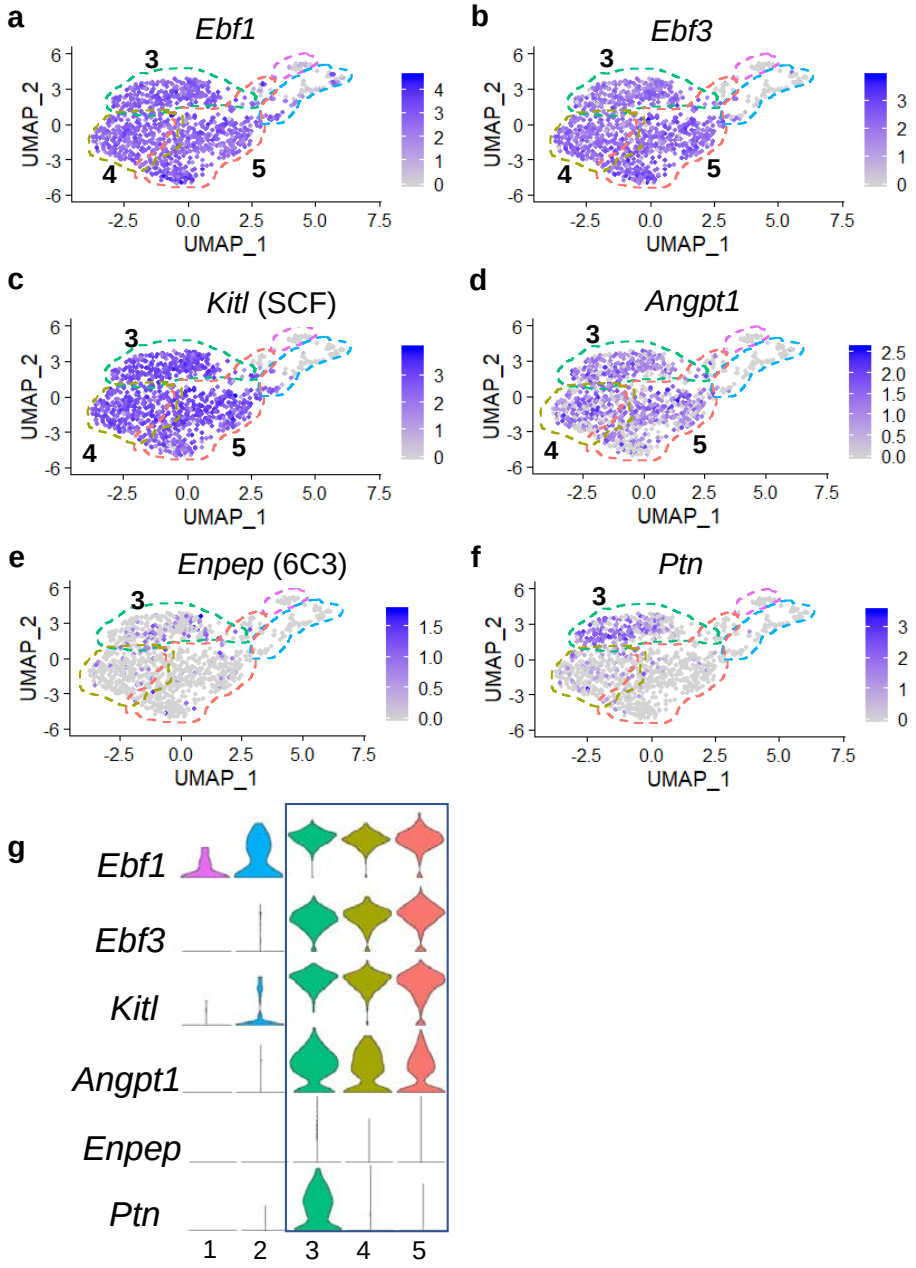


Figure S5. Several hematopoietic supportive genes are expressed in SSPC clusters 3, 4, and 5. (a-d) Multiple genes associated with support of hematopoietic cells are expressed throughout clusters 3, 4, and 5. (e-f) Two genes that maintain HSC stemness are more uniquely expressed in cells of cluster 3. (g) Violin plots of genes in (a-f).

Supplemental Figure 6.

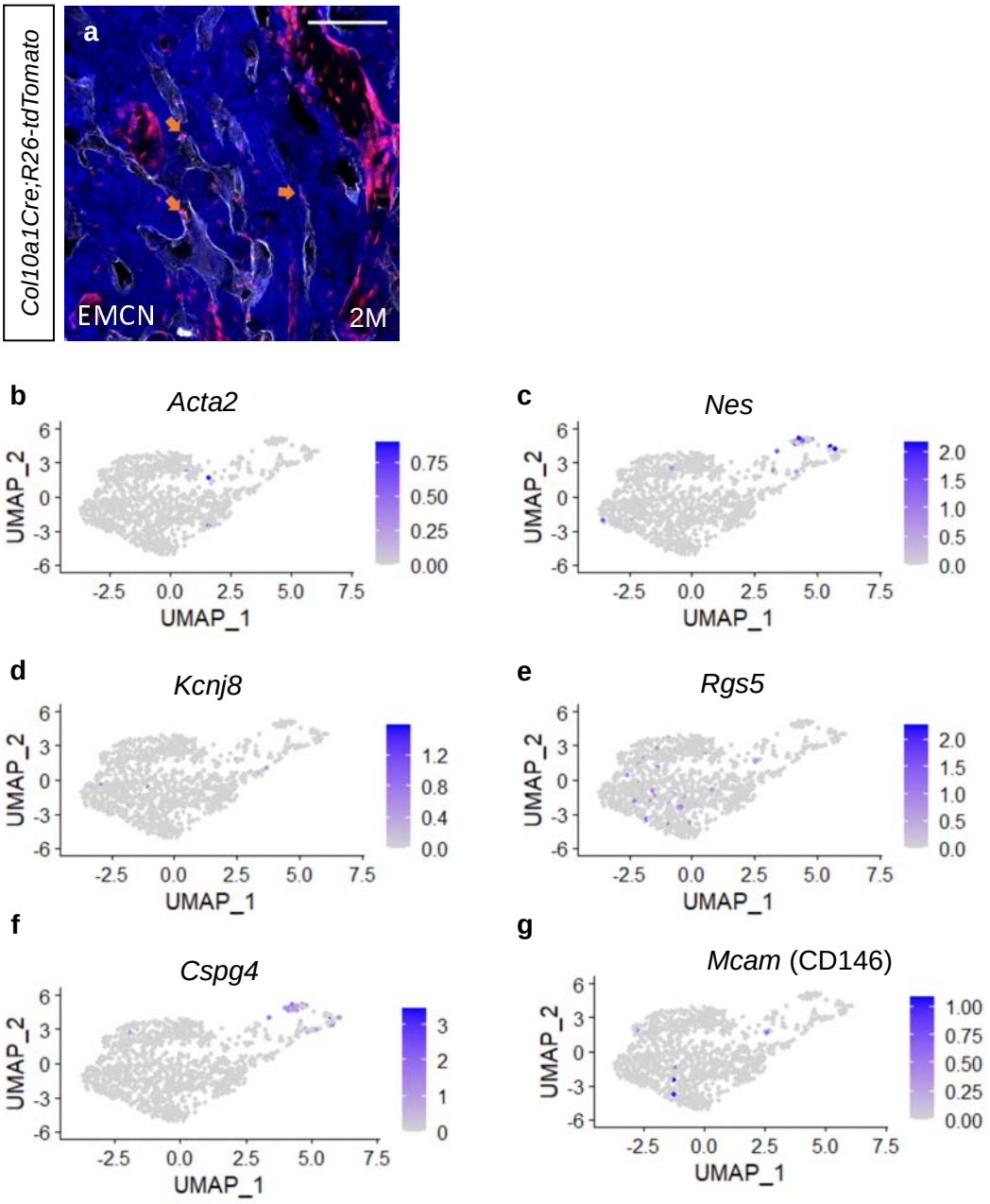


Figure S6. Hypertrophic chondrocyte derived cells can associate with blood vessels; however, do not express genes associated with pericytes or vascular smooth muscle cells. (a) Immunostaining for endothelial cell associated protein, ENDOMUCIN, on 2M old *Col10a1Cre;Rosa26-tdTomato* bone sections show adjacent tdTOMATO^{+} cells indicated by orange arrows. Feature plots for (b-c) vascular smooth muscle cell associated genes and (d-g) pericyte associated genes indicate that these genes are rarely expressed in any cell cluster. Scale bar = 100um.

Supplemental Figure 7.

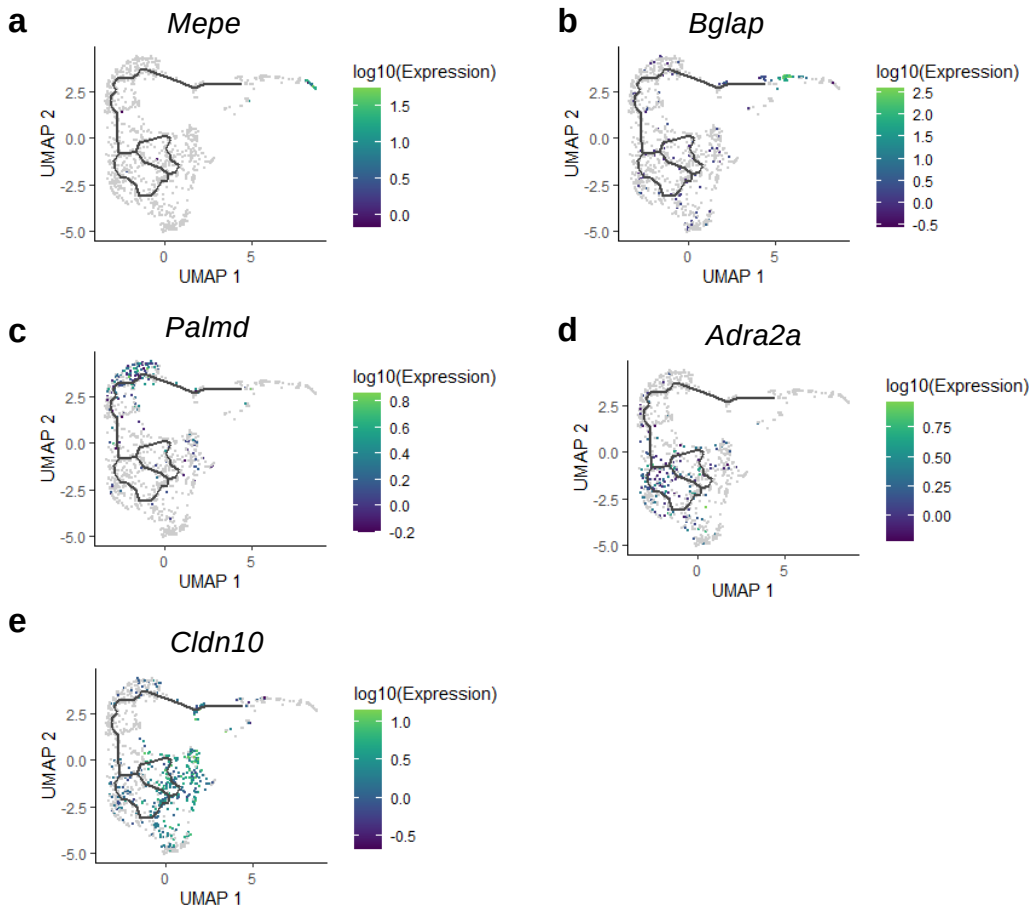


Figure S7. Monocle 3 trajectory map overlayed with genes identified in each cluster. (a) Cluster 1 associated gene *Mepe*. (b) Cluster 2 associated gene *Bglap*. (c) Cluster 3 associated gene *Palmd*. (d) Cluster 4 associated gene *Adra2a*. (e) Cluster 5 associated gene Cldn10.

Supplemental Figure 8.

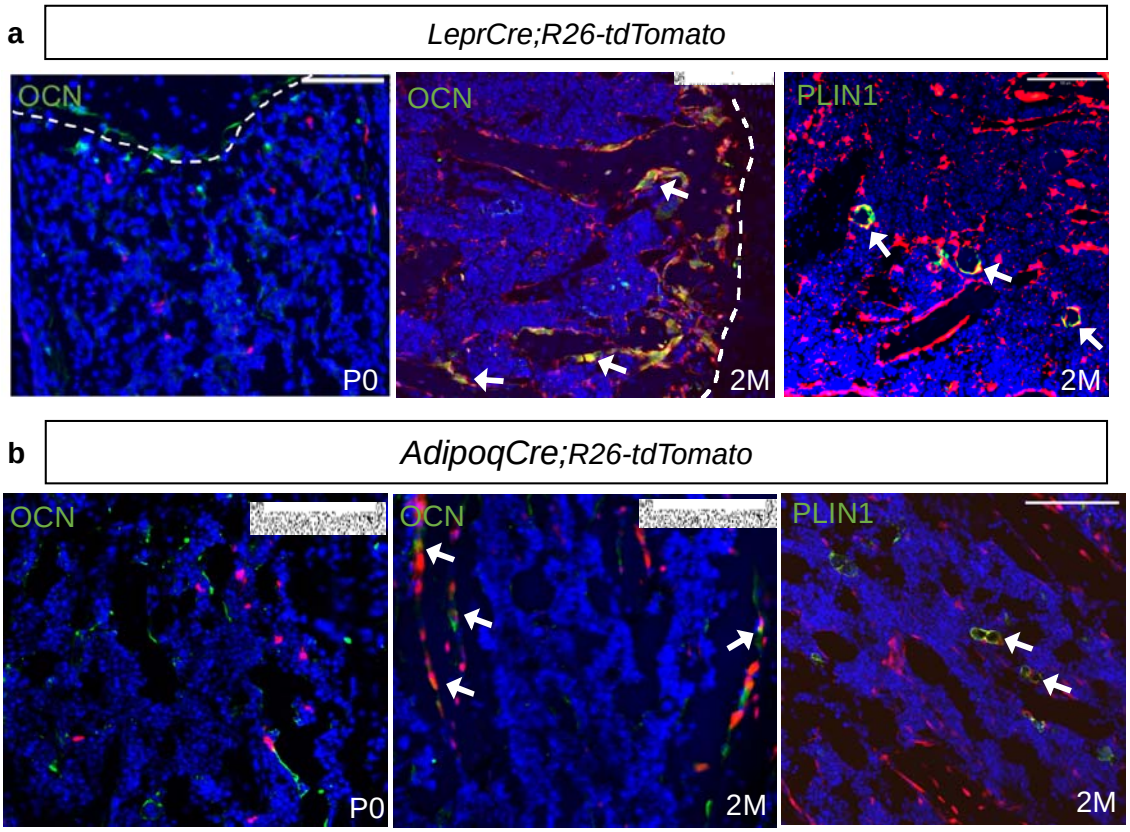


Figure S8. *LeprCre; R26-tdTomato* and *AdipoqCre; R26-tdTomato* mice exhibit reporter expression in marrow associated cells, osteoblasts, and adipocytes with age. (a) Immunostaining of bone sections from *LeprCre;R26-tdTomato* mice exhibit tdTOMATO$^+$ marrow associated cells, however do not display tdTOMATO$^+$, OCN$^+$ osteoblasts at P0. Immunostaining at 2M of age reveals both tdTOMATO$^+$, OCN$^+$ osteoblasts and tdTOMATO$^+$, PERILIPIN$^+$ adipocytes. (b) Immunostaining of bone sections from *AdipoqCre;R26-tdTomato* mice also exhibit tdTOMATO$^+$ marrow associated cells, however do not display tdTOMATO$^+$, OCN$^+$ osteoblasts at P0. Immunostaining at 2M of age reveals both tdTOMATO$^+$, OCN$^+$ osteoblasts and tdTOMATO$^+$, PERILIPIN$^+$ adipocytes.

Supplemental Figure 9.

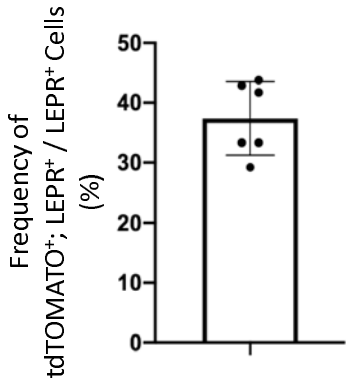


Figure S9. Immunostaining analysis of LEPR$^+$ and TOMATO$^+$ marrow associated cells. Immunostaining for LEPR on bone sections from 2M old *Col10a1Cre;R26-tdTomato* mice exhibit 37.4% LEPR$^+$; tdTOMATO$^+$ as compared to total LEPR+ marrow associated cells. N= 2 technical replicates of 3 biologic replicates, SD ± 6.2%.

Supplemental Figure 10.

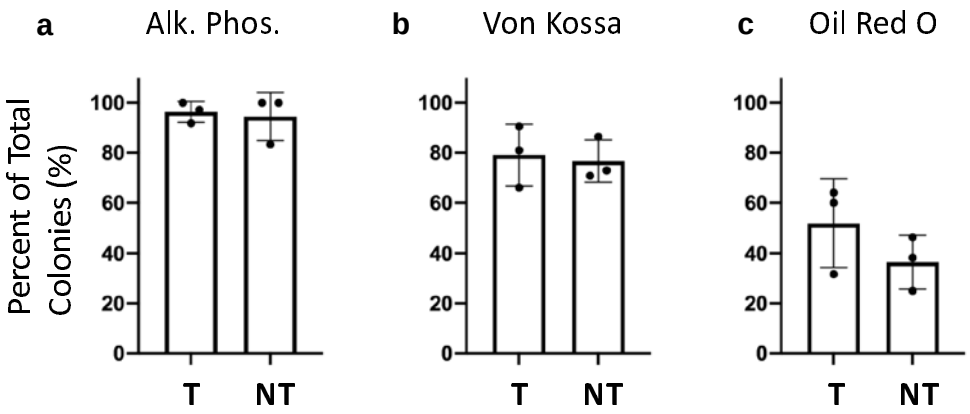


Figure S10. *In vitro* differentiation of colonies reveals relatively similar osteogenic and adipogenic differentiation capacities. (a-b) CFUs from *Col10a1Cre;R26-tdTomato* bone marrow differentiate into osteoblasts stained with alkaline phosphatase (a) and von kossa (b) (CFU-OB). alkaline phosphatase: SD Tomato^{+} (T) ± 4.2%, SD Tomato^{-} (NT) ± 9.6%, p-value = 0.63. von kossa: SD Tomato^{+} ± 12.3%, SD Tomato^{-} ± 8.4%, p-value = 0.67. (c) CFUs from *Col10a1Cre;R26-tdTomato* marrow differentiate into adipocytes stained with oil red o (CFU-AD). SD Tomato^{+} ± 17.6%, SD Tomato^{-} ± 10.8%, p-value = 0.11.