Osteocytes regulate senescence of bone and bone marrow

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Abstract
The skeletal system contains a series of sophisticated cellular lineages arisen from the mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), that determine the homeostasis of bone and bone marrow. Here we reasoned that osteocyte may exert a function in regulation of these lineage cell specifications and tissue homeostasis. Using a mouse model of conditional deletion of osteocytes by the expression of diphtheria toxin subunit α (DTA) in dentin matrix protein 1 (DMP1) positive osteocytes, we demonstrated that partial ablation of DMP1 positive osteocytes caused severe sarcopenia, osteoporosis and degenerative kyphosis, leading to shorter lifespan in these animals. Osteocytes reduction altered mesenchymal lineage commitment resulting in impairment of osteogenesis and induction of osteoclastogenesis. Single cell RNA sequencing further revealed that hematopoietic lineage was mobilized towards myeloid lineage differentiation with expanded myeloid progenitors, neutrophils and monocytes, while the lymphopoiesis was impaired with reduced B cells in the osteocyte ablation mice. The acquisition of a senescence-associated secretory phenotype (SASP) in both osteoprogenic and myeloid lineage cells was the underlying cause. Together, we showed that osteocytes play critical roles in regulation of lineage cell specifications in bone and bone marrow through mediation of senescence.

Key words: Osteocytes, SASP, osteogenesis, osteoclastogenesis, myelopoiesis
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Introduction

The skeletal system is an elaborate organ mainly containing bone, bone marrow and other connective tissues, whose function includes movement, support, hematopoiesis, immune responses and endocrine regulation (Karsenty and Ferron 2012; Katsnelson 2010; Quarles 2011). The skeletal system hosts at least more than 12 types of cell lineages arisen from HSCs and MSCs (Mendez-Ferrer et al. 2010). During hematopoiesis, HSCs give rise to lymphoid and myeloid lineage cells including B cell, neutrophil and monocytes as well as osteoclasts. Meanwhile, MSCs differentiate into osteoblastic lineage cells, bone marrow adipocytes and form fibroconnective tissues. The sophisticated processes of differentiation and interaction of these cell lineages are critical not only to skeletal development, but also to the integrity of hematopoietic, immune and endocrine systems (Mendez-Ferrer, et al. 2010; Le, Andreeff, and Battula 2018; Yu and Scadden 2016). During aging, these cell lineage commitments change rigorously and cause imbalance between myeloid-lymphoid hematopoiesis and adipo-osteogenic differentiation (Chen et al. 2016; Sinha et al. 2022), which lead to the increased myelopoiesis and adipogenesis as opposed to lymphopoiesis and osteogenesis. While the complex communications between these cell lineages have been documented, it is still unclear what determines these cell lineages to survive and how their cell fates are maintained during development and aging. It has been speculated that cellular senescence, characterized by cell proliferation arrest, altered metabolism and apoptosis resistance (Gorgoulis et al. 2019; Tchkonia et al. 2013), may be responsible for the regulation of lineage cell fates. However, the precise role in aging and age-related diseases remains unclear.

Osteocytes, as the long living terminally differentiated cells and the most abundant cells within the bone matrix (Tresguerres et al. 2020), play vital roles in maintaining the skeletal homeostasis. Apart from mechanical transduction (Long 2011; Sato et al. 2020), osteocytes have been shown to regulate bone formation, bone resorption, bone marrow hematopoiesis (Asada et al. 2013; Azab et al. 2020; Fulzele et al. 2013; Xiao et al. 2021) and generate endocrine signals to mediate function of other organs (Razzaque 2009; Fulzele et al. 2017; Cain et al. 2012). Osteocytes regulate both the osteoblast and osteoclast activities during bone remodeling (Delgado-Calle and Bellido 2022; Tresguerres, et al. 2020). Sclerostin, one of the key inhibitors of Wnt signaling pathway, is mainly produced by osteocytes (Tresguerres, et al. 2020). NO and PGE2 secretion by osteocyte in respond to mechanical stimulation have anabolic effects on osteoblasts (Rochefort, Pallu, and Benhamou 2010). Receptor activation of nuclear factor-κ B ligand (RANKL), the osteoclast differentiation factor, is mainly produced by osteocytes (Nakashima et al. 2011). Osteocytes regulate neutrophil development through secretion of soluble factors like IL19 (Xiao, et al. 2021) and osteocytes can also regulate myelopoiesis through Gsα-dependent and independent pathways (Fulzele, et al. 2013; Azab, et al. 2020). In addition, studies have shown that aging is associated with dysfunction of osteocytes. Degeneration of osteocytes lacunacanalicular network had been observed in in older adult (Busse et al. 2010) and in the aging animal model (Tiede-Lewis et al. 2017). Senescent osteocytes and their SASP
have been shown to contribute to age-related bone loss (Farr et al. 2016; Kim et al. 2020). Together, current data suggest that osteocyte is a single cell that co-ordinate activities of bone and bone marrow during skeletal aging (Sfeir et al. 2022).

Here we hypothesize that co-ordination of bone and bone marrow homeostasis requires the presence of functional osteocytes. Reduction of osteocytes and their function may result in the detrimental impact in altering lineage cell fates and specifications in bone marrow. Using a mouse model of conditional deletion of osteocytes by the expression of DTA in DMP1 positive osteocytes, we showed that osteocytes regulated bone and bone marrow lineage cell specification. Ablation of osteocytes in these mice caused impairment of osteogenesis and lymphopoiesis, increased osteoclastogenesis and mobilization of myelopoiesis toward myeloid lineage differentiation with expanded myeloid progenitors, neutrophils and monocytes. These have resulted in the induction of accelerated skeletal aging. Mice with osteocyte ablation have severe sarcopenia, osteoporosis and kyphosis at the early stage of 13 weeks resulting in shorter lifespan. Together, we demonstrated that osteocytes play a critical role in regulation of the HSC and MSC lineage cell differentiations by mediation of senescence.

**Results**

**Mice with less osteocytes have severe osteoporosis, kyphosis, sarcopenia and shorter lifespan**

To delineate the role of osteocyte in skeletal tissue development and maturation, we established a mouse model based on diphtheria toxin subunit α-mediated cell knockout using the promoter of DMP1 (Breitman et al. 1990). The latter is a protein highly expressed in late-stage osteocytes but has been shown not to be essential for early skeletal development (Feng et al. 2003). The results showed that complete ablation of DMP1 positive osteocytes (osteocyte_\text{DMP1}^\text{DM1cre} \text{Rosa26}^{\text{em1Cin(SA-IRES-Loxp-ZsGreen-stop-Loxp-DTA)}}^\text{homozygotes} \text{DTA}^\text{ho}) caused lethality of mice before birth. This has led us to investigate the impact of partial ablation of osteocytes using Dmp1_\text{cre} \text{Rosa26}^{\text{em1Cin(SA-IRES-Loxp-ZsGreen-stop-Loxp-DTA)}}^\text{heterozygotes} \text{DTA}^\text{het}). Interestingly, Alizarin red/Alcian blue staining of whole mount skeleton at E19.0 showed no apparent differences of craniofacial, long bones or spines between WT and DTA_\text{het} mice (Figure 1 - figure supplement 1A). As shown in Figure 1A and B, DTA_\text{het} mice had more empty lacunae without the presence of osteocytes within cortical and trabecular bone matrix as compared to WT mice. Further, reduced dendrites were also observed in residual osteocytes of DTA_\text{het} mice (Figure 1C and D), indicating the impairment of osteocyte network. To define how osteocyte partial ablation was achieved, we performed the quantification of empty lacunae ratio of DTA_\text{het} mice at 13 weeks. About 80% empty lacunae was observed in DTA_\text{het} mice at 13 weeks which increased about 20% compared to 4 weeks (Figure 1 – figure supplement 1B and C), indicating diphtheria toxin (DT) had an accumulative effect with age in DTA_\text{het} mice. Together, these results indicated that although there was partial ablation of osteocyte_\text{DMP1} in DTA_\text{het} mice, the embryonic development of skeletal tissue appeared
Next, we investigated if reduction of osteocyte DMP1 in DTA\textsuperscript{het} mice had an impact of postnatal maturation of bone tissue. Micro-computed tomography (\(\mu\)CT) examination of the appendicular skeleton revealed a significant decrease in femur bone mineral density (BMD), bone volume fraction (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th), as well as greater trabecular separation (Tb.Sp) in DTA\textsuperscript{het} mice as compared to those in WT mice at 4 weeks (Figure 2A and B). Moreover, ablation of osteocytes also led to cortical bone loss with decreased cortical thickness (Ct.Th) and increased cortical porosity (Ct.Po) (Figure 2A and C). At 13 weeks, DTA\textsuperscript{het} mice exhibited more bone loss in both trabecular and cortical bone compared to those in WT mice (Figure 2D-G). The progressive bone loss was observed through the life of DTA\textsuperscript{het} mice. The phenotype observed is unique and gender insensitive (Figure 2 - figure supplement 1A-C). Similarly, \(\mu\)CT observation of axial skeleton also revealed the significant bone loss in vertebral bodies (Figure 2H and I, Figure 2 - figure supplement 1D and E). Furthermore, there was no increase of bone mass of vertebral bodies from 4 to 13 weeks in DTA\textsuperscript{het} mice (Figure 2J and K), suggesting the retardation of vertebral body maturation. At 13 weeks, obvious kyphosis occurred in DTA\textsuperscript{het} mice (Figure 2L) due to serve osteoporosis and vertebral body compression. Whole-body \(\mu\)CT scan revealed that there was giant increase of thoracic and lumbar curvature of DTA\textsuperscript{het} mice (Figure 2M). At the age of 30 weeks almost all of DTA\textsuperscript{het} mice developed severe kyphosis (Figure 2N). In consistent with development of kyphosis, gait analysis revealed that DTA\textsuperscript{het} mice at 4 weeks have abnormal steps when running (Figure 2 - figure supplement 2A and B). The font and hind stride length were much shorter in DTA\textsuperscript{het} mice (Figure 2 - figure supplement 2C). Also, the swing speed of DTA\textsuperscript{het} mice was much slower than WT mice (Figure 2 - figure supplement 2D, Video 1-6).

Whole body examination of DTA\textsuperscript{het} mice revealed there was a continual body weight loss and muscle weight loss (Figure 3A, B and C) from 4 weeks. Histology examination of gastrocnemius muscles revealed focal muscle atrophy with mild inflammation at 4 weeks (Figure 3D and E). Many myonuclei were mispositioned and became centralized as contrast to those in WT mice. No muscle fibrosis was observed. At 13 weeks, there was continual muscle atrophy, rimmed vacuoles and inclusion bodies were seen within the muscle fibers (Figure 3F-G). To preclude the direct target of DMP1 on muscle, we quantified the number of muscle fibers and the results showed that there was no reduction of numbers of muscle fibers after osteocyte ablation at 4 weeks (Figure 3 - figure supplement 1A) and 13 weeks in DTA\textsuperscript{het} mice as compared to the WT mice (Figure 3 - figure supplement 1B). Measurement of \(Dmp1\) expression in WT muscle showed that the level of \(Dmp1\) expression in muscle was very weak and far less than bone (Figure 3 - figure supplement 1C). Together these results suggested that DTA\textsuperscript{het} mice had systemic muscle atrophy and sarcopenia. It is most likely that sarcopenia was caused by the impairment of osteocyte-muscle crosstalk. Analyses of lifespan in these mice further revealed the average lifespan of
DTAhet mice was about 20-40 weeks, which was much shorter than WT mice (Figure 3H). Together, these data demonstrated that osteocytes ablation caused severe osteoporosis and kyphosis, as well as sarcopenia, which occurred at the very early stage compared to normal aging process. These age-related skeletal phenotypes combined with shortened lifespan demonstrated that osteocyte ablation led to the accelerated skeletal aging.

Ablation of osteocytes alters mesenchymal lineage commitment and promoted osteoclastogenesis

To explore the potential mechanism on why reduction of osteocytes has caused severe osteoporosis and kyphosis, RNA sequencing was performed on whole bone with bone marrow flushed out from DTAhet and WT mice at 4 weeks. Selected skeleton related gene ontology (GO) analysis revealed that downregulated genes by osteocyte ablation were enriched in ossification, osteoblast differentiation, positive regulation of osteoblast differentiation, endochondral ossification and bone morphogenesis (Figure 4 - figure supplement 1A and Supplementary File 1). Heatmap of significantly differentiated genes (fold change > 2.0-fold, WT average FPKM > 10, FDR < 0.05) and subsequent RT-qPCR verified that genes that are critical for osteogenesis, including Alpl, Bglap, Col1a1, Spp1, Sp7 and Runx2, were affected by the ablation of osteocytes (Figure 4 - figure supplement 1B and C). Also, gene set enrichment analysis (GSEA) revealed that osteogenesis related pathways including Wnt signaling pathway, Hedgehog signaling pathway and Notch signaling pathway were downregulated (Figure 4 – figure supplement 1D, E and F). In addition, numbers of osteoblasts and osteoid surface were remarkably reduced in DTAhet mice compared to WT mice (Figure 4A and B). Also, bone marrow fat accumulation in DTAhet mice was observed (Figure 4C and D). Together these results suggested that DTAhet mice displayed increased adipogenesis and decreased osteogenesis. To further evaluate the dynamics of bone formation in DTAhet mice, a 7-day dynamic histomorphometric analysis using calcein labeling was performed. The result showed that mineralized surface, mineral apposition rate (MAR) and bone formation rate (BFR) were significantly decreased in DTAhet mice (Figure 4E and F). Serum procollagen type 1 N-terminal propeptide (P1NP), a bone formation index, was also reduced after osteocyte ablation (Figure 4G). Intriguingly, in vitro osteogenesis showed that there were also decreased osteogenesis and mineralization in DTAhet mice as compared to WT mice at both time points of 4 and 13 weeks and the impairment of osteogenesis was greater in DTAhet mice at 13 weeks as compared to 4 weeks (Figure 4H and I). And the mRNA level of osteogenic markers at 4 weeks including Alpl, Bglap, Runx2 was also decreased (Figure 4J).

In the aspect of osteoclastogenesis, histomorphometry analysis revealed that osteoclast numbers and surface were significantly increased after osteocytes deletion (Figure 4K and L). Circulatory RANKL was also increased in DTAhet mice (Figure 4M). In contrast, circulatory osteoprotegrin (OPG), a decoy receptor of RANKL, was decreased, leading to the elevated ratio of RANKL/OPG (Figure 4M). Serum collagen
type I c-telopeptide (CTX), a bone resorption index, was also significantly augmented in DTA<sup>het</sup> mice compared to WT mice (Figure 4N), which implicated a high level of osteoclast activity of DTA<sup>het</sup> mice in vivo. Also flow cytometry analysis revealed that there was slightly increases (less than 1%) of osteoclast progenitors (B220<sup>-</sup>CD11b<sup>+</sup>Ly-6C<sup>hi</sup>) in DTA<sup>het</sup> mice at 4 weeks as compared to WT mice (Figure 4 - figure supplement 1G and H). To assess the effects of osteocyte ablation on osteoclastogenesis, bone marrow derived macrophages (BMMs) from WT and DTA<sup>het</sup> mice at both time points of 4 and 13 weeks were collected and plated at the same density for the examination of osteoclastogenesis in vitro. The results showed that osteoclastogenesis was increased in DTA<sup>het</sup> mice as compared to WT mice at both time points (Figure 4O and Q). Interestingly, the induction of osteoclastogenesis was greater in DTA<sup>het</sup> mice at 13 weeks as compared to 4 weeks (Figure 4P and Q), suggesting the time-dependent accumulative effect of osteoclastogenesis in DTA<sup>het</sup> mice. Also, the expression of the signature genes of osteoclasts including Acp5, Calcr, Ocstamp at the mRNA level was significantly upregulated in DTA<sup>het</sup> mice (Figure 4Q). Together, osteocytes ablation impaired osteogenesis and promoted osteoclastogenesis.

Alteration of hematopoietic lineage commitment by osteocyte ablation

As a part of the skeletal system, bone marrow has its vital functions in maintaining bone homeostasis (Divieti Pajevic and Krause 2019; Fulzele, et al. 2013; Asada, et al. 2013). HSCs give rise to lymphoid and myeloid lineage cells to establish the hematopoietic and immune system. To gain a full insight into the role of osteocyte in bone marrow homeostasis, single cell RNA sequencing (scRNA-seq) was performed using 10× Genomics Chromium platform. After rigorous quality control, gene expression data from 26562 cells (13835 and 12727 cells from 4-week littermate WT and DTA<sup>het</sup> mice respectively) were compiled for clustering analysis, and there revealed 10 distinct populations visualized with uniform manifold approximation and projection (UMAP) embeddings (Figure 5A, B and C). These 10 distinct populations included B cell, hematopoietic stem cell and progenitor cell (HSPC), megakaryocyte, neutrophil, erythrocyte, monocyte, dendritic cell (DC), macrophage, T cell and MSC (Figure 5A and C). Proportion analysis revealed a significant expansion of neutrophils in DTA<sup>het</sup> mice (Figure 5D and E). Also, the number of B cells was significantly less in DTA<sup>het</sup> mice than that in WT mice (Figure 5D and E), which implicated that osteocytes ablation induced lymphoid-myeloid malfunction in the bone marrow. To further dissect the differences in the bone marrow development between two groups, RNA velocity was performed. The result showed that DTA<sup>het</sup> mice had stronger directionality of velocity vectors from the HSPC population to the neutrophil population compared to WT mice (Figure 5F), implying that osteocytes deletion altered HSPC differentiation. Meanwhile, myeloid trajectory analysis revealed that there was a significantly higher pseudotime density distribution in G4 cell (a subcluster of neutrophil) in DTA<sup>het</sup> mice (Figure 5G). In contrast, lymphoid trajectory analysis demonstrated a relatively lower pseudotime density distribution in pre-B cell and immature B cell (subclusters of B cell) in DTA<sup>het</sup> mice (Figure 5H).
To corroborate the results observed from scRNA-seq, flow cytometry and further analysis were performed after removing adherent cells (Figure 5 - figure supplement 1A and B). Although there was no significant change of HSCs (Lin-c-Kit\textsuperscript{-}Sca\textsuperscript{1}, LSK\textsuperscript{-} cell) numbers between DTA\textsuperscript{het} and WT mice (Figure 5 - figure supplement 2A and B), DTA\textsuperscript{het} mice demonstrated significantly increased number of short-term HSCs (ST-HSCs) with decreased number of long-term HSCs (LT-HSCs), indicating that HSCs in DTA\textsuperscript{het} mice bone marrow were mobilized (Figure 5 - figure supplement 2C and D). Further flow cytometry analysis revealed that the number of myeloid progenitors including common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP) and common monocyte progenitors (cMoP) were substantially increased after osteocyte ablation (Figure 5I and J, Figure 5 - figure supplement 2E and F), and megakaryocyte erythroid progenitors (MEP) numbers were decreased (Figure 5I and J). Meanwhile, total CD11b\textsuperscript{+} myeloid cells were also increased (Figure 5K and L) in DTA\textsuperscript{het} mice, in which both neutrophils and monocytes significantly expanded (Figure 5M and N, Figure 5 - figure supplement 2G and H). In addition, the proportion of common lymphoid progenitors (CLP) was not altered in DTA\textsuperscript{het} mice (Figure 5I and J), total B220\textsuperscript{+} lymphoid cells reduced remarkably after osteocyte ablation (Figure 5O and P), in which DTA\textsuperscript{het} mice showed a relatively lower proportion of early B cell (pro-B pre-B, immature B and transitional B cell) and a relatively higher proportion of late B cell (early mature B and late mature B) (Figure 5K and L), which suggested that B cell development was impaired along the immature B to mature B cell transition in DTA\textsuperscript{het} mice. As scRNA-seq revealed that neutrophils underwent a significant change after osteocyte ablation, neutrophil population were further reclusted into four subclusters from G1 to G4 (Figure 5 - figure supplement 3A and B) and G4 population was significantly increased in DTA\textsuperscript{het} mice compared to WT mice (Figure 5 - figure supplement 3C and D), which implied that osteocyte ablation accelerated neutrophil maturation. Consistent with this observation, neutrophil functions including activation, chemotaxis were all upregulated in DTA\textsuperscript{het} mice (Figure 5 - figure supplement 3E and F). Genes related to glycolysis and necroptosis were also upregulated (Figure 5 - figure supplement 3G and H), indicating that osteocyte ablation altered neutrophil functions. Together, these results demonstrated that osteocyte ablation altered hematopoietic lineage, characterized by the shift from lymphopoiesis to myelopoiesis.

**Senescence of osteoprogenitors and myeloid lineage cells leads to the accelerated skeletal aging**

Senescence occurred during development as a precise programmed cellular process, contributes to cell fate specification, tissue patterning and transient structure removal (Munoz-Espin and Serrano 2014; Rhinn, Ritschka, and Keyes 2019). Given that DTA\textsuperscript{het} mice had accelerated skeletal phenotype of aging including increased myelopoiesis, osteoporosis, kyphosis and sarcopenia with shortened lifespan, we hypothesized that osteocyte ablation may be associated with senescence of osteoprogenitors and myeloid lineage cells. ScRNA-seq revealed that total bone
marrow had increased senescence with a higher SASP score in DTA^{het} mice compared to WT mice (Figure 6A). DTA^{het} mice also had increased maturity in bone marrow reflected from RNA velocity (Figure 6B). Meanwhile, circulatory SASP including Tnf-α, Il1β and Il6 were also elevated in DTA^{het} mice (Figure 6C). Further scRNA-seq analysis uncovered that MSC, CMP, monocyte and its subcluster Ly6c2 monocyte, neutrophil and its subcluster G2, G3 and G4 had increased SASP scores (Figure 6D-G) and higher-level expressions of senescence related genes in DTA^{het} mice (Figure 6H). RT-qPCR also verified the elevated senescence with increased gene expressions including Cdkn2a and Cdkn1a in DTA^{het} mice (Figure 6I and J). Further, senescence associated β -galactosidase (SA-β Gal) staining revealed that there were obvious increased numbers of SA-βGal+ cell in the primary spongiosa, bone marrow and cortical bone in DTA^{het} mice compared to WT mice (Figure 6 - figure supplement 1A). Together, these results suggested that cell senescence of osteoprogenitors and myeloid lineage cells was associated with ablation of osteocyte.

Owing to the fact that osteoblast derived from mesenchymal stem cell lineage, we next investigated whether accumulation of osteoprogenitor cell senescence impaired osteogenesis. GO analysis revealed that downregulated genes after osteocyte ablation were enriched in ossification and biomineral tissue development (Figure 6 - figure supplement 1B), which was consistent with the finding of impaired osteoblast differentiation (Figure 4H-J). Meanwhile, the mRNA level of adipogenic markers including Adipoq, Fabp4, Pparγ and Cebpa significantly was increased (Figure 6 - figure supplement 1C), indicating increased adipogenesis and alteration of MSC commitment after osteocyte ablation. In addition, the mRNA levels of cartilage anabolism related genes (Col1a2, Acan, Sox9 and Prg4) and catabolism related genes (Mmp3, Mmp13, Adamts1 and Adamts5) were not significantly changed (Figure 6 - figure supplement 1D), indicating that chondrogenesis was not altered after osteocyte ablation. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the subcluster 2 and 4 of Ly6c2+ monocytes demonstrated the enrichment of osteoclast differentiation related genes after osteocyte ablation (Figure 6 - figure supplement 1E and F), which was corroborated in our enhanced in vitro osteoclast differentiation (Figure 4O-Q). Together, our data suggested that senescence in osteoprogenitors and myeloid lineage cells led to the impaired osteogenesis and increased osteoclastogenesis, respectively.

**Discussion**

In this study, we showed that co-ordination of bone and bone marrow homeostasis requires the presence of functional osteocytes. Reduction of osteocytes number results in the detrimental impact of lineage cell fate and specifications in bone and bone marrow. Partial ablation of osteocytes^{DMP1} caused severe sarcopenia, osteoporosis and degenerative kyphosis, which led to shorter lifespan. Acquisition of SASP in both osteoprogenic and myeloid lineage cells may be an underlying cause that led to the accelerated skeletal aging phenotype of impaired osteogenesis, increased osteoclastogenesis and myelopoiesis.
Sarcopenia usually occurs concurrently with osteoporosis during aging (Clynes et al. 2021). Our study has showed for the first time that osteocyte ablation caused severe sarcopenia and muscle atrophy. In consistent with our observation, previous studies have reported that osteocyte-specific ablation of Cx43 impaired muscle formation (Shen et al. 2015). Osteocyte-derived factors has also been shown to stimulate myogenic differentiation in vitro (Huang et al. 2017). On the contrary, specific deletion of Mbtps1 in osteocyte promotes soleus muscle regeneration and increase its size with age (Gorski et al. 2016). Sclerostin, an osteocyte-derived circulating protein, is negatively correlated with skeletal muscle mass (Kim et al. 2019). Previously there has been a study showing weak DMP1 expression in skeletal muscle fibers (Lim et al. 2017). This has led us to suggest that sarcopenia may be caused directly by the DMP1 expression in muscle. However, our histology finding of no obvious changes in the total number of nuclei of muscle in partial ablation of DMP1 positive osteocytes suggested that the sarcopenia and muscle atrophy phenotype is most likely caused by the disturbance of osteocyte-muscle crosstalk. Certainly, further studies based on a more specific osteocyte ablation model are needed to understand the link of osteocytes between osteoporosis and sarcopenia. Nevertheless, severe kyphosis observed in these osteocyte ablation mice, support our hypothesis of direct osteocyte-muscle crosstalk, as kyphosis is the direct result of the significant bone loss and sarcopenia (Wijshake et al. 2012; Woods et al. 2020).

Osteocytes regulate the process of bone resorption and coupled bone formation via secreting factors including sclerostin and RANKL (Tresguerres, et al. 2020; van Bezooijen et al. 2005; Nakashima, et al. 2011). Theoretically, osteocyte ablation may lead to lower expression of sclerostin and RANKL which in term increased osteogenesis and impaired osteoclastogenesis. However, our results demonstrated that osteocyte ablation impaired osteogenesis and induced osteoclastogenesis. In mice with partial ablation of osteocytes, expression of sclerostin was reduced but the serum RANKL was increased. In addition, osteogenesis related pathways including Wnt signaling pathway, Hedgehog signaling pathway and Notch signaling pathway were also downregulated. We speculated that induction of SASP in both osteoprogenitors and myeloid progenitors may account for the underlying cause. Senescent osteoprogenitors have reduced self-renewal capacity and predominantly differentiate into adipocytes as opposed to osteoblasts (Chen, et al. 2016; Li et al. 2017; Rosen et al. 2009). Consistently, our model indicated an increased adipogenesis after osteocyte ablation. Also, fat-induction factors inhibit osteogenesis during adipogenesis (Chen, et al. 2016). Thus, senescence accumulation in osteoprogenitors led to the impaired osteogenesis. As for enhanced osteoclastogenesis, besides the production of RANKL from osteogenic cells like osteocytes and osteoblasts (Nakashima, et al. 2011; Fumoto et al. 2014), other cells like adipocyte, T cell also secret RANKL to regulate bone metabolism (Yu et al. 2021; Hu et al. 2021; Djaafer et al. 2010; Takayanagi et al. 2000). Also, B cell can produce OPG to regulate RANKL/OPG axis (Li et al. 2007). In our model, increased adipogenesis, T cell expansion (data not shown) and
decreased B cell number may compensate for the altered RANKL/OPG axis. Intriguingly, we also found that even under in vitro condition in which osteocyte ablation is no longer existed, impairment of osteogenesis and induction of osteoclastogenesis were still observed. Our study has suggested that osteoprogenitors and BMMs have been primed by the altered bone microenvironment in DTA\textsuperscript{het} mice before in vitro differentiation. In support of this, previous studies have suggested that progenitor cells can receive a long-lasting impact from the in vivo local microenvironment, where these cells are situated. Isolation of cells for in vitro cell differentiation or even transferring cells to health mice would not alter their original in vivo phenotypes (Cao et al. 2020; Isaac et al. 2014; Ding et al. 2022; Edgar et al. 2021; Li et al. 2022).

Bone marrow, embedded in the skeletal system, has a close link with matrix-embedded osteocyte. Previous studies have reported that osteocyte regulates myelopoiesis via Gs\textsubscript{\alpha} dependent and -independent signaling (Fulzele, et al. 2013; Azab, et al. 2020). Recent study also reported that osteocyte mTORC1 signaling regulates granulopoiesis via secreted IL-19 (Xiao, et al. 2021). Meanwhile, sclerostin secreted by osteocyte adversely affects B cell survival (Horowitz and Fretz 2012). In our study, when osteocytes were partially depleted, myelopoiesis especially granulopoiesis was significantly induced, but B cell development was significantly impaired. Further studies demonstrated that HSCs were mobilized and shifted to myelopoiesis with increased CMP, GMP, eMoP and CD11b\textsuperscript{+} myeloid cells, in which monocytes and neutrophils were increased, and neutrophil function was also activated after osteocyte ablation. While B cell number was severely reduced with altered development pattern. Interestingly, previous study has shown that osteoblastic cell support megakaryopoiesis and platelet formation (Xiao et al. 2017). In our study, the number of MEP (erythrocyte and platelet precursors) was also reduced, and scRNA-seq analysis showed no significant change in erythrocyte population (data not shown), inferring that osteocyte may also participate in regulating platelet formation.

Bone and bone marrow harbor different cell lineages and form specific niches to maintain complex, delicate and extensive communications between them (Hu et al. 2016). Previous studies have shown that osteocyte controls bone remodeling, regulates hematopoiesis and even remote organ function (Divieti Pajevic and Krause 2019; Asada, Sato, and Katayama 2015), via secretion of factors including sclerostin, RANKL, FGF23 and IL19 (Xiao, et al. 2021). Although our study has shown that osteocytes also influence cell lineage commitments of bone and bone marrow via the mediation of cell senescence, it is still not clear what factors that osteocytes produce to regulate this process. Further study is required to identify the mechanism in which osteocytes regulate the homeostasis of bone and bone marrow. Furthermore, as our study only focus on the effect of osteocyte ablation in muscle, bone and bone marrow, it is still not clear what is the impact of osteocyte ablation in other organs. Nevertheless, previous study showed that osteocyte ablation induces lymphoid organ
atrophy, thymocyte depletion and altered fat metabolism in ‘osteocyte-less’ mice model (Sato et al. 2013), suggesting the role of osteocytes in the extra-skeletal system.

In conclusion, we demonstrated a critical role of osteocytes in regulating senescence of bone, and bone marrow (Figure 7). Ablation of osteocytes induced SASP accumulation in bone marrow osteoprogenitors and myeloid lineage cells, which altered MSC and HSC lineage commitments with impaired osteogenesis, promoted myelopoiesis and osteoclastogenesis, leading to the accelerated skeletal aging phenotype with severe sarcopenia, osteoporosis, degenerative kyphosis and bone marrow myelopoiesis, thus shortened lifespan of mice. Targeting osteocyte function and cell fate may shed light on the therapeutic regimens for aging associated bone diseases.

Materials and methods

Mice
All mouse lines were maintained on a C57BL/6J background. Dmp1cre mice were provided by J. Q. (Jerry) Feng from Texas A&M College of Dentistry, USA (Jackson Laboratory stock number, 023047). Rosa26em1Cin(SA-IRES-Loxp-ZsGreen-stop-Loxp-DTA) heterozygotes were from GemPharmatech (strain ID, T009408). Osteocyte ablation mice model during development was established by crossing Dmp1cre mice with Rosa26em1Cin(SA-IRES-Loxp-ZsGreen-stop-Loxp-DTA) homozygotes to obtain Dmp1cre Rosa26em1Cin(SA-IRES-Loxp-ZsGreen-stop-Loxp-DTA) heterozygotes (DTAhet). All mice experiments were approved by the Animal Care and Use Committee of Shanghai Sixth People’s Hospital (Permit number: 2021-0935, 2021-0936). All surgery was performed under isoflurane anesthesia, and every effort was made to minimize suffering.

Bone histomorphometry analysis
Mice femur was dissected and fixed in 4% paraformaldehyde (PFA) for two days and further decalcified with 10% EDTA (pH=7.2) in 4°C for about 2 weeks. Then specimens were embedded in paraffin and sectioned at 4 μm thickness. TRAP staining was performed for osteoclast analysis. H&E staining was performed for adipocyte and osteocyte analysis. For osteoblast analysis, undecalciﬁed femur was embedded in plastic and sectioned at 5 μm thickness and Goldner trichrome staining was performed. For dynamic histomorphometry analysis, double calcein-labeling was used. Briefly, each mouse was given 30 μg/gram body weight Calcein (Sigma) on day 1 and day 7 by intraperitoneal injection before sacriﬁce. Bones were then ﬁxed, dehydrated, embedded in plastic and cut into 5 μm slices and calculated using the software under ﬂuorescence. Bioquant Osteo software (Bioquant) was used for histomorphometry analysis. Accepted nomenclature was used to report the results (Dempster et al. 2013). ImageJ was used to measure the number of osteocyte lacunae.

Immunofluorescence staining
Both ends of the mice tibias/femurs were removed. Then they were embedded in OCT
for frozen sectioning and cut parallel to the long axis of the long bones. Stop cutting when the maximum cross section of the long bones was observed. The OCT around the rest of the bones were melted at room temperature. The bone samples remained were washed 3 times in PBS for 10 minutes and fixed in 4% paraformaldehyde (PFA) for 2 hours. Then, they were immersed in 0.1% Triton X-100 for 1 hour, blocked using 3% BSA and stained using Alexa Fluor™ 568 Phalloidin (Invitrogen) for 48 hours at 4°C in the dark with gentle shake. The samples were washed 3 times with PBS for 10 minutes. The cross section of the sample was inverted in the confocal dish. Pictures were captured using confocal microscopy (Olympus) and ImageJ was used to measure the number dendrites per osteocyte.

**SA-β Gal staining**

For SA-β Gal staining, mice femur was dissected and fixed in 4% PFA for two days and further decalcified with 10% EDTA (pH=7.2) in 4°C for about 2 weeks. Then specimens were dehydrated in 30% sucrose and embedded in OCT and frozen sectioned at 10 μm thickness. Then SA-β Gal staining was performed according to the manufacturer’s instructions (Beyotime).

**Bone density measurements**

Mice femurs and L3 lumbar were stripped of soft tissue and fixed in 4% PFA overnight at 4 °C, then stored in 70% ethanol until scanned using the μCT instrument (SkyScan 1176). Relevant structure parameters of the μCT instrument were as previous reported (Ding, et al. 2022): scanning voxel size, 9×9×9 μm³; X-ray tube potential, 50 kV and 450 μA; integration time, 520 ms; rotation Step, 0.4° for 180° scanning. CTAn micro-CT software version 1.13 (Bruker) was used to analyze the images. The threshold value (grayscale index) for all trabecular bone was 75. For all cortical bone the threshold value (grayscale index) was 110. The femurs were analyzed at a resolution of 9 μm. The volumetric regions for trabecular analyses include the secondary spongiosa located 1 mm from the growth plate and extending 1.8 mm (200 sections) proximally. For cortical bone analysis, the volumetric regions include 600 μm long at mid-diaphysis of the femur (300 μm extending proximally and distally from the diaphyseal midpoint between the proximal and distal growth plates). For vertebrae, the volumetric regions include the entire trabecular region without the primary spongiosa (300 μm below the cranial and above the caudal growth plate). Morphometric parameters including BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp, Ct.Th and Ct.Po were calculated.

**Gait analysis**

CatWalk automated gait analysis system (Noldus Information Technology) was used to analyze gait. Mice were expected to run along a special glass plate with a green LED lit and a high-speed video camera under it. Their paws were captured by the camera. Before the formal experiments, the mice were habituated in the plate to achieve an unforced locomotion. Three compliant runs without stopping, changing direction and turning around were analyzed with Catwalk Software. Relevant data
were generated by Catwalk Software after each footprint was checked manually. Data including stride length, swing speed and normal step sequence ratio were analyzed.

**Whole mount alcian blue/alizarin red staining**

The skin and viscera of the intact fetal mice (E19.0) were removed. The embryos were fixed in 95% ethanol overnight and then degreased in absolute acetone overnight with gentle agitation. The embryos were stained overnight in 0.015% alcian blue (Sigma) /0.005% alizarin red (Sigma) in 70% ethanol with gentle agitation. They were washed in 70% ethanol for 30 min three times and digested using 1% KOH solution. When most of the soft tissue was digested, the embryos were immersed in 75% (vol/vol)1% KOH/glycerol solution for further clearing. Graded glycerol was changed according to the degree of embryos digestion and relevant pictures were obtained under the microscope (Leica).

**Whole-body µCT scan**

13- and 37-week-old DTAhet and wild-type mice were deeply anesthetized and carefully positioned with a dedicated cradle and holder to capture the whole-body (excluding the tail) radiographs at a resolution of 35 µm using the µCT instrument (SkyScan 1176). Scanning details were listed as following: X-ray tube potential, 65 kV and 375 μA; exposure time, 150 ms; rotation step, 0.5° for 180° scanning. CTAn micro-CT software version 1.13 (Bruker) was used to reconstruct pictures.

**RNA-seq**

Total RNA of whole bone with bone marrow flushed out from 4 weeks WT and DTAhet mice was extracted using Trizol reagent (Thermofisher), quantified and purified using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent). Following purification, mRNA library was constructed, fragmented, amplified and loaded into the nanoarray and sequencing was performed on Illumina Novaseq™ 6000 platform following the vendor’s recommender protocol. After sequencing, generated reads were filtered and mapped to the reference genome using HISAT2 (v2.0.4) and assembled using StringTie (v1.3.4d) with default parameters. Then, all transcriptomes from all samples were merged to reconstruct a comprehensive transcriptome using gffcompare software (v0.9.8) and the expression levels of all transcripts were calculated by Stringtie and ballgown. Differential gene analysis was performed by DESeq2 software and then subjected to enrichment analysis of GO functions. GSEA was performed using GSEA software (ver. 4.1.0; Broad Institute, MIT). Genes were ranked according to their expression; Gene set were searched from website (www.gsea-msigdb.org). The data were deposited into the GEO repository (GSE202356, secure token for reviewer: ipqryuycnloznsz)

**Cell culture**

*In vitro osteoclastogenesis assay*

The bone marrow of mice femurs and tibias were flushed to get bone marrow cells. Cells were cultured overnight by using α-MEM (Hyclone) which contains 10% FBS
(Gibco), 100 μg/ml streptomycin (Gibco) and 100 U/ml penicillin (Gibco). The non-adherent cells were collected, layered on Ficoll-Paque (GE Healthcare) and separated through density gradient centrifugation at 4 °C and 2000 rpm for 20 min. The BMMs in the middle layer of the separation were collected and washed twice with ice-cold PBS. To induce osteoclast differentiation, BMMs (2.5×10⁴ cells per well for 96-well plates and 8×10⁵ per well for 6-well plates) were cultured by using α-MEM which contains 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 100 ng/ml M-CSF (Peprotech) and 100 ng/ml RANKL (Peprotech) for 5 days before TRAP staining. Cells were cultured at 37°C in a humidified incubator at 5% CO₂. The medium was changed every 2 days. At the end of assay (the fifth day), the cells were fixed and stained with Tartrate-resistant acid phosphatase (TRAP) kit according to the manufacturer’s instructions (Sigma) to quantify osteoclast numbers, or RNA was extracted as recommended protocol. TRAP-positive cells which contains more than three nuclei were counted as mature osteoclast-like cells (OCLs). The assay was repeated three times and number of OCLs per well were recorded for each biological replicate.

Isolation of mesenchymal stem cells and tri-lineage differentiation
Bone marrow cells were collected by flushing femur and tibia from WT and DTA<sup>het</sup> mice and were cultured in DMEM (Hyclone) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. After 48 hours, non-adherent cells were removed and fresh medium were added. The adherent spindle-shaped cells were further cultured for 2 days. After culturing the cells to 70-80% confluence, they were replated at a density of 5000 cells per well for 96-well plates or 2×10⁵ cells per well for 6-well plates. When the cells were cultured to 70-80% confluence, the medium was replaced with osteogenic differentiation medium (Cyagen) for osteogenesis or with adipogenic differentiation medium (Cyagen) for adipogenesis or with chondrogenic differentiation medium (Cyagen). RNA extraction was performed after two days of differentiation. After three weeks of differentiation, alizarin red staining was performed.

RT-qPCR
Total RNA was isolated using RNeasy® Mini Kit (Qiagen). 500 ng of total RNA was reverse transcribed into cDNA using PrimeScript<sup>TM</sup> RT Master Mix (Takara, RR036A). qPCR analyses were performed using SYBR Premix Ex Taq<sup>TM</sup> II (Takara, RR820L) and samples were run on the ABI HT7900 platform (Applied Biosystems). SYBR Green PCR conditions were 1 cycle of 95°C for 30 seconds, and 40 cycles of 95°C for 5 seconds and 34°C for 60 seconds. Melting curve stage was added to check primers specificity. Relative gene expression levels were calculated using the threshold cycle (2^-ΔΔCT) method. Relevant primers were listed as below: Gapdh: 5′-ACC CAG AAG ACT GTG GAT GG-3′ and 5′-CAC ATT GGG GGT AGG AAC AC-3′; Cdkn1a: 5′-GTC AGG CTG GTC TGC CTC CG-3′ and 5′-CGG TCC CGT GGA CAG TGA GCA G-3′; Cdkn2a: 5′-GTC AGG CTG GTC TGC CTC CG-3′ and 5′-CGG TCC CGT GGA CAG TGA GCA G-3′; Ccl2: 5′-GCA TCC ACG TGT TGG

（Gibco），100 μg/ml streptomycin （Gibco） and 100 U/ml penicillin （Gibco）。The non-adherent cells were collected, layered on Ficoll-Paque （GE Healthcare） and separated through density gradient centrifugation at 4 °C and 2000 rpm for 20 min. The BMMs in the middle layer of the separation were collected and washed twice with ice-cold PBS。To induce osteoclast differentiation, BMMs （2.5×10⁴ cells per well for 96-well plates and 8×10⁵ per well for 6-well plates） were cultured by using α-MEM which contains 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 100 ng/ml M-CSF （Peprotech） and 100 ng/ml RANKL （Peprotech） for 5 days before TRAP staining。Cells were cultured at 37°C in a humidified incubator at 5% CO₂。The medium was changed every 2 days。At the end of assay （the fifth day）, the cells were fixed and stained with Tartrate-resistant acid phosphatase （TRAP） kit according to the manufacturer’s instructions （Sigma） to quantify osteoclast numbers, or RNA was extracted as recommended protocol。TRAP-positive cells which contains more than three nuclei were counted as mature osteoclast-like cells （OCLs）。The assay was repeated three times and number of OCLs per well were recorded for each biological replicate。

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CTC A-3' and 5'-CTC CAG CCT ACT CAT TGG GAT CA-3'; Tnf: 5'-ATG AGA
AGT TCC CAA ATG GC-3' and 5'-CTC CAC TTG GTG GTT TGC TA-3'; Il1b: 5'-
GCC CAT CCT CTG TGA CTC AT-3' and 5'-AGG CCA CAG GTA TTT TGT CG-
3'; Alpl: 5'-TCA GGG CAA TGA GGT CAC AT-3' and 5'-CCT CTG GTG GCA TCT
CGT TA-3'; Bglap: 5'-GCC TCC TGA TGA GTC TGA CAA AGC CT-3' and 5'-GCG GTC TCC TTC
AAG CCA AT-3' and 5'-CAA TAG GAG AGA GGC AGG GG-3'; Runx2: 5'-GCC
CAG TAT TTC AGA TG-3' and 5'-GGT AAA GGT GGC TGG GTA GT-3';
Dmp1: 5'-CAG TGA GGA TGA GGC AGA CA-3' and 5'-CGA TCG CTC CTG GTA
CTC TC-3'; Sost: 5'-GCC GGA CCT ATA CAG GAC AA-3' and 5'-CAC GTA GCC
CAA CAT CAC AC-3'; Acp5: 5'-TGG ACA TGA CCA CAA CCT GCA GTA-3' and
5'-TGC CAC AGA GGG A TC CA T GAA GTT-3'; Calcr: 5'-AGC C AC AGC CT A
TCA GCA CT-3' and 5'-GTA GGG AAG AAG CCA AT-3' and 5'-GTT AGT CAA GAG ACC
GCC TCC ATA TGA CCT CGA GTA G-3' and 5'-TAA AAG GCT TGT AAA TTG
GAG GAC T-3'; Atp6v0d2: 5'-ACA TGT CCA CTG GAA GCC CAG TAA-3' and 5'-
ATG AAC GTA TGA GGC CAG TGA-3'; Tyrobp: 5'-CTG GTG TAC TGG
CTG GGA TT-3' and 5'-CGA TCG CTC CTG GTA CTC TC-3' and 5'-TCC TGA GCC CTT TGT GTG TC-3';
Fabp4: 5'-GAT GAA ATC ACC GCA GAC GAC A-3' and 5'-ATT GTG GTG GAC
TTT CCA TCC C-3'; Ppar: 5'-GGA AAG ACA ACG GAC AAA TCA C-3' and 5'-
AGG TGG AAA ATA CTT CCC GTC-3'; Prg4: 5'-GGA GGG GAT CTC GGG GTT G-3'; Sox9: 5'-CGG AAC AGA
TCC AC A CTACA TCC C-3' and 5'-GCT TGC TGC TGC-3'; Prg4: 5'-GGG GAT CTC GGG AAA CTA CCT CTC C-3' and 5'-CAG GAC
AGC ACT CCA TGT AGT-3'; Mmp3: 5'-ACA TGG AGA CTT TGT CTC CCC TTT TG-3' and 5'-TTG GAG TGG TGA
AGT AGC TTG C AC-3'; Mmp13: 5'-CTT CTG TGT GAG TCA G-3' and 5'-CAT AAC ATG GAG TCC GCC
GTT CTG G-3'; Prg4: 5'-GGG GAT CTC GGG AAA CTA CCT CTC C-3' and 5'-CAG GAC
AGC ACT CCA TGT AGT-3'; Mmp3: 5'-ACA TGG AGA CTT TGT CTC CCC TTT TG-3' and 5'-TTG GAG TGG TGA
AGT AGC TTG C AC-3'; Mmp13: 5'-CTT CTG TGT GAG TCA G-3' and 5'-CAT AAC ATG GAG TCC GCC
GTT CTG G-3'; Prg4: 5'-GGG GAT CTC GGG AAA CTA CCT CTC C-3' and 5'-CAG GAC
AGC ACT CCA TGT AGT-3'; Mmp3: 5'-ACA TGG AGA CTT TGT CTC CCC TTT TG-3' and 5'-TTG GAG TGG TGA
AGT AGC TTG C AC-3'; Mmp13: 5'-CTT CTG TGT GAG TCA G-3' and 5'-CAT AAC ATG GAG TCC GCC
GTT CTG G-3'; Prg4: 5'-GGG GAT CTC GGG AAA CTA CCT CTC C-3' and 5'-CAG GAC
Flow cytometry
Bone marrow cells were isolated by flushing the bone marrow of mice femurs and
tibias with PBS and were dissociated into a single cell suspension by gently filtering
them through 70 μm nylon mesh. After red blood cells lysis, the isolated cells were
blocked by anti-mouse CD16/32 antibody (Biolegend, 101302) for 15 min and stained
with fluorescence-conjugated antibodies for 30 min at 4°C in the dark. Relevant
antibodies were listed as below and their catalog numbers were provided in the
brackets: anti-Ly-6C-Pacific Blue™ (128013), anti-Ly-6C-PE (128007), anti-Ly-6G-
Pacific Blue™ (127611), anti-Ly-6G-PE/Cy7 (127617), anti-CD16/32-FITC (101305), anti-CD115-PE (135505), anti-CD117-PE (105808), anti-CD117-APC/Cy7 (105825), anti-CD45R-PE/Cy5 (103209), anti-CD45R-APC (103212), anti-Ly-6A/E-APC (108111), anti-Ly-6A/E-Alexa Fluor®700(108142), anti-CD34-PerCP/Cyannine5.5 (128607), anti-CD127-PE (121111), anti-CD127-APC (135011), anti-CD11b-FITC (101205) and anti-CD24-Pacific Blue™ (101819). All these antibodies were purchased from Biolegend. Samples were analyzed using cytomter CytoFlex (Beckman Coulter) and FlowJo software version 10.4. 50000 events were collected for each sample.

Preparation of mice serum

For serum collection, mice were anesthetized with isoflurane and blood samples were collected from the ophthalmic vein. Samples were then centrifuged at 5000 rpm for 5 min. Supernatants were transferred to a new tube and centrifuged at 5000 rpm for 5 min again. Supernatants were collected to a new tube and treated with liquid nitrogen fast and then stored at -80 °C.

Enzyme-linked immunosorbent assay (ELISA)

Elisa was performed as kit instructions (Jianglai). Briefly, working standards and diluted samples were prepared and added to each well. Plates were sealed and incubated for 1 hour at 37 °C. After washing three times, 100 μl enzyme-labeled reagents were added and plates were incubated for 1 hour at 37 °C. Finally, TMB substrates were added and incubated for 15-30 min at 37 °C followed by Stop solution addition. Then plates were read at 450 nm within 5 min.

Singe cell collection, library construction and sequencing

Bone marrow cells from WT and DTA<sup>het</sup> mice were flushed and sieved through a 70 µm cell strainer. After red blood cell analysis, dissociated single cells were stained with AO/PI for viability assessment. Single-cell RNA sequencing (scRNA-seq) was performed using 10<sup>x</sup> Genomics Chromium platform. Related operations including Generation of gel beads in emulsion (GEMs), barcoding, GEM-RT cleanup, complementary DNA amplification and library construction were all carried out following the manufacturer’s protocol. By using 150-base-pair paired-end reads, the final libraries were sequenced on the Illumina NovaSeq 6000 platform. The scRNA-seq data could be accessed from GEO database (GSE202516, secure token for reviewer: ihudckqqxvopruz)

Data processing, dimension reduction, unsupervised clustering and annotation

ScRNA-seq data analysis was performed by NovelBio Co.,Ltd with NovelBrain Cloud Analysis Platform (www.novelbrain.com). Fastp was applied with default parameters filtering the adaptor sequence and the low-quality reads were removed to achieve the clean data. Then the feature-barcode matrices were obtained by aligning reads to the mouse genome (mm10 Ensemble: version 92) using CellRanger v3.1.0.
Down sample analysis among samples sequenced was applied according to the mapped barcoded reads per cell of each sample and finally achieved the aggregated matrix. Cells contained over 200 expressed genes and mitochondria UMI rate below 20% passed the cell quality filtering and mitochondria genes were removed in the expression table.

Seurat package (version: 3.1.4, https://satijalab.org/seurat/) was used for cell normalization and regression based on the expression table according to the UMI counts of each sample and percent of mitochondria rate to obtain the scaled data. PCA was constructed based on the scaled data with top 2000 high variable genes and top 10 principals were used for tSNE construction and UMAP construction. Utilizing graph-based cluster method, the unsupervised cell cluster results based the PCA top 10 principal were acquired, and the marker genes by FindAllMarkers function with wilcox rank sum test algorithm was calculated under following criteria: lnFC > 0.25, p value < 0.05 and min.pct > 0.1. To identify the cell type detailed, the clusters of same cell type were selected for re-tSNE analysis, graph-based clustering and marker analysis.

Identification of differential gene expression and gene enrichment analysis

To identify differentially expressed genes among samples, the function FindMarkers with wilcox rank sum test algorithm was used under following criteria: lnFC > 0.25, p value < 0.05 and min.pct > 0.1. GO analysis was performed to facilitate elucidating the biological implications of marker genes and differentially expressed genes. The GO annotations from NCBI (http://www.ncbi.nlm.nih.gov/), UniProt (http://www.uniprot.org/) and the Gene Ontology (http://www.geneontology.org/) were downloaded. Fisher’s exact test was applied to identify the significant GO categories and FDR was used to correct the p-values. Pathway analysis was used to find out the significant pathway of the marker genes and differentially expressed genes according to KEGG database. Fisher’s exact test was applied to select the significant pathway, and the threshold of significance was defined by P-value and FDR. To characterize the relative activation of a given gene set such as pathway activation, QuSAGE (2.16.1) analysis was performed, and related gene sets involving neutrophil function and SASP were according to the publications (Xie et al. 2020; Zhang et al. 2021) and listed in Supplementary File 2. Briefly, based on the gene set, the gene set variation analysis (GSVA) software package (Hanzelmann, Castelo, and Guinney 2013) was used to calculate the score of SASP in each cells. Ggpubr R package via the Wilcoxin test (https://github.com/kassambara/ggpubr) (version 0.2.4) was used to analyze changes in the scores between WT and DTA het mice.

Developmental trajectory inference and RNA velocity analysis

The Single-Cell Trajectories analysis was applied utilizing Monocle2 (http://cole-trapnell-lab.github.io/monocle-release/) using DDR-Tree and default parameter. Before Monocle analysis, marker genes of the Seurat clustering result and raw expression counts of the cell passed filtering were selected. Based on the pseudo-time...
analysis, branch expression analysis modeling (BEAM Analysis) was applied for branch fate determined gene analysis. To estimate the cell dynamics, RNA Velocity analysis was performed through scVelo package (Version0.2.3) based on ScanPy package (Version1.5.0) with default parameters.

**Statistical analysis**

All data were analyzed using GraphPad Prism (v8.2.1) software for statistical significance. P value was determined by the student’s t test for two-group or one-way ANOVA test for multiple group comparisons. Gehan-Breslow-Wilcoxon test was used for analyzing Kaplan-Meier curve of WT and DTA\textsuperscript{het} mice.

**Acknowledgements**

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**Data and materials availability**

SeRNA-Seq and RNA-seq data have been deposited into GEO repository with accession codes GSE202516 and GSE202356 respectively. Source data have been deposited in Dryad database (https://datadryad.org/stash/share/OZrZgJpj1zyIRuuATH0ulO7Orzbe0fg_gEXD2yryW0).

**Conflict of Interest**

The authors declare no conflict of interest.

**Reference**


Li, H., P. Liu, S. Xu, Y. Li, J. D. Dekker, B. Li, Y. Fan, Z. Zhang, Y. Hong, G. Yang, T.


Figure 1. DTAhet mice display partial osteocyte ablation. (A-B) Hematoxylin-eosin staining of WT and DTAhet mice femur at 4 weeks (A) and 13 weeks (B) and quantification of the ratio of empty lacunae (arrows) (C) (n=8-12 per group), indicating reduced osteocyte number in DTAhet mice. Scare bar, 20 μm. (D-E) Immunofluorescence staining of femoral cortical bone of 4-week-old WT and DTAhet mice (D) and quantification of dendrites per osteocyte based on the images (E) (n=152 osteocytes in WT group and n=64 osteocytes in DTAhet group). Scare bar, 20 μm. Error bar represents the standard deviation.

Figure 2. Osteocyte ablation induces severe osteoporosis and kyphosis. (A-C) Representative μCT reconstructive images of male WT and DTAhet mice femur at 4 weeks (A) and trabecular microstructural parameters (bone mineral density, BMD; bone volume fraction, BV/TV; trabecular number, Tb.N; trabecular separation, Tb.Sp; and trabecular thickness, Tb.Th); (B) and cortical microstructural parameters (cortical thickness, Ct.Th; and cortical porosity, Ct.Po) (C) derived from μCT analysis (n=4-7 per group). (D-G) Representative μCT reconstructive images of male WT and DTAhet mice femur at 13 weeks (D) and trabecular microstructural parameters (BMD, BV/TV, Tb.N, Tb.Sp and Tb.Th) (E-F) and cortical microstructural parameters (Ct.Th and Ct.Po) (G) derived from μCT analysis (n=3 per group), demonstrating severe bone loss in DTAhet mice. (H-I) Representative μCT reconstructive images of male WT and DTAhet mice third lumbar at 4 weeks (H) and trabecular microstructural parameters (BMD, BV/TV, Tb.N, Tb.Sp and Tb.Th) (I) derived from μCT analysis. (J-K) Representative μCT reconstructive images of male WT and DTAhet mice third lumbar at 13 weeks (J) and trabecular microstructural parameters (BMD, BV/TV, Tb.N, Tb.Sp and Tb.Th) (K) derived from μCT analysis, showing vertebral body bone loss in the spine of DTAhet mice. (L) Gross images of male WT and DTAhet mice at 13 weeks. (M) Representative whole-body μCT reconstructive and sagittal images of male WT and DTAhet mice at 13 weeks. (N) Representative whole-body μCT reconstructive and sagittal images of male DTAhet mice at 37 weeks, noting that severe kyphosis occurred in DTAhet mice. Error bar represents the standard deviation.

Figure 3. Osteocyte ablation leads to severe sarcopenia and shorter lifespan. (A-B) Gross images (A) and weight (B) of male WT and DTAhet mice at 4 weeks (n=5-8 per group). (C) The ratio of gastrocnemius muscle weight male WT and DTAhet mice at 4 weeks (n=3 per group). (D-E) Hematoxylin-eosin staining of WT and DTAhet mice gastrocnemius muscle at 4 weeks (D) and quantification of myonuclei per area fiber (n=11 per group) and centralized nucleus per field (E) (n=5 per group). Scale bar, 20 μm. Showing focal muscle atrophy, increased centralized myonuclei and mild inflammation in DTAhet mice. (F-G) Hematoxylin-eosin staining of WT and DTAhet mice gastrocnemius muscle at 13 weeks (F) and quantification of myonuclei per area fiber (n=11 per group) and centralized nucleus per field (G) (n=6 per group). Noting muscle atrophy, rimmed vacuoles and inclusion bodies within the muscle fibers in
DTA\textsuperscript{het} mice. Scale bar, 20 µm. (H) Kaplan-Meier survival curve of WT and DTA\textsuperscript{het} mice (n=4-5 per group), showing that DTA\textsuperscript{het} mice had shorter lifespan than that of wild type. Error bar represents the standard deviation.

**Figure 4. Ablation of osteocytes alters mesenchymal lineage commitment and promoted osteoclastogenesis.** (A-B) Goldner trichrome staining of male WT and DTA\textsuperscript{het} mice femur at 4 weeks (A) and histomorphometry analysis of osteoblast numbers (N.Ob/BS) (arrows) and osteoid-covered surface (OS/BS) (B) (n=6 per group). Scale bar, 20 µm. (C-D) Hematoxylin-eosin staining of WT and DTA\textsuperscript{het} mice femur at 4 weeks (C) and histomorphometry analysis of adipocyte (arrows) volume (Ad.V/TV) (D) (n=6 per group). Scale bar, 50 µm. (E-F) Representative images of calcein double labeling of the mineral layers of male WT and DTA\textsuperscript{het} mice femur at 4 weeks (E) and histomorphometry analysis of the mineral surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) (F) (n=4 per group). Scale bar, 50 µm. (G) ELISA of the concentration of bone formation index P1NP in the serum (n=6-7 per group). (H-I) Alizarin red staining of osteogenesis from 4-week (H) and 13-week mice (I). Scale bar, 250 µm. (J) RT-qPCR analysis of osteoblast signature genes expression at the mRNA levels of osteogenesis from 4-week mice (n=3 per group from three independent experiments), indicating impaired osteogenesis in DTA\textsuperscript{het} mice. (K-L) TRAP staining of WT and DTA\textsuperscript{het} mice femur at 4 weeks (K) and histomorphometry analysis of osteoclast (arrows) surface (Oc.S/BS) and osteoclast numbers (N.Oc/BS) (L) (n=6 per group). Scale bar, 20 µm. (M) ELISAs of the concentration of RANKL, OPG and the ratio of RANKL/OPG in the serum (n=6-7 per group). (N) ELISA of the concentration of bone resorption index CTX in the serum (n=6-7 per group). (O-P) TRAP staining of osteoclastogenesis from 4-week (O) and 13-week mice (P) and quantitative analysis (Q) of TRAP positive cells (nucleus > 3) per well (n=3 per group from three independent experiments). Scale bar, 250 µm. (R) RT-qPCR analysis of osteoclast signature genes expression at the mRNA level of osteoclastogenesis from 4-week mice (n=3 per group from three independent experiments), showing increased osteoclastogenesis in DTA\textsuperscript{het} mice. Error bar represents the standard deviation.

**Figure 5. Alteration of hematopoietic lineage commitment by osteocyte ablation.** (A-B) The UMAP plot of cells isolated from the bone marrow of 4 weeks WT and DTA\textsuperscript{het} mice and inferred cluster identity (A) and number of mRNA per cell (B). (C) Dot plot showing the scaled expression of selected signature genes for each cluster. Dot size represents the percentage of cells in each cluster with more than one read of the corresponding gene and dots are colored by the average expression of each gene in each cluster. (D-E) The UMAP plot of cells shown by sample (D) and proportions of each cluster in two samples (E). (F) RNA velocity analysis of clusters of WT and DTA\textsuperscript{het} mice shown by the UMAP embedding, showing stronger directionality of velocity vectors from HSPC cluster to neutrophil cluster in DTA\textsuperscript{het} mice. (G) Trajectory analysis of myeloid clusters of WT and DTA\textsuperscript{het} mice, demonstrating myeloid-biased hematopoiesis in DTA\textsuperscript{het} mice. (H) Trajectory analysis of lymphoid
clusters of WT and DTA<sup>het</sup> mice, demonstrating impaired lymphopoiesis in DTA<sup>het</sup> mice. (I-J) Representative image of flow cytometry (I) and analysis of proportions of myeloid progenitors (CMP, GMP and MEP) (J) of 4 weeks WT and DTA<sup>het</sup> mice (n=3-4 per group). (K-L) Representative image of flow cytometry (K) and analysis of proportions of CD11b<sup>+</sup> myeloid cells (L) of 4 weeks WT and DTA<sup>het</sup> mice (n=3 per group). (M-N) Representative image of flow cytometry (M) and analysis of proportions of neutrophils (N) of 4 weeks WT and DTA<sup>het</sup> mice (n=3-4 per group). (O-P) Representative image of flow cytometry (O) and analysis of proportions of B220<sup>+</sup> lymphoid cells (P) of 4 weeks WT and DTA<sup>het</sup> mice (n=3 per group). (Q-R) Representative image of flow cytometry (Q) and analysis of proportions of ProB PreB, immature B, transitional B, early mature B and late mature B (R) of 4 weeks WT and DTA<sup>het</sup> mice (n=3-4 per group), indicating altered B cell development pattern in DTA<sup>het</sup> mice. Error bar represents the standard deviation.

**Figure 6. Senescence of osteoprogenitors and myeloid lineage cells leads to the accelerated skeletal aging.** (A) Comparisons of total bone marrow cells SASP score between 4 weeks WT and DTA<sup>het</sup> mice. (B) Latent time of RNA velocity analysis of WT and DTA<sup>het</sup> mice shown by the UMAP embedding. (C) ELISAs of the concentration of TNF-α, IL-1β and IL-6 of 4 weeks WT and DTA<sup>het</sup> mice in the serum (n=5-6 per group). (D) Comparisons of MSCs SASP score between 4 weeks WT and DTA<sup>het</sup> mice, indicating the senescence of osteoprogenitors in DTA<sup>het</sup> mice. (E) Comparisons of CMP SASP score between 4 weeks WT and DTA<sup>het</sup> mice. (F) Comparisons of monocytes and its subcluster Ly6c<sup>+</sup> monocytes SASP score between 4 weeks WT and DTA<sup>het</sup> mice. (G) Comparisons of neutrophils and its subcluster (G2, G3 and G4) SASP score between 4 weeks WT and DTA<sup>het</sup> mice, indicating the senescence of myeloid lineage cells. (H) Bubble plot of the expression of senescence related genes in subclusters of WT and DTA<sup>het</sup> mice. (I) RT-qPCR analysis of senescence related genes expression at the mRNA level of 4 weeks WT and DTA<sup>het</sup> mice cortical bone (n=3 per group). (J) RT-qPCR analysis of senescence related genes expression at the mRNA level of 4 weeks WT and DTA<sup>het</sup> mice bone marrow (n=3 per group). Error bar represents the standard deviation.

**Figure 7. Schematic diagram of osteocyte ablation induced skeletal senescence.** Ablation of osteocytes induced SASP accumulation in bone marrow osteoprogenitors and myeloid lineage cells, which altered MSC and HSC lineage commitments with promoted adipogenesis, myelopoiesis and osteoclastogenesis at the expense of osteogenesis and lymphopoiesis, leading to the skeletal premature aging phenotype with severe sarcopenia, osteoporosis, degenerative kyphosis and bone marrow myelopoiesis, thus shortened lifespan of mice.
Figure supplement legend

Figure 1 - figure supplement 1. Osteocyte ablation has no impact on embryonic skeletal development. (A) Whole mount skeleton staining of WT and DTA<sup>het</sup> mice at E19.0 by Alizarin red/Alcian blue. (B-C) Hematoxylin-eosin staining of WT and DTA<sup>het</sup> mice femur at 13 weeks (B) and quantification of the ratio of empty lacunae (C) (n=6 per group). Scare bar, 20 µm. Error bar represents the standard deviation.

Figure 2 - figure supplement 1. Osteocyte ablation induces severe osteoporosis and kyphosis. (A-C) Representative µCT reconstructive images of female WT and DTA<sup>het</sup> mice at 4 weeks (A) and trabecular microstructural parameters (BMD, BV/TV, Tb.N, Tb.Sp and Tb.Th) (B) and cortical microstructural parameters (Ct.Th and Ct.Po) (C) derived from µCT analysis (n=3-5 per group). (D-E) Representative µCT reconstructive images of female WT and DTA<sup>het</sup> mice third lumbar at 4 weeks (D) and trabecular microstructural parameters (BMD, BV/TV, Tb.N, Tb.Sp and Tb.Th) (E) derived from µCT analysis (n=3-5 per group). Error bar represents the standard deviation.

Figure 2 - figure supplement 2. Osteocyte ablation induces severe osteoporosis and kyphosis. (A) Gait analysis of normal step sequence ratio of male WT and DTA<sup>het</sup> mice at 4 weeks (n=6 per group). (B-D) Representative gait images and foot pattern of male WT and DTA<sup>het</sup> mice (B) at 4 weeks and gait analysis of stride length and swing speed of each paw (C and D) (n=6 per group). Error bar represents the standard deviation.

Figure 3 - figure supplement 1. Osteocyte ablation leads to severe sarcopenia and shorter lifespan. (A-B) The quantification of muscle fiber number of WT and DTA<sup>het</sup> mice at 4 weeks (A) and 13 weeks (B) (n=5-6 per group). (C) RT-qPCR verification of Dmp1 expression of muscle and bone (n=3 per group). Error bar represents the standard deviation.

Figure 4 - figure supplement 1. Ablation of osteocytes alters mesenchymal lineage commitment and promoted osteoclastogenesis. (A) Selected osteogenesis related gene ontology (GO) analysis of downregulated genes by osteocyte ablation. (B) Heatmap of significantly differentiated genes (fold change > 2.0-fold, WT FPKM > 10, FDR < 0.05) (n=2 per group). (C) Indicated gene expression analysis of the cortical bones of WT and DTA<sup>het</sup> mice (n=3 per group). (D-F) GSEA analysis of osteogenesis related pathways including Wnt signaling pathway (D), Hedgehog signaling pathway (E) and Notch signaling pathway (F). (G-H) Representative image of flow cytometry (G) and analysis of proportions of osteoclast progenitors (B220<sup>CD11b<sup>Lo-Ly-6C<sup>hi</sup></sup></sup>) (H) of 4 weeks WT and DTA<sup>het</sup> mice (n=3-4 per group). Error bar represents the standard deviation.

Figure 5 - figure supplement 1. Flow cytometry gating strategy. (A) Flow
Flow cytometry of gating HSC (Lin−Sca1+c-Kit+), LT-HSC (Lin−Sca1+c-Kit+Flk2+), ST-HSC (Lin−Sca1+c-Kit−Flk2−), CMP (Lin−Sca1−c-Kit−IL7Rα CD34+Fcγ RI/IIIhi), GMP (Lin−Sca1−c-Kit−IL7Rα−CD34−Fcγ RII/IIIlo), MEP (Lin−Sca1−c-Kit−IL7Rα−CD34−Fcγ RII/IIIlo), cMoP (Lin+c-Kit+CD115+Ly-6Chi), CLP (Lin+IL7Rα Flk2+Sca1−c-Kit−), ProB PreB (B220−CD24+IgM−IgD−), immature B (B220−CD24+IgMloIgD−), transitional B (B220−CD24+IgM+IgD−), early mature B (B220−CD24+IgM−IgD+) and late mature B (B220−CD24+IgMlo−IgD−).

Figure 5 - figure supplement 2. Alteration of hematopoietic lineage commitment by osteocyte ablation. (A-B) Representative image of flow cytometry (A) and analysis of proportions of HSC (B) of 4 weeks WT and DTAhet mice (n=3-4 per group). (C-D) Representative image of flow cytometry (C) and analysis of proportions of LT-HSC and ST-HSC (D) of 4 weeks WT and DTAhet mice (n=3-4 per group). (E-F) Representative image of flow cytometry (E) and analysis of proportions of cMoP (F) of 4 weeks WT and DTAhet mice (n=3-4 per group). (G-H) Representative image of flow cytometry (G) and analysis of proportions of monocyte (H) of 4 weeks WT and DTAhet mice (n=3-4 per group). (I-J) Representative image of flow cytometry (I) and analysis of proportions of CLP (J) of 4 weeks WT and DTAhet mice (n=3-4 per group). Error bar represents the standard deviation.

Figure 5 - figure supplement 3. Increased granulopoiesis after osteocyte ablation. (A) The UMAP plot of neutrophils of 4 weeks WT and DTAhet mice and inferred subcluster identity. (B) Dot plot showing the scaled expression of selected signature genes for each cluster. Dot size represents the percentage of cells in each cluster with more than one read of the corresponding gene and dot are colored by the average expression of each gene in each cluster. (C-D) The UMAP plot of cells shown by sample (C) and proportions of each subcluster in two samples (D). (E-H) Comparisons of neutrophil activation score (E), chemotaxis score (F), glycolysis score (G) and necroptosis score (H) between 4 weeks WT and DTAhet mice.

Figure 6 - figure supplement 1. Senescence of osteoprogenitors and myeloid lineage cells leads to the accelerated skeletal aging. (A) SA-β Gal staining of frozen sections of femur of WT and DTAhet mice. Scale bar, 20 µm. (B) Bar plot of GO analysis of MSC cluster. (C-D) RT-qPCR analysis of the mRNA expression of adipogenesis (C) and chondrogenesis (D) related maker genes (n=3 per group). (E-F) Bar plot of KEGG analysis of subcluster 2 and 4 of Ly6c2_monocytes. Error bar represents the standard deviation.
Supplementary file legend

Supplementary File 1. Selected skeleton related GO analysis.

Supplementary File 2. Gene sets involving neutrophil function and SASP.
Video legend

**Video 1.** Representative movie showing movement in WT mice at 4 weeks.

**Video 2.** Representative movie showing movement defects in DTA$^{het}$ mice at 4 weeks.

**Video 3.** Representative movie showing movement in WT mice at 13 weeks.

**Video 4.** Representative movie showing movement defects in DTA$^{het}$ mice at 13 weeks.

**Video 5.** Representative movie showing movement in WT mice at 37 weeks.

**Video 6.** Representative movie showing movement defects in DTA$^{het}$ mice at 37 weeks.
Figure 1

A. WT and DTA^{het} tissue sections at 4 weeks.

B. Graph showing empty lacunae percentage for WT and DTA^{het}.

C. Immunofluorescent images of WT and DTA^{het} tissue with Philodine staining.

D. Scatter plot showing dendrites per axon for WT and DTA^{het} with P-values 0.0001 and 0.0045, respectively.
Figure 2 - figure supplement 1

A. 

WT | DTA<sup>het</sup> | WT | DTA<sup>het</sup>
---|-----------------|---|-----------------|
4w female femur

B. 

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>DTA&lt;sup&gt;het&lt;/sup&gt;</th>
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<tr>
<td>BMD (g/cm²)</td>
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<td>P=0.0008</td>
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<td>BV/TV (%)</td>
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<td>P=0.1083</td>
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<tr>
<td>Tb.S (1/mm)</td>
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<td></td>
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<tr>
<td>Tb.Th (1/mm)</td>
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C. 

<table>
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<th></th>
<th>WT</th>
<th>DTA&lt;sup&gt;het&lt;/sup&gt;</th>
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<tr>
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<td>Cl.Po (%)</td>
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D. 

4w female spine

E. 

<table>
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<th>WT</th>
<th>DTA&lt;sup&gt;het&lt;/sup&gt;</th>
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<td>Tb.Th (1/mm)</td>
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Figure 5 - figure supplement 3

A) UMAP plot with clusters G1 to G4 colored differently.
B) Dot plot showing percentage expressed and average expression.
C) UMAP plot with clusters G1 to G4.
D) Bar chart showing distribution of clusters G1 to G4.

E) Box plot showing Neutrophil activation score with G1 to G4.
P-values: 0.0442, 0.457, 0.0056.

F) Box plot showing Chemokinesis score with G1 to G4.
P-values: 0.83, 0.0087, 6.36e-06, 6.63e-07.

G) Box plot showing Glycolysis score with G1 to G4.
P-values: 0.0874, 0.0392, 0.000672, 0.0947.

H) Box plot showing Necrosis score with G1 to G4.
P-values: 0.0387, 0.064, 4.08e-06, 0.000455.