# Title: Aging Negatively Impacts the Ability of Megakaryocytes to Stimulate Osteoblast Proliferation and Bone Mass

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Abstract

Osteoblast number and activity decreases with aging, contributing to the age-associated decline of bone mass, but the mechanisms underlying changes in osteoblast activity are not well understood. Here, we show that the age-associated bone loss critically depends on impairment of the ability of megakaryocytes (MKs) to support osteoblast proliferation. Co-culture of osteoblast precursors with young MKs is known to increase osteoblast proliferation and bone formation. However, co-culture of osteoblast precursors with aged MKs resulted in significantly fewer osteoblasts compared to co-culture with young MKs, and this was associated with the downregulation of transforming growth factor beta. In addition, the ability of MKs to increase bone mass was attenuated during aging as transplantation of GATA1$^{low/low}$ hematopoietic donor cells (which have elevated MKs/MK precursors) from young mice resulted in an increase in bone mass of recipient mice compared to transplantation of young wild-type donor cells, whereas transplantation of GATA1$^{low/low}$ donor cells from old mice failed to enhance bone mass in recipient mice compared to transplantation of old wild-type donor cells. These findings suggest that the preservation or restoration of the MK-mediated induction of osteoblast proliferation during aging may hold the potential to prevent age-associated bone loss and resulting fractures.

## 1. Introduction

The traditional view regarding the regulation of bone modeling/remodeling focuses on the cells involved directly with bone formation (osteoblasts) and resorption (osteoclasts), and how the actions of these cells regulate the reciprocal process. [1, 2]. Not surprisingly, much of the research on the effects of aging on bone health (e.g. senile osteoporosis) focuses on molecules directly involved in osteoblast-to-osteoclast or osteoclast-to-osteoblast signaling [3, 4]. However, studies in animals have shed light on the role of other cells within the bone environment (e.g. endothelial cells, B-lymphocytes, MKs, etc.) in regulating the activity of osteoblasts and osteoclasts [5-8]. One cell type of particular interest is the MK, which plays a role in the regulation of bone formation and bone resorption within the marrow cavity [7-21].

Numerous mouse models have been made where MK numbers are increased. These mice also have greatly increased bone growth within the marrow cavity [9, 10, 20-27]. Importantly, osteosclerosis has been documented in patients with megakaryocytoses [28, 29]. Additional studies have shown that MKs have direct effects on osteoblast proliferation through direct cell-to-cell contact [7, 12, 13, 16-18] and

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the inhibition of osteoclastogenesis, which is contact independent [8, 16, 19]. At least one critical aspect of the function of MKs is the formation and remodeling of the hematopoietic stem cell niche [9, 15, 30]. Aging results in a striking loss of cancellous bone within the marrow cavity and on the endosteal surface of cortical bone, which is known to be caused by an increase in osteoclast activity that outpaces the ability of osteoblasts to replace the lost bone [31, 32]. What is not known is whether the loss of steady state MK activity in bone cell regulation changes during aging. A better understanding of the role of MKs in the regulation of bone mass, and how this role changes with aging, could lead to the identification of novel therapies for osteoporosis that are able to both increase osteoblast activity and inhibit osteoclast activity indirectly by targeting the MK.

To better understand MK regulation of bone cell activity during aging, we used a combination of in vitro experiments with osteoblasts and MKs derived from old or young mice. We observed reduced ability of old MKs to increase osteoblast proliferation, as well as a reduced response of old osteoblasts to the stimulatory effects of MKs. We then compared the ability of wild-type and megakaryocytotic hematopoietic progenitors ($GATA1^{low/low}$) from young and old mice to increase bone mass in an adoptive transfer model. While young $GATA1^{low/low}$ cells increased bone mass in recipient mice compared to wild-type cells, there were no differences observed in the bone mass of recipients of old $GATA1^{low/low}$ cells vs. old wild-type cells, suggesting that even when MKs were increased, their ability to regulate bone cell function was diminished with age.

## 2. Materials and Methods

### 2.1 Animals

C57BL/6J and $GATA1^{low/low}$ mice were used in this study. Generation and breeding of mutant mice with selective loss of MK-expressed GATA1 were described previously [22, 33]. These mice have been backcrossed more than 16 generations, have been maintained on the C57BL/6 background, and were kindly provided by Dr. Stuart Orkin. C57BL/6 and $GATA\text{-}1^{low/low}$ mice were bred and housed in the animal facility at the Indiana University School of Medicine. All animal procedures were in compliance with protocols approved by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine and were in compliance with the National Institutes of Health guide on the care and use of laboratory animals.

### 2.2 Adoptive Transfer Studies

For transplantation studies, recipient C57BL/6 mice received 1,100cGy ionizing radiation from a cesium source (split dose, both at 550cGy, 4 hours apart) and donor cells ($10^6$ spleen cells isolated from young $GATA\text{-}1^{low/low}$ or C57BL/6 wild-type mice were resuspended in 100 µl 1X phosphate buffered saline) were injected retro-orbitally within 2-3 hours after the second dose. Mice were monitored daily for signs of morbidity and mortality. Mice were euthanized 8 weeks post-transplantation.

### 2.3 Complete Blood Count

Mice were tail-bled and blood was analyzed for complete blood count (CBC) values using a validated HEMAVET® 950FS Hematology System (Drew Scientific, Waterbury, CT) at least 10 min after collection, but within 24 hours, as previously described [34, 35].

### 2.4 Megakaryocyte Generation, Isolation, and Conditioned Media

Murine fetal liver derived MKs were prepared as previously described [19, 21]. In brief, fetuses were dissected from pregnant C57BL/6 mice at E13–15. The livers were removed and single cell suspensions made by forcing cells through an 18 g needle in complete media: Dulbecco Modified Eagle Medium (DMEM; 12100; Gibco, Dublin, Ireland) containing 5% fetal bovine serum (FBS Hyclone; GE Healthcare Life Sciences, Pittsburgh, PA) and 1,000 U/mL Penicillin/Streptomycin (Invitrogen, Waltham, MA). Young (3-4 mo) and old (22-24 mo) adult spleen derived MKs were prepared by mechanical digestion of the spleens by crushing the tissue. The cells were filtered and pelleted by centrifugation (500 *xg* for 6 min) and the pellet was resuspended in fresh complete DMEM containing 1% murine thrombopoietin (TPO) conditioned media [19, 27].

After 3–5 days for fetal liver MKs or 7-10 days for adult spleen MKs, MKs cells were enriched by separating them from the lymphocytes and other cells using a one-step albumin gradient to obtain an approximately 95% pure MK population [36]. The bottom layer of the gradient was 3% albumin in PBS (Bovine Albumin, protease free, fatty acid poor, Serologicals Proteins Inc., Kankakee, IL), the middle layer was 1.5% albumin in PBS, and the top layer was alpha minimum essential media (αMEM; 12000-063; Gibco, Dublin, Ireland) containing the cells to be separated. The MK fraction was collected from the bottom of the tube and live cells were counted using trypan blue exclusion and a hemocytometer.

2.5 RNA preparation and Real time PCR

Total RNA was isolated with RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) with total RNA (1 µg). Quantitative real-time PCR was performed according to the instructions provided for the StepOnePlus system (Applied Biosystems, Foster City, CA) with the SYBR Green PCR Mater Mix assay (Applied Biosystems, Foster City, CA). The amplification reaction was performed for 40 cycles with denaturation at 95 °C for 10 min, followed by annealing at 95 °C for 15 s and extension and detection at 60 °C for 1 min. All reactions were run in triplicate. Primers were as follows: For *Gapdh* Fwd 5'-CTTTGGCATTGTGGAAGGGC-3'; Rev 5'-CAGGGATGATGTTCTGGGCA-3', for *Tgfb1* Fwd 5'-TACGTCAGACATTCGGGAAGCAG-3'; Rev 5'- AAAGACAGCCACTCAGGCGTATC-3', for *Bmp2* Fwd 5'-TGAGGATTAGCAGGTCTTTG-3'; Rev 5'-CACAACCATGTCCTGATAA-3'

2.6 Bone Cell Preparation and Isolation

Neonatal murine calvarial osteoblast cells were prepared as previously described [37] from C57BL/6 mice. Our technique was a modification of the basic method described by Wong and Cohn [38]. Briefly, calvaria were dissected from neonatal mice and then incubated with 4 mM EDTA in PBS for 10 min with gentle shaking at 37 °C. The EDTA solution was removed by vacuum and this step was repeated an additional time. Calvaria were then subjected to sequential collagenase-II digestions (200 U/mL; Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C with gentle shaking. Fractions 1 and 2 were from 10 min incubations and were discarded. Fractions 3-5 were from 15 min incubations and were pooled after passing the solution through a 100 µm filter to remove debris. A PBS wash of the calvaria was also performed between each incubation and these fractions were added to the pooled calvarial cells (CCs). This protocol results in ~90-95% osteoblast or osteoblast precursors based on previously reported criteria [37, 39, 40]. The same method was used to isolate adult bone cells from femurs and tibias, but with an additional step of first flushing the bones using PBS to remove the bone marrow.

2.7 Megakaryocyte and Bone Cell Co-Culture

Bone cells and MKs were co-cultured by adding freshly isolated bone cells with freshly isolated MKs in a 2:1 ratio. For CCs co-cultured with young vs. old adult spleen derived MKs, the cells were plated at 80,000 live CCs ± 40,000 live MKs per well in a 6-well plate. For old vs. young adult bone cells co-cultured with fetal liver MKs, the cells were plated at 10,000 live bone cells ± 5,000 live MKs per well in a 96-well plate. It should be noted that fetal liver derived MKs were used when possible as they are an enriched source of MKs. After 5 days, the wells were washed 4x with PBS to remove loosely adherent MKs, then the adherent cells were removed by trypsinization (0.25% trypsin in EDTA; 25200-056; Gibco, Dublin, Ireland), pelleted by centrifugation (500 x *g* for 6 minutes), resuspended in complete DMEM media, and live cells were counted by trypan blue exclusion and hemocytometer. Results are presented from 3 separate experiments.

2.8 Cell staining, flow cytometry, and cell sorting

Cells were washed with stain wash (PBS, 1% bovine calf serum, and 1% penicillin/streptomycin) and stained for 15 minutes on ice. Freshly isolated bone marrow cells from the hind limbs were stained with CD41 antibody (APC-Cy7; Clone MWReg30; BioLegend, San Diego, CA) and analyzed for $CD41^+$ (MKs) using the BD LSRFortessa (BD Biosciences, San Jose, CA).

2.9 Micro-computed Tomography

Right lower limbs were defleshed and fixed in 10% neutral buffered formalin for 72 h and stored in 70% ethanol at 4 °C. Whole femurs were imaged using a desktop SkyScan 1172 µCT imaging system (SkyScan, Kontich, Germany). Scans were acquired at 60kV using an 8 µm voxel size. The femoral trabecular volume of interest in the distal femur encompassed regions from 0.25-0.75 mm from the distal growth plate. Cortical and trabecular measurements from the midshaft were obtained from a 0.6 mm segment that was 45% of the distance proximal of the length of the diaphysis from the growth plate (diaphysis length = distance of femoral head - distance of growth plate).

2.10 Statistics

All experiments were performed with technical triplicates with a minimum of three biological replicates. Statistical tests were determined based upon the number and types of comparisons made and are mentioned in the corresponding figure legends. For statistical significance we used an $\alpha$ value = 0.05.

3. Results

3.1 Megakaryocyte Numbers were Elevated in Aged Bone Marrow in C56BL/6 Mice

Due to the positive role that MKs have on bone mass in mouse models with high MK numbers, we determined if age-related bone loss might be correlated with a decrease in bone marrow MKs. To do this, we compared the relative number of $CD41^+$ MKs in the bone marrow of young (3-4 mo), middle-aged (11-14 mo), and old-aged (22-24 mo) C57BL/6 mice by flow cytometric analysis of $CD41^+$ cells. Interestingly, we observed an increasing frequency of MKs in the bone marrow cavity with age that reached statistical significance by 24 mos compared to 3 mos (Figure 1A; 3.1-fold enrichment). Coincident with the increased MKs during aging, we observed significantly increased platelets in the blood of old compared to young animals (Figure 1B; +41.6%; $p < 0.05$). In order to determine whether MK progenitors from aged mice are sensitized to differentiate into MKs, spleen cell suspensions from

young or old mice were cultured with TPO (the main MK growth factor). Following BSA gradient isolation, we found significantly more MKs generated in vitro from old spleens, compared to young spleens (+144% per spleen; $p < 0.05$; Figure 1C), suggesting that the increased MKs in old animals may be due to enhanced megakaryopoiesis.

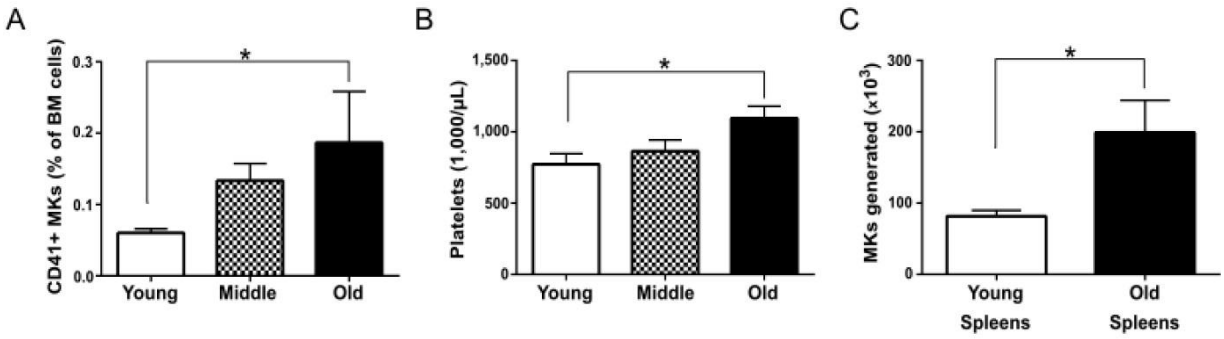


**Figure 1. The effect of aging on megakaryocyte formation.** Young (3-4 mo), middle-aged (11-14 mo), and old-aged (22-24 mo) C57BL/6 mice were characterized for MK phenotype. (A) Frequency of total bone marrow cells that stain positive for the MK marker CD41 by FACS analysis (n = 3 mice per age cohort). (B) Circulating platelet counts from whole blood (n = 21-31 mice per age cohort). (C) Total number of MKs generated per spleen from young or old spleens cultured in the presence of TPO and isolated by BSA gradient (n = 4 independent experiments). Data are expressed as means ± S.E.M. Kruskal-Wallis test with Dunn's post hoc analysis (A), 1-Way ANOVA with Holm-Sidak post hoc analysis (B), and Unpaired t-test (C). * $p < 0.05$. MK = megakaryocyte.

3.2 Aging Negatively Impacts the Ability of MKs to Stimulate the Proliferation of Osteoblasts

Because we did not see a decrease in MKs during aging and osteoblast activity is known to be decreased in old mice, this suggested that old MKs are either inferior at stimulating osteoblasts, or that old osteoblasts are less able to respond to MK stimulation of bone formation. To test these hypotheses, we used a variety of in vitro co-culture experiments (Figure 2). First, we took cultured MKs derived from either young (3-4 mo) or old (22-24 mo) mice and co-cultured them with neonatal calvarial bone cells. As can be seen in Figure 2A, while old MKs trended toward increasing bone cell numbers (2.9-fold increase; $p = 0.1422$), young MKs had a stronger effect (10.1-fold increase; $p < 0.001$). To identify whether two MK secreted pro-osteoblastic growth factors, TGFβ1 and BMP2 [41], were changed in old MKs, we compared their expression by qPCR (Figure 2B). Our results showed that there was a strongly significant downregulation of *Tgfb1* mRNA levels in MKs from old compared to young mice. To address whether old osteoblasts might be less responsive than young osteoblasts to MK stimulation, we co-cultured bone cells derived from the long bones of young and old mice with young MKs. As shown in Figure 2C, there was a significant increase in young bone cell number in the presence of MKs (9.9-fold increase; $p < 0.01$), whereas the old bone cells did not appreciably change in the presence of MKs. Taken together these results suggest that the ability of bone cells to proliferate in response to MK stimulation is decreased in aging.

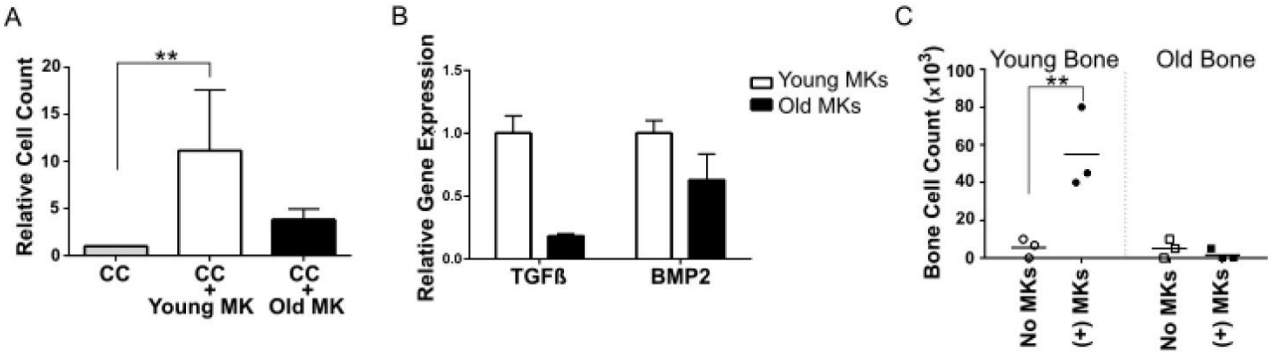


**Figure 2. Megakaryocytes from aged mice are ineffective at stimulating bone cell proliferation.** (A) MKs derived from young (3 mo) or old-aged (24 mo) mice were co-cultured (1:2 ratio) for 5 days with calvarial cells isolated from newborn pups by collagenase digestion. Results are from 6 separate experiments and normalized to within experiment CC only cell counts. (B) mRNA was isolated from lysates of MKs derived from young or old mice, reverse transcribed, and analyzed by qPCR. *Gapdh* was used as a normalization control. (n = 4-6) (C) Long bone cells were isolated by collagenase digestion from young or old-aged mice and co-cultured (10,000 cells per well) for 5 days with fetal-liver derived MKs (5,000 cells per well). (n = 3 mice per age group). Data are expressed as means ± S.E.M. Kruskal-Wallis with Dunn's post hoc analysis (A), Student's T-test (B), or 2-Way ANOVA with Holm-Sidak post hoc analysis (C). ** $p < 0.01$; *** $p < 0.001$. CC = calvarial cell. MK = megakaryocyte.

3.3 Adoptive Transfer of Aged GATA1$^{low/low}$ Cells Failed to Recapitulate High Bone Mass Phenotype

GATA1 is a transcription factor that critically regulates the development of both MKs and erythrocytes. Mice with a mutation in a GATA1 enhancer (GATA1$^{low/low}$) that drives GATA1 expression in MKs, but not erythrocytes, show an overabundance of immature MKs and osteosclerosis, coupled with reduced platelets (due to immaturity of MKs) and increased spleen mass (due to marrow overcrowding causing extramedullary hematopoiesis) [21, 22].

Notably, Vannuchi et al (2002) previously characterized many of the hematopoietic cell populations contained within the spleen of young and old wild-type and GATA-1$^{low/low}$ mice [43]. Of interest for this study, their data show that MK progenitors increase with age irrespective of genotype, but that GATA-1$^{low/low}$ spleens contain higher numbers of MK/MK progenitors than wild-type mice of the same age. Supplementary Figure 1 provides additional information regarding the hematopoietic and MK/MK progenitor composition of the C57BL/6 and GATA-1$^{low/low}$ spleen cells. We confirm that there is an age-associated increase in MK/MK progenitors contained within C57BL/6 spleens, and that GATA-1$^{low/low}$ spleen cells contain high numbers of MKs/MK progenitors. Importantly, as detailed in Supplementary Figure 1C&D, the number of hematopoietic stem cells (HSCs) within the spleens of young and old C57BL/6 and young GATA-1$^{low/low}$ mice is unlikely high enough to support stem cell-based hematopoiesis. Therefore, most likely our data were derived from progenitor activity.

Previously we showed that the MK, spleen, and bone mass phenotypes of GATA1$^{low/low}$ mice could be adoptively transferred to wild-type mice following irradiation and transplantation with young spleen derived hematopoietic progenitors [42]. We used this model to test whether the ability of GATA1$^{low/low}$ cells to enhance bone mass changes with age.

If old MKs lose the ability to enhance bone mass, then we would not expect that adoptive transfer of old (18 mo) GATA1$^{low/low}$ hematopoietic progenitors to differ from the transfer of old wild-type hematopoietic progenitors. As expected, when hematopoietic progenitors were adoptively transferred from young (3 mo) GATA1$^{low/low}$ mice into young (3 mo) C57BL/6 mice (recipient mice), there was a significantly higher bone volume fraction of trabecular bone in the distal femur (BV/TV; +150%; Figure 3A,B) when compared to adoptive transfer of young wild-type C57BL/6 hematopoietic progenitors. This change was primarily driven by a greater number of trabeculae (Tb.N; +120%; Table 1). We also observed the presence of trabecular bone in the midshaft of mice receiving the young GATA1$^{low/low}$ cells, which is a characteristic trait of this mutation (Figure 3C,D). Consistent with a loss of the ability of old MKs to enhance bone mass, we did not observe any differences between C57BL/6 mice receiving old GATA1$^{low/low}$ mutant or old wild-type cells (Figure 3 and Table 1). Unlike young mutant cells, old GATA1$^{low/low}$ cells failed to induce midshaft trabecular bone accumulation (Figure 3C,D and Table 1). To verify that the adoptive transfer of the old GATA1$^{low/low}$ cells indeed worked, we also compared spleen weight and platelet counts (Figure 3E,F). Indeed adoptive transfer of GATA1$^{low/low}$ cells significantly increased spleen mass for both young (+124%; $p < 0.001$) and old (+37%; $p < 0.05$) cells when compared to adoptive transfer of wild-type cells. There were also significantly fewer circulating platelets in mice that received either young (-75%; $p < 0.001$) or old (-90%; $p < 0.001$) GATA1$^{low/low}$ cells.

3.4 Adoptive Transfer of Aged Hematopoietic Cells Results in Cortical Expansion at the Femoral Midshaft and Increased Trabecular Bone Fraction in the Distal Femur

Interestingly, adoptive transfer with old hematopoietic cells resulted in a significant increase in cortical expansion compared to that observed with young spleen cells, independent of genotype. Specifically, as detailed in Table 1, transplantation of old wild-type cells significantly increased cortical tissue area (T.Ar; +31%; $p < 0.001$), bone area (B.Ar; +22%; $p < 0.001$), and marrow area (M.Ar; +37%; $p < 0.001$) compared to that observed with transplantation of young wild-type cells. Likewise, transplantation of old GATA1$^{low/low}$ hematopoietic cells significantly increased cortical tissue area (T.Ar; +26%; $p < 0.001$), bone area (B.Ar; +21%; $p < 0.001$), and marrow area (M.Ar; +32%; $p < 0.001$) compared to that observed with transplantation of young GATA1$^{low/low}$ cells.

With regard to trabecular bone parameters (Table 1), transplantation of old wild-type cells were better than young wild-type cells at increasing trabecular bone fraction (BV/TV; +129%; $p < 0.001$). This appears to be a result of old wild-type cells increasing trabecular thickness (Tb.Th; +35%; $p < 0.001$) and number (Tb.N; +66%; $p < 0.001$), and a decrease in trabecular spacing (Tb.Sp; -9%; $p=0.002$). As noted above, the transplantation of old GATA1$^{low/low}$ cells resulted in a trabecular bone phenotype which was not different from that of transplantation of old wild-type cells.

**Table 1. Bone mass parameters as measured by µCT in recipient mice following 8wk adoptive transfer of young or old, wild-type or GATA1$^{low/low}$ hematopoietic cells[a]**

| | Young Donors | | Old Donors | |
|---|---|---|---|---|
| Variables compared[b] | Wild-type | GATA1$^{low/low}$ | Wild-type | GATA1$^{low/low}$ |
| **Distal trabecular** | | | | |
| BV/TV (%) | 3.53 ± 0.31 | **8.67 ± 1.34*** ** | 8.10 ± 0.71 | 7.34 ± 1.01 |
| Tb.Th (mm) | 0.051 ± 0.001 | 0.055 ± 0.001 | 0.069 ± 0.002 | 0.070 ± 0.005 |
| Tb.Sp (mm) | 0.336 ± 0.007 | 0.318 ± 0.021 | 0.306 ± 0.005 | 0.328 ± 0.013 |
| Tb.N ($mm^{-1}$) | 0.693 ± 0.055 | **1.541 ± 0.227*** ** | 1.148 ± 0.073 | 1.006 ± 0.093 |
| Conn.D ($\mu m^{-3}$) | 0.020 ± 0.002 | **0.110 ± 0.023*** ** | 0.030 ± 0.003 | 0.030 ± 0.004 |
| **Midshaft trabecular** | | | | |
| BV/TV (%) | 0.02 ± 0.01 | **7.00 ± 2.57*** ** | 0.35 ± 0.14 | 0.79 ± 0.43 |
| Tb.Th (mm) | 0.013 ± 0.003 | **0.051 ± 0.007*** ** | 0.041 ± 0.007 | 0.053 ± 0.007 |
| Tb.Sp (mm) | 0.475 ± 0.002 | 0.415 ± 0.030 | 0.730 ± 0.011 | 0.754 ± 0.022 |
| Tb.N ($mm^{-1}$) | 0.008 ± 0.004 | **1.083 ± 0.420*** ** | 0.061 ± 0.017 | 0.107 ± 0.044 |
| **Midshaft cortical** | | | | |
| Diaphysis length (mm) | 12.41 ± 0.07 | 12.27 ± 0.04 | 13.62 ± 0.13 | 13.68 ± 0.09 |
| T.Ar ($mm^2$) | 1.57 ± 0.02 | 1.59 ± 0.02 | 2.05 ± 0.05 | 2.01 ± 0.07 |
| B.Ar ($mm^2$) | 0.73 ± 0.01 | 0.71 ± 0.04 | 0.89 ± 0.02 | 0.86 ± 0.03 |
| B.Ar/T.Ar (%) | 46.5 ± 0.4 | 45.2 ± 2.1 | 43.8 ± 0.8 | 42.9 ± 1.0 |
| M.Ar ($mm^2$) | 0.84 ± 0.01 | 0.88 ± 0.05 | 1.15 ± 0.04 | 1.16 ± 0.05 |
| Ct.Th (mm) | 0.17 ± 0.01 | 0.17 ± 0.01 | 0.18 ± 0.01 | 0.17 ± 0.01 |
| MMI polar ($mm^4$) | 0.29 ± 0.01 | 0.28 ± 0.02 | 0.49 ± 0.02 | 0.46 ± 0.03 |

[a] Values are expressed as mean ± S.E.M. (n = 10-13). Bolded values represent statistically significant differences following 2-way ANOVAs and Holm-Sidak post-hoc analysis. Comparisons were between age-matched wild-type and mutant parameters. (*** $p < 0.001$).

[b] BV/TV = bone volume / total volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular spacing; Tb.N = trabecular number; Conn.D = connectivity density; T.Ar = tissue area; B.Ar = bone area; M.Ar = marrow area; Ct.Th = cross-sectional thickness; MMI = mean moment of inertia.

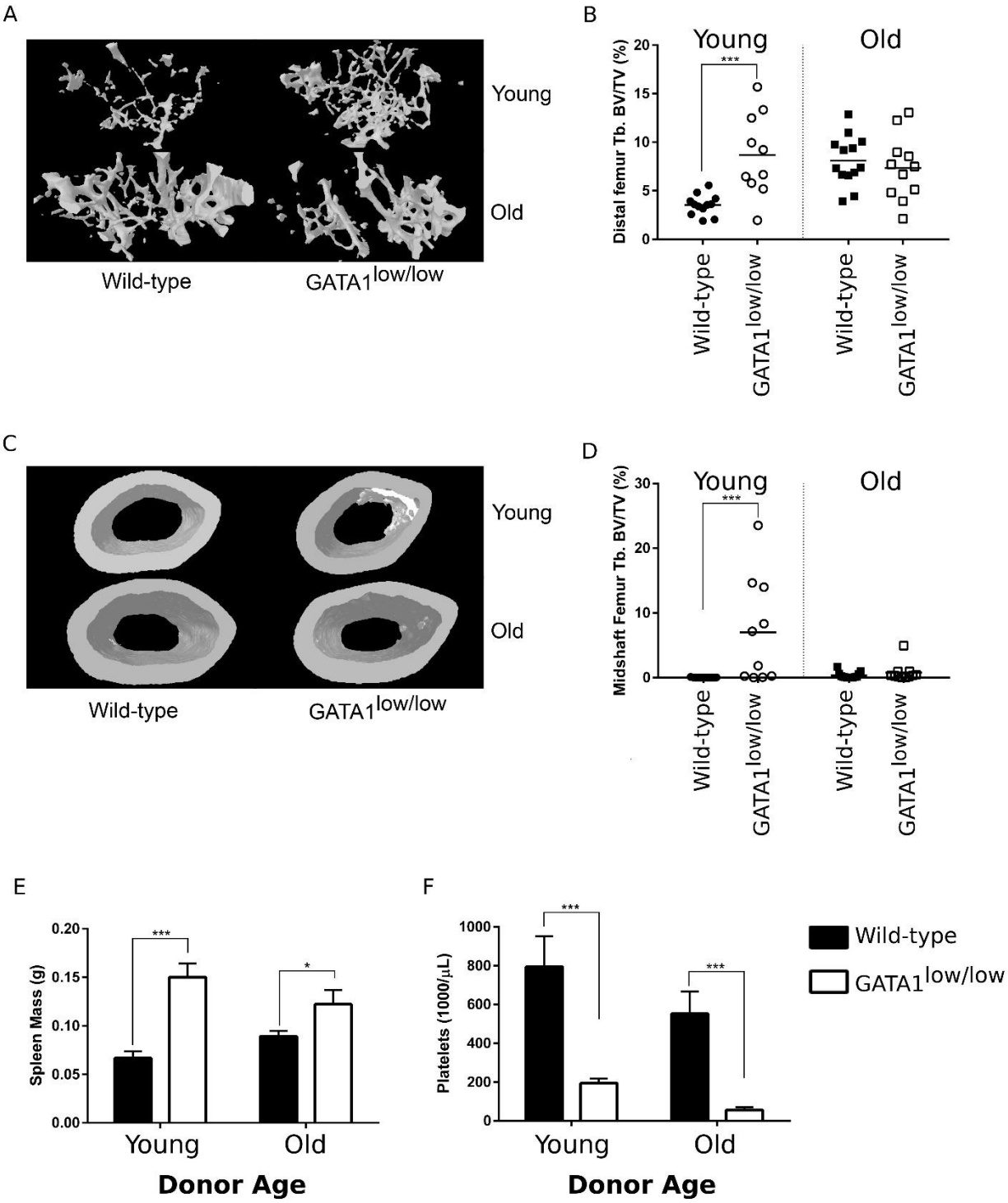


**Figure 3. Adoptive transfer of young, but not old megakaryocyte skewed hematopoietic cells, increased bone mass in young wild-type recipient mice.** Young C57BL/6 male mice were lethally irradiated and transplanted with young (3 mo) or old (18 mo) wild-type or GATA1$^{low/low}$ spleen cells by retro-orbital injection. Adoptively transferred (recipient) mice were euthanized and analyzed for femoral bone phenotype by μCT after 8 weeks. (A) Representative 3D renderings of distal trabecular bone from each group. (B) μCT analyses of bone volume fraction (BV/TV) of distal trabecular region. (C)

Representative 3D renderings of midshaft cortical bone with trabecular bone highlighted in white. (D) μCT analyses of BV/TV of midshaft trabecular bone region. (E) Comparisons of spleen mass at sacrifice. (F) Platelet counts taken from whole blood at sacrifice. (B,D) Each data point represents a unique animal. Black bars indicate average of population. (E,F) Bars are averages with S.E.M. Two-way ANOVA with Holm-Sidak post-hoc analyses. * $p < 0.05$,*** $p < 0.001$. Tb = trabecular.

## 4. Discussion

The work outlined in this paper extends from our previous publications showing how MKs orchestrate increased bone mass through actions on osteoblasts [9, 12, 16-19]. To better understand how MKs might be involved with age-related bone loss, we sought to determine how the bone marrow MK population changes during aging, and what effect age has on the ability of MKs to alter osteoblast activities.

To determine how aging affects the MK population, we assessed the frequency of MKs in the bone marrow of C57BL/6 mice. We observed an age-related increase in the frequency of MK cells in the bone marrow and an increase in the number of MKs generated from aged spleens in vitro (Figure 1). Further, as shown in Supplementary Figure 1 and as reported by Vannuchi et al (2002), spleens from old wild-type and old GATA-$1^{low/low}$ mice contained more MK/MK progenitors than did genotype matched young mice [43]. While it is unclear whether the increased number of MKs was due to an increase in MK progenitors or an increased sensitivity to TPO, an age-associated skewing to the MK/erythrocyte progenitor (MEP) population has been observed in both humans and mice [43, 44].

We then explored the interaction between MKs and osteoblasts during aging by comparing both the ability of young and old MKs to stimulate osteoblast proliferation, as well as, by comparing the ability of young and old osteoblasts to respond to MKs. While old MKs trended toward reduced ability to stimulate the proliferation of osteoblasts, the most striking difference we observed was the inability of old osteoblasts to respond to MKs (Figure 2).

MKs have been shown to produce TGF-β isoforms 1, 2, and 3 [41]. TGF-β is a major bone cytokine [45] and stimulates the proliferation of osteoblast progenitors, but inhibits their maturation [46]. Our data shown in Figure 2B demonstrate that TGF-β1 mRNA expression was markedly reduced in MKs derived from old mice. These data are consistent with those of Gazit et al (1998) showing that there is a decrease in TGF-β availability and signaling in bone from aged male mice. One of the co-receptors for TGF-β, TGFβR-1a was shown to be downregulated in aged osteoblasts [47], and old rat and human osteoblasts showed a lack of response to TGF-β [48, 49], which suggests that old osteoblasts may be less responsive to TGF-β from MKs. While these data demonstrate distinct differences with age, they do not conclusively demonstrate the role of MK-mediated TGF-β production in this process. Should future studies confirm this mechanism of action, increasing TGFβR-1a expression and/or activity or downstream TGFβR signaling in aged osteoblasts may serve as an important therapeutic approach to increasing bone mass in aged individuals.

To confirm that aged MKs would have a reduced capacity to stimulate anabolic bone responses in vivo, we used the adoptive transfer model in which the hematopoietic cells of young C57BL/6 mice were replaced with either young or old GATA$1^{low/low}$ hematopoietic cells, and findings were compared to the bone phenotype of mice receiving age-matched wild-type cells (Figure 3 and Table 1). Consistent with a reduced ability to enhance bone mass, mice receiving spleen cells from old GATA$1^{low/low}$ donors showed

no enhancement of trabecular bone in either the distal femur or cortical midshaft. This is in stark contrast to the trabecular bone enhancement that was observed in mice receiving spleen cells from young GATA1$^{low/low}$ donors. Further, our in vitro results suggested that aging had a negative effect on both the ability of MKs to stimulate osteoblast cell counts as well as a reduced ability of osteoblasts to respond to MKs (Figure 2). While not proof of cause, taken together our data suggest that the increase in MK/MK progenitors seen in old C57BL/6 donors, young GATA-1$^{low/low}$ donors, and old GATA-1$^{low/low}$ donors compared to young C57BL/6 donors is responsible for the bone phenotype observed in recipient mice.

Interestingly, adoptive transfer of old hematopoietic cells (irrespective of genotype), resulted in a significant increase in cortical expansion. This cortical expansion may be explained by transplantation of increased numbers of osteoclast progenitors (myeloid skewing with aging), resulting in increased numbers of osteoclasts forming in recipient mice. Additionally, adoptive transfer of old C57BL/6 hematopoietic cells resulted in a significant increase in trabecular bone volume fraction compared to transfer of young C57BL/6 hematopoietic cells. The increase in trabecular bone volume fraction was likely due to an increase in both trabecular number and thickness, suggesting increased osteoblast activity. It is intriguing that transfer of old hematopoietic cells into young recipient mice results in striking changes in bone phenotype compared to transfer of young wild-type cells. Indeed, as inflammation is a well-documented aging phenomenon ("inflammaging"), it is possible that transplantation of any old hematopoietic cells into young mice could result in an initial stimulation of osteoblast activity, due to increased expression of numerous inflammatory cytokines. Alternatively, the increase in osteoclast progenitors in mice transplanted with old donor cells, could themselves stimulate the recipient osteoblast cells. Understanding the mechanisms responsible for the increased trabecular bone mass with transplantation of old donor cells, and whether the bone phenotype would persist over time, remains to be determined.

In conclusion, we demonstrated that aging negatively affects the ability of MKs to enhance bone mass and that this was due to a reduction in MK-stimulated osteoblast proliferation. Because the age-related decline of bone mass is driven by osteoclastic bone resorption outpacing osteoblastic bone formation [1, 2], the decline in the ability of MKs to orchestrate osteoblastic activity may be a contributing factor.

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Author Statement

KAM, ERH, APP, HLC, PS, JG, SFM, IA, AF, CS, J-MH, PC, and MA were responsible for data curation, formal analysis, investigation, and methodology. KAM prepared the original draft. EFS, AB, LMP, CMO, MAK were responsible for conceptualization, supervision, funding acquisition, project administration, formal analysis, and manuscript preparation. All authors assisted in reviewing/editing the manuscript and approve of the final version.

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**Highlights**

- Megakaryocytes and platelets increase with age but bone mass declines
- Megakaryocyte increase of osteoblasts and bone formation declines with age
- Aging decreases response of bone cells to megakaryocytes
- Transplantation of old hematopoietic cells increases cortical bone expansion
- Transplantation of old hematopoietic cells increase trabecular bone parameters