Megakaryocyte and Osteoblast Interactions Modulate Bone Mass and Hematopoiesis

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Emerging evidence demonstrates that megakaryocytes (MK) play key roles in regulating skeletal homeostasis and hematopoiesis. To test if the loss of MK negatively impacts osteoblastogenesis and hematopoiesis, we generated conditional knockout mice where *Mpl*, the receptor for the main MK growth factor, thrombopoietin, was deleted specifically in MK (*Mpl^{ff};PF4^{cre}*). Unexpectedly, at 12 weeks of age, these mice exhibited a 10-fold increase in platelets, a significant expansion of hematopoietic/mesenchymal precursors, and a remarkable 20-fold increase in femoral midshaft bone volume. We then investigated whether MK support hematopoietic stem cell (HSC) function through the interaction of MK with osteoblasts (OB). LSK cells (Lin[−]Sca1⁺CD117⁺, enriched HSC population) were co-cultured with OB+MK for 1 week (1wk OB+MK+LSK) or OB alone (1wk OB+LSK). A significant increase in colony-forming units was observed with cells from 1wk OB+MK cultures. Competitive repopulation studies demonstrated significantly higher engraftment in mice transplanted with cells from 1wk OB+MK+LSK cultures compared to 1wk OB+LSK or LSK cultured alone for 1 week. Furthermore, single-cell expression analysis of OB cultured±MK revealed adiponectin as the most significantly upregulated MK-induced gene, which is required for optimal long-term hematopoietic reconstitution. Understanding the interactions between MK, OB, and HSC can inform the development of novel treatments to enhance both HSC recovery following myelosuppressive injuries, as well as bone loss diseases, such as osteoporosis.

Keywords: megakaryocytes, osteoblasts, hematopoietic stem cells, bone regeneration, hematopoiesis

Introduction

MEGAKARYOCYTES (MK) ARE HEMATOPOIETIC stem cell (HSC)-derived cells and are the largest and one of the rarest cells (0.05%–0.1%) found in the bone marrow (BM) [1]. They produce platelets, which stop bleeding and minimize blood vessel injury. Within the BM, they are primarily located centrally, near sinusoidal endothelial cells, where they release platelets into the circulation [2]. MK also play significant roles in the maintenance/quiescence of HSC and bone formation [3,4].

Recent whole-mount imaging and computational studies in mice show that HSC are frequently located adjacent to MK. This suggests a functional relationship, and MK have been postulated to regulate HSC quiescence and pool size [3,4]. Using genetic strategies to deplete MK, two groups observed that in the absence of MK, HSC proliferation increased, suggesting that MK maintain HSC quiescence in the niche [3,4]. MK expression of PF4 (platelet factor 4, also known as *CXCL4*) and transforming growth factor β was

implicated in this process. Of importance, these studies genetically regulated MK *in vivo*; therefore, they could result from direct MK activities on HSC or through indirect interactions between MK and other components of the niche.

Work from Nilsson's laboratory describes an indirect effect of MK on HSC quiescence [5]. This group has shown that mature MK produce factors that generate thrombin, which in turn cleaves osteopontin (an extracellular matrix protein found at the endosteal surface), opening a cryptic binding site for $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins. HSC binding to this site resulted in maintenance of HSC quiescence through inhibiting proliferation and differentiation [5]. These studies elucidate the role of MK in supporting HSC maintenance.

The endosteal niche is a complex structure lining the interiors of cortical bone and trabecular bone surfaces that contain several mesenchymal lineage cells. Endosteal osteoblasts (OB) are derived from mesenchymal precursors and are involved in the regulation of bone formation and HSC function [6,7]. Our group and others have demonstrated that less mature OB-lineage cells better support hematopoiesis

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than more mature cells [8–10]. Thus, it is likely that, increased osteoblastogenesis alters the BM microenvironment/HSC niche. Indeed, our group has previously demonstrated that MK regulate bone formation by increasing osteoblastogenesis through direct cell-to-cell contact [11]. A portion of this effect is mediated by $\beta 1$ integrin and Pyk2 signaling pathways [12,13].

MK regulation of bone formation *in vivo* was demonstrated when mice, deficient in transcription factors required for normal MK development (GATA-1 or NF-E2), exhibited significant increases in immature MK numbers and trabecular bone [11]. In addition, mice overexpressing thrombopoietin (TPO), the major growth factor for MK, also have increased numbers of MK and present with an osteosclerotic bone phenotype [14]. Therefore, mouse models with increased numbers of MK in BM consistently induce a high bone mass phenotype. Paradoxically, MK inhibit OB differentiation *in vitro*. Specifically, OB-lineage cells in long-term co-cultures (14 days) showed that MK inhibited the expression of OB differentiation markers [15]. These data demonstrate that MK regulate OB proliferation and differentiation [11,15].

To further understand the impact that interactions between MK and OB-lineage/osteoprogenitor cells have on bone formation and hematopoiesis, we generated mice where the TPO receptor, the cellular homologue of the myeloproliferative leukemia virus oncogene (*Mpl*), was specifically deleted in MK (*Mpl^{fl/fl};PF4cre*) to examine the effect of the loss of MK on OB-mediated hematopoiesis. Our results are consistent with work by Ng et al. [16] who recently characterized the hematopoietic phenotype of a similar mouse model. Although some of our data confirm what Ng et al. documented, we demonstrate a previously unknown interaction between MK and OB that leads to enhanced OB-mediated support of hematopoiesis. This effect is likely mediated through MK-induced expression of adiponectin by OB-lineage cells. Unexpectedly, we also confirm the ability of MK to regulate bone formation by generating a mouse model of megakaryocytosis, resulting in a high bone mass phenotype.

Materials and Methods

Animals

Commercially available, C57BL/6, B6.SJL-Pt_{cq}Pep3b/BoyJ (BoyJ), C57BL/6 X BoyJ (F1), and C57BL/6-Tg (PF4-icre) Q3Rsko/J (The Jackson Laboratory, Bar Harbor, ME) mice were used in this study. Mice were bred and housed in the animal facility at Indiana University. For transplantation studies, recipient mice received 1,000 cGy ionizing radiation from a cesium source (650 and 350 cGy, split dose). All procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine and followed National Institutes of Health guidelines.

Generation of *Mpl* conditional knockout mice

The conditional knockout strategy used in preparing these mice involved the introduction of loxP sites flanking coding exon 3 of the *Mpl* gene by homologous recombination (Ingenious Targeting Laboratory, Ronkonkoma, NY). Briefly, a 7.78 kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 BAC clone

(RP23: 118J14) into the backbone vector (pSP72; Promega, Madison, WI). The 5' homology arm extends about 5.31 kb and contains the first two exons. The 5' loxP site, containing an engineered EcoRV, Msc I, and Sca I site for Southern blot analysis, was inserted upstream of exon 3. The loxP-FRT-flanked pGK-gb2-Neo cassette was inserted downstream of exon 3 with the 3' arm extending 1.9 kb. The targeting construct was verified by restriction analysis and sequencing after each modification step.

Targeting of the *Mpl* locus was confirmed by Southern blot of EcoRV-digested genomic DNA hybridized with a 5' probe from targeted iTL BA1(C57BL/6 $\times$ 129/SvEv) hybrid embryonic stem cells microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage agouti coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. Mice were genotyped by polymerase chain reaction (PCR) using the following primers: (1) 5'-ctgcctctctacgtctcaagtc-3' and (2) 5'-gaggaaggtgctgtcctg-3'. Mice homozygous for the *Mpl* floxed allele (*Mpl^{fl/fl}*) on a mixed C57BL/6-Sv129 genetic background were crossed with *PF4Cre* on a C57BL/6 background. Unless specified, male and female *Mpl^{fl/fl};PF4cre* and their littermate counterparts (*Mpl^{fl/fl}*, *Mpl^{fl/+}*; *PF4cre*) were used at 8, 12, and 17 weeks of age.

Quantitative real-time PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and was used to generate complementary DNA (cDNA) by reverse transcription according to the manufacturer's instructions (Transcriptor First Strand cDNA Synthesis kit; Roche, Indianapolis, IN). PCR were performed in an MX3000 detection system using SYBR green PCR reagents following the manufacturer's instructions (Life Technologies, Warrington, UK). For *Mpl*, and GAPDH expression, their specific pairs of primers were used, a calibration curve was performed, and all oligonucleotides were tested to ensure specificity and sensitivity. For each sample, arbitrary units obtained using the standard curve and the expression of GAPDH was used to normalize the amount of the investigated transcript.

Mpl 5' tcacctgggtgactgctctg 3' 5' ggacttagggtgcagtgctc 3'
GAPDH 5' accacgtccatgccatcac 3' 5' tccaccacctgttgctgta 3'

TPO serum concentrations

Whole blood was collected, serum separated, and stored at -80°C until assayed. The Mouse Thrombopoietin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) was used to determine the TPO serum concentration, following the manufacturer's instructions.

Complete blood counts

Whole blood was collected and complete blood counts were performed using a validated HEMAVET[®] 950FS Hematology System (Drew Scientific, Waterbury, CT).

Histology

Femurs were dissected and fixed in 10% neutral buffered formalin for 48 h before being transferred to 70% ethanol for storage. Static histomorphometric measurements were performed on von Kossa stained (AgNO₃; Sigma-Aldrich, St.

Louis, MO) and toluidine blue (Sigma-Aldrich) counter-stained sections to determine OB and MK number [17]. Tartrate-resistant alkaline phosphatase (TRAP) staining was performed to visualize osteoclasts (OC).

Ex vivo microcomputed tomography

Microcomputed tomography (μ CT, Skyscan 1172; Sky-Scan, Kontich, Belgium) was used to determine properties of the trabecular and cortical bone from both the distal and midshaft of the right femur. Parameters used were as follows: 60 kV, 0.5 mm aluminum filter, and 6 μ m pixel size [18]. Trabecular bone values from the distal femur were obtained from a 0.5 mm thick region, 0.5 mm proximal from the distal growth plate, and included the following: bone volume/tissue volume (BV/TV; %), trabecular number (Tb.N; mm^{-1}), trabecular thickness (Tb.Th; mm), and trabecular separation (Tb.Sp; mm). Cortical and midshaft trabecular bone values were obtained from a 1 mm thick region covering an area 0.5 mm proximal and distal from the midpoint of the femur. Cortical parameters included were as follows: bone area (B.Ar; mm^2), cortical area (Ct.Ar; mm^2), marrow area (M.Ar; mm^2), BV/TV, mean polar moment of inertia (MMI polar; mm^4), and cortical thickness (Ct.Th; mm). Midshaft trabecular parameters measured were the same as for the distal femur.

Preparation of OB and MK

Calvarial OB were prepared after a modification of published methods [19,20]. Briefly, calvariae from C57BL/6 mice <48 h old were dissected, pretreated with EDTA (4 mM; Sigma-Aldrich) in phosphate-buffered saline (PBS) for 30 min, and then subjected to sequential collagenase digestions (200 U/mL; Worthington Biomedical, Lakewood, NJ) at 37°C. Fractions 3–5 (collected between 45–60, 60–75, and 75–90 min through the digestion) were collected and used as OB. These cells are ~95% OB or OB precursors and 5% osteal macrophages. Freshly prepared OB were used for all studies. To isolate MK, livers from E13 to E15 fetal mice were collected and single-cell suspensions were prepared and cultured in Dulbecco's modified Eagle's medium (Life Technologies) with 10% fetal calf serum (Hyclone, Logan, UT) and 1% conditioned medium from a murine TPO-secreting fibroblast cell line. MK were separated from other cells using a one-step albumin gradient (Probumin; Millipore, Kankakee, IL) [21].

Flow cytometry for HSC, mesenchymal stem cell, and OB phenotyping

Low-density BM cells were washed once with stain wash (PBS, 1% bovine calf serum, and 1% penicillin-streptomycin), followed by antibody staining for 15 min on ice with phycoerythrin (PE)-conjugated CD3, CD4, CD45R, Ter119, and Gr1; Alexa Fluor 700 (AF700)-conjugated c-Kit (CD117); (PE)-Cy7-conjugated Sca1; fluorescein isothiocyanate (FITC)-conjugated CD34; Allophycocyanin (APC)-conjugated Flk2 (CD135); PerCP-Cy5.5-conjugated IL7Ra (CD127); and APC-Cy7 conjugated anti-Fc- γ R (CD16/32). All monoclonal antibodies were obtained from BD Biosciences (San Jose, CA), except CD127 from eBioscience (San Diego, CA) and anti-Fc- γ R from BioLegend (San Diego,

CA). Cells were washed once more with stain wash buffer, and data were acquired on BD LSRII (BD Biosciences). Progenitor cell content was gated and analyzed as described before [22], using FlowJo software (FlowJo, LLC, Ashland, OR).

For mesenchymal stem cell (MSC) phenotyping, BM cells were washed once with stain wash followed by antibody staining for 15 min on ice with PE-Cy7-conjugated CD45; FITC-conjugated CD31 and Ter119; PE-conjugated CD51 (BD Biosciences); and APC-conjugated PDGFRa (BioLegend). Lineage (CD45, CD31, and Ter119)-negative cells were analyzed for the presence of CD51 and PDGFRa [23]. For MK phenotyping, BM cells were treated as above, stained with PE-conjugated CD41 and FITC-conjugated CD61 (BD Biosciences), and analyzed for the presence of CD41 and CD61. Cell sorting was performed on a BD fluorescence-activated cell sorting (FACS) Aria and flow cytometric analysis was done on a BD LSRII.

For OB phenotyping, OB were generated from femurs and the epiphyses were removed from the diaphysis, forming two separate groups for analysis. The BM from the diaphysis was then flushed. After flushing, the bone pieces from both the groups were minced into segments (<1-mm), and then the bone segments were washed once with PBS. Bone segments were then subjected to five consecutive 15 min collagenase digestions. Cells were harvested after each digestion, pooled, and then stained with FITC-conjugated CD45, Ter119, and CD31 (BioLegend); APC: Cy7-conjugated Sca-1 (BioLegend); and PE-conjugated CD166 (eBioscience). CD45⁺Ter119⁺CD31⁺Sca-1⁺ cells were gated as OB and then analyzed for the presence or absence of CD166 on a BD Fortessa (BD Biosciences). Data were analyzed and plots were made using FlowJo software.

Cell co-culture and colony-forming unit assay

Neonatal 2-day calvarial OB and fetal liver MK were prepared as detailed above and as previously described [19,20]. Calvarial OB were cultured alone (60,000 cells/well) or with MK (100,000 cells/well) for 7 days in α -minimum essential medium (α -MEM; Life Technologies) with 10% fetal bovine serum (FBS; Hyclone), supplemented with ascorbic acid (50 μ g/mL; Sigma-Aldrich). MK were removed from the wells (four washes) as described before [19,20]. Freshly isolated OB, OB cultured for 1 week, OB co-cultured with MK for 1 week, and MK alone were seeded in 12-well plates for ~24 h before co-culture with 1,000 freshly sorted murine LSK (Lin⁺Sca1⁺CD117⁺, enriched HSC population) cells from BoyJ (CD45.1) mice.

The medium consisted of 1:1 mix of Iscove's modified Dulbecco's medium (Life Technologies) and α -MEM supplemented with a cocktail of cytokines containing recombinant murine stem cell factor (SCF) and IL-3 (10 ng/mL; PeproTech, Rocky Hill, NJ), IGF-1 and TPO (20 ng/mL; PeproTech), IL-6 and fms-related tyrosine kinase 3 (Flt3; 25 ng/mL; PeproTech), and osteopontin (50 ng/mL; R&D Systems). Cultures were maintained for 7 days and hematopoietic cells were then harvested and plated in duplicate in 3 cm Petri dishes containing 1 mL of methylcellulose with included cytokines (recombinant human IL-6, erythropoietin, and insulin; recombinant mouse SCF, IL-3; MethoCultTM GF M3434; STEMCELL Technologies, Vancouver, Canada) for colony-forming unit (CFU) assay. Cultures were maintained at 37°C in a humidified

incubator at 5% CO₂, and colonies were counted on an inverted microscope after 7 days.

Bone marrow repopulating assay

Cultures of 1wkOB+LSK, 1wkOB+MK+LSK, and LSK were maintained in 12-well plates for 5 days and then co-transplanted with 100,000 C57BL/6 (CD45.2) competitor cells through intravenous injection into lethally irradiated CD45.2×CD45.1 F1 recipients (4–5 recipients/group). Chimerism was assessed monthly for 4 months using peripheral blood (PB). After red blood cells were lysed with RBC lysis buffer (BioLegend), cells were stained with FITC-conjugated CD45.1 and PE-conjugated CD45.2, and chimerism was assessed as [CD45.2/(CD45.2 + CD45.1)]/100. This calculation was therefore independent of any residual host-derived HSC activity. The mice were euthanized at 4 months and marrow flushed from the femurs was stained as described above with CD45.1 and CD45.2 antibodies, and chimerism was determined.

Microfluidic chip-based single-cell analysis

Calvarial OB cultures were plated±MK and were collected at day 4. Co-cultures were washed four times with PBS to remove MK, trypsinized, and FACS sorted. CD45⁺ OB-lineage single cells from both cultures were used for

single-cell quantitative PCR (qPCR) on the Fluidigm platform (San Jose, CA). Briefly, on a C1 integrated fluidic circuit chip (IFC), 56–84 individual cells were captured, cDNA synthesized, and product preamplification was performed. Individual cell qPCR was done using a Biomark 96.96 dynamic array. Initial data analysis was completed using Fluidigm real-time PCR analysis software. Differential gene expression was determined and violin plots generated with the FluidigmSC R-package software.

Statistical analysis

Data are presented as mean±standard deviation and where applicable, triplicate samples were measured. Two-tailed Student's *t*-tests were performed when two groups were compared. One-way and two-way analysis of variance (ANOVA) were used for multiple group comparisons, followed by Tukey's honest significant difference (HSD) or Bonferroni's post hoc *t*-tests. Significance was set at 0.05.

Results

Development of *Mpl*^{fl} MK conditional knockout mice

We generated conditional knockout mice where *Mpl* was deleted specifically in MK (*Mpl*^{fl};PF4cre). Our strategy involved the introduction of loxP sites flanking coding exon 3 of the *Mpl* gene by homologous recombination (Fig. 1A),

FIG. 1. Gene targeting of exon 3 in the *Mpl* gene. **(A)** Schematic of the targeting strategy. The 5' loxP site was inserted upstream of exon 3, followed by the insertion of an *Frt*-pGK-loxP-Neo-*Frt*-loxP selection cassette. The expected *EcoR*V-digested fragments for wild-type and targeted alleles are indicated by large arrows. The 5' external probe is indicated by a short line and the 5' and 3' homology arms are shown in gray. LoxP and *Frt* sites are shown by filled triangles and empty triangles, respectively. Genotyping primers are shown as small arrows. *Flp*, *F* protein recombinase; *PF4cre*, Platelet factor 4 cre recombinase mice. **(B)** Southern blot analysis with the 5' probe. 1–5, *Mpl*^{fl/+} mouse lines; HYB, C57BL/6/129 hybrid; B6, C57BL/6; 129, 129/SvEv mice. **(C)** PCR analysis of mouse tail DNA obtained from *Mpl*^{fl/+} intercrosses, indicating transmission of the targeted (KO) allele. **(D)** qPCR of cDNA from MK showing expression of *Mpl*, *n* = 3–5 mice per genotype, Student's *t*-test, **P* < 0.001. cDNA, complementary DNA; KO, knockout; MK, megakaryocytes; qPCR, quantitative polymerase chain reaction.

similar to the strategy used to generate the global *Mpl* knockout mice (*Mpl*^{-/-}) [24]. Southern blot analysis of embryonic stem cell clones (Fig. 1B) and PCR of mouse tail DNA (Fig. 1C) were used to confirm homologous recombination. We mated homozygous *Mpl*^{fl/fl} mice with *PF4cre* mice; PF4 is a platelet-specific chemokine that is expressed by MK and platelets [25]. qPCR was used to evaluate the expression of *Mpl* in MK isolated from *Mpl*^{fl/fl} and *Mpl*^{fl/fl}; *PF4cre*, which confirmed a decrease of 90%–95% in MK from *Mpl*^{fl/fl}; *PF4cre* mice (Fig. 1D).

Mpl^{fl/fl}; *PF4cre* mice exhibit thrombocytosis and megakaryocytosis

Complete blood cell counts revealed that *Mpl*^{fl/fl}; *PF4cre* mice did not exhibit thrombocytopenia as is seen in global *Mpl*^{-/-} mice [24,26]. Instead, there was a significant 10-fold increase in the number of platelets compared to *Mpl*^{fl/fl} and *Mpl*^{fl/+}; *PF4cre* mice (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). In *Mpl*^{fl/+}; *PF4cre*, one wild-type *Mpl* allele appeared to rescue the increase in platelet levels. MK and platelets are thought to be the major consumers of TPO through Mpl and

a decrease in the expression of Mpl on MK shifts the steady state of TPO levels higher in the circulation as shown in *Mpl*^{-/-} mice [24]. Therefore, we analyzed the serum levels of TPO in *Mpl*^{fl/fl}; *PF4cre* mice. Unexpectedly, the concentration of TPO in the serum of *Mpl*^{fl/fl}; *PF4cre* was not significantly different than in *Mpl*^{fl/fl} or the *Mpl*^{fl/+}; *PF4cre* mice (Supplementary Fig. S1) [16]. HSC express Mpl [27] and recently, we have shown that OB and OC precursors also express Mpl and are responsive to TPO [28]. As the TPO serum concentrations between the three genotypes were not different, we surmise that TPO serum levels are regulated, in part, by a process independent of MK/platelet consumption.

While *Mpl*^{fl/fl}; *PF4cre* mice are healthy and appear no different from controls, they displayed significant splenomegaly compared to *Mpl*^{fl/fl} and *Mpl*^{fl/+}; *PF4cre* mice (Supplementary Table S2). FACS analysis of BM cells indicated a sevenfold increase in the percentage of MK (CD41⁺CD61⁺) in the *Mpl*^{fl/fl}; *PF4cre* mice compared to *Mpl*^{fl/fl} mice (Fig. 2A), and is consistent with the high numbers of platelets in the mutant mice (Supplementary Table S1). Histological staining also confirmed the expansion of the MK pool in the mutant BM (Fig. 2B).

FIG. 2. Skeletal and mesenchymal stem cell phenotype of the *Mpl*^{fl/fl}; *PF4cre* mice. (A) The percentage of MK in the BM. (B) Histological sections of the BM stained with von Kossa and toluidine blue. Yellow arrows indicate MK. Scale bar = 100 μ m. (C, D) Longitudinal and cross-sectional μ CT images of *Mpl*^{fl/fl} and *Mpl*^{fl/fl}; *PF4cre* femurs. BV/TV in the femoral midshaft (E) and near the growth plate (F). $n = 6$ –8 mice/genotype, Student's t -test, $*P < 0.001$ (Table 1). (G) FACS analysis of the mesenchymal stem cell populations in the BM. $N = 3$ –5 mice/genotype. One-way ANOVA, $*P < 0.002$. μ CT, microcomputed tomography; ANOVA, analysis of variance; BM, bone marrow; BV/TV, bone volume/tissue volume. FACS, fluorescence-activated cell sorting.

TABLE 1. SKELETAL PHENOTYPE OF 12-WEEK-OLD *MPL^{ff};PF4^{Cre}* FEMALE MICE

	Genotype	μ CT				Static histomorphometry	
		BV/TV	Tb.Th	Tb.N	Tb.Sp	N.Ob/mm	N.Oc/mm
Epiphysis	<i>Mpl^{ff}</i>	17 ± 2	0.03 ± 0.001	5.8 ± 0.7	0.17 ± 0.03	10.0 ± 2.5	2.9 ± 0.3
	<i>Mpl^{ff};PF4^{Cre}</i>	20 ± 3	0.02 ± 0.003 ^a	8.4 ± 1.8 ^a	0.15 ± 0.02	5.8 ± 0.6 ^a	1.9 ± 0.6 ^a
Diaphysis	<i>Mpl^{ff}</i>	0.02 ± 0.04	0.02 ± 0.01	0.03 ± 0.0	0.72 ± 0.04	N/D	N/D
	<i>Mpl^{ff};PF4^{Cre}</i>	25 ± 6 ^a	0.03 ± 0.01 ^a	4.6 ± 1.1 ^a	0.10 ± 0.02 ^a	12.0 ± 2.7	2.7 ± 0.8

μ CT $n=6-8$, static histomorphometry $n=3-5$ mice. Results are presented as mean ± SEM. Significant difference compared to *Mpl^{ff}*. ^a $p < 0.05$.

μ CT, microcomputed tomography; BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; N.Ob/mm, number of osteoblasts/mm; N.Oc/mm, osteoclast number/mm; SEM, standard error of the mean.

High bone mass phenotype in *Mpl^{ff};PF4^{Cre}* mice

To determine if the increased BM megakaryopoiesis impacted bone volume, we investigated the skeletal phenotype of *Mpl^{ff};PF4^{Cre}* mice. μ CT of femurs from female *Mpl^{ff};PF4^{Cre}* mice revealed a medullary canal replete with trabecular bone at 12 weeks of age, compared to the *Mpl^{ff}* mice (Fig. 2C, D). This striking phenotype represents a 20-fold increase in femoral BV/TV in *Mpl^{ff};PF4^{Cre}* animals at the diaphysis (Fig. 2E, F and Table 1). The skeletal phenotype of the *Mpl^{ff};PF4^{Cre}* was similar to the *Mpl^{ff}* mice (data not shown). The Tb.Th, Tb.N, and Tb.Sp were also significantly different in the *Mpl^{ff};PF4^{Cre}* compared to the *Mpl^{ff}* mice at the diaphysis (Table 1). At the distal epiphysis, there were no differences in the BV/TV, and Tb.Sp; however, Tb.Th and Tb.N were significantly different between the genotypes (Table 1).

In addition, histomorphometric analysis indicated that there was a significant 42% and 34% reduction in the number of OB/bone surface (N.Ob/BS) and OC/bone surface (N.Oc/BS), respectively, in the *Mpl^{ff};PF4^{Cre}* mice compared to controls, suggesting low bone turnover (Table 1). That said, the total number of OB and OC in the distal femur (N.Ob/T.Ar and N.Oc/T.Ar) was higher in the mutant *Mpl^{ff};PF4^{Cre}* mice because of the increased bone surface in these animals (Table 1). We also analyzed the N.Ob/BS and N.Oc/BS at the epiphysis and diaphysis in only the *Mpl^{ff};PF4^{Cre}* mice, and found no differences in their numbers (Supplementary Table S3). It should be noted that N.Ob/T.Ar and N.Oc/T.Ar were not reported as the defined regions, were different due to differences in bone geometry.

Next, we examined the cortical bone phenotype at the midshaft using μ CT. As shown in Fig. 2D and as reported in Table 2, at 12 weeks of age, female *Mpl^{ff};PF4^{Cre}* mice had a small, but significantly higher amount of cortical bone, as reflected by BV/TV and bone area at this site. Because there was no significant increase in cortical area (Ct.Ar), this in-

dicates that the increase in bone mass was primarily driven by increased endosteal, not periosteal bone mass.

Due to the increased amount of bone present in the marrow space, we performed FACS analysis on the MSC fraction (CD51⁺ PDGFR α ⁺) in the BM (which contains OB-lineage cells) and found a fourfold increase in the MSC population in *Mpl^{ff};PF4^{Cre}* mice (Fig. 2G). As we have shown that MK maintain OB in an immature state [11,15], this raised the question, why was there increased bone with increased numbers of MK in the marrow space? Therefore, we investigated variation in the maturity levels of OB at the epiphyses and the diaphyses in *Mpl^{ff};PF4^{Cre}* mice. We have previously demonstrated that Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166) is positively associated with immature OB [29]. FACS analysis was performed on OB from the femoral diaphyses and the epiphyses of 8- and 17-week-old *Mpl^{ff}* and *Mpl^{ff};PF4^{Cre}* mice (Fig. 3A). The percentage of Lin[−] Sca1[−]CD166⁺ cells was significantly higher at the epiphysis of the *Mpl^{ff};PF4^{Cre}* mice compared to the *Mpl^{ff}* mice at 8 and 17 weeks of age (Fig. 3B). This may be due to higher numbers of CD51⁺PDGFR α ⁺ osteoprogenitors in the *Mpl^{ff};PF4^{Cre}* mice. However, there were no differences in Lin[−] Sca1[−]CD166⁺ cell number within the diaphysis between the genotypes (Fig. 3C). There were also no significant differences in Lin[−] Sca1[−]CD166⁺ cell number between the epiphysis and diaphysis within either genotype (Fig. 3B, C and Supplementary Fig. S3A–C).

In an effort to assess the divergence in the development of the high bone mass phenotype between females and males, we examined the percentage of males and females at 8 and 12 weeks of age, which presented with atypical bone (>1% BV/TV) within the marrow cavity at the midshaft (Table 3). At 8 weeks, 40% of females had high bone mass at the midshaft, while this was not observed in the males. At 12 weeks, 100% of the females assessed demonstrated high bone mass at the midshaft compared to 30% of the males. It appears that male mice develop this bone phenotype 4–5 weeks later than females.

TABLE 2. CORTICAL BONE PHENOTYPE FOR 12-WEEK-OLD FEMALE *MPL^{ff};PF4^{Cre}* MICE

Genotype	N	BV/TV	Ct.Ar.	B.Ar.	M.Ar.	MMI (polar)	Ct.Th.
<i>Mpl^{ff}</i>	6	45.39 ± 0.99	1.75 ± 0.041	0.792 ± 0.021	0.954 ± 0.033	0.350 ± 0.0146	0.178 ± 0.00442
<i>Mpl^{ff};PF4^{Cre}</i>	7	49.80 ± 1.08 ^a	1.85 ± 0.071	0.919 ± 0.031 ^a	0.930 ± 0.049	0.418 ± 0.0300	0.191 ± 0.00544

Results are presented as mean ± SEM. Significant difference compared to *Mpl^{ff}*.

^a $p < 0.05$.

B.Ar., bone area; Ct.Ar., cortical area; Ct.Th., cortical thickness; M.Ar., marrow area; MMI (polar), polar moment of inertia.

FIG. 3. FACS analysis of OB maturity in *Mpl^{fl/fl};PF4^{cre}* mice femurs. (A) Dotplots showing the Lin⁻Sca1⁻CD166⁺ cells within the epiphyseal region of femurs from 17-week-old *Mpl^{fl/fl};PF4^{cre}* and *Mpl^{fl/fl}* mice. (B) Percentage of CD166⁺ OB within the femoral epiphyses. YCE, young (8-week old) control (*Mpl^{fl/fl}*) epiphysis; YME, young (8-week old) mutant (*Mpl^{fl/fl};PF4^{cre}*) epiphysis; OCE, old (17-week old) control (*Mpl^{fl/fl}*) epiphysis; OME, old (17-week old) mutant (*Mpl^{fl/fl};PF4^{cre}*) epiphysis. Student's *t*-test **P* < 0.05. (C) Percentage of CD166⁺ OB within the femoral diaphyseal region. YCD, young (8-week old) control (*Mpl^{fl/fl}*) diaphysis; YMD, young (8-week old) mutant (*Mpl^{fl/fl};PF4^{cre}*) diaphysis; OCD, old (17-week old) control (*Mpl^{fl/fl}*) diaphysis; OMD, old (17-week old) mutant (*Mpl^{fl/fl};PF4^{cre}*) diaphysis. *n* = 3–5 mice per genotype, One-way ANOVA, **P* < 0.05. OB, osteoblasts.

TABLE 3. DEVELOPMENT OF HIGH BONE MASS PHENOTYPE IN FEMALE AND MALE *MPL^{fl/fl};PF4^{cre}* MICE

Age (weeks)	Sex	N	High bone mass phenotype (%)
8	Female	2/5	40
	Male	0/5	0
12	Female	6/6	100
	Male	2/7	30

The altered HSC phenotype in *Mpl^{fl/fl};PF4^{cre}* mice

Erythroid cell number was normal in the PB of all genotypes; however, lymphocyte and monocyte numbers were significantly increased in *Mpl^{fl/fl};PF4^{cre}* mice (Supplementary Table S1). To investigate these increases, we analyzed the HSC phenotype using FACS analysis. *Mpl^{fl/fl};PF4^{cre}* mice exhibited a two-to-fivefold significant increase in LSK, long- and short-term HSC, multipotent progenitors (MPP), common myeloid progenitors (CMP), and granulocyte/macrophage progenitors (GMP), compared to *Mpl^{fl/fl}* and *Mpl^{fl/fl};PF4^{cre}* mice (Supplementary Fig. S2A). Common lymphoid progenitors (CLP) were not different (Supplementary Fig. S2B). There was a trend for increased MK/erythroid progenitor (MEP; Lin[−]Il7rα[−]Sca1[−]c-Kit⁺CD16/32[−]CD34[−]) population, although this did not reach statistical significance. Of

note, Ng et al. used alternate cell differentiation markers CD150⁺FcyR⁺ [30] to distinguish the MEP population and found this population to be significantly increased [16].

MK enhances OB-lineage cell support of hematopoiesis

We have previously shown that MK augment OB-lineage proliferation and suppress maturation [11,14]. We have also shown that immature OB have higher hematopoiesis-enhancing activity (HEA) than mature OB [8,9]. We therefore sought to determine whether MK can promote OB-mediated HEA. To accomplish this, we cultured OB for 1 week either alone (1wkOB) or with MK (1wkOB+MK) (Fig. 4A). On day 7, 1wkOB and 1wkOB+MK were reseeded at the same concentrations into separate wells, and fresh MK were added to the latter group. At this time, fresh OB and MK were prepared and seeded into separate wells. A day later (day 8), all wells were seeded with freshly isolated LSK cells. After 1 week (day 15), LSK progeny were harvested and several hematopoietic parameters were analyzed.

Figure 4B shows that the highest percentage of cells expressing Lin[−]Sca1⁺ phenotype was detected in the cultures with 1wkOB+MK+LSK, indicating that MK can preserve the HEA of OB matured in vitro for 1 week. It should be noted that c-Kit expression is lost when cells are exposed to SCF in vitro [31]. The CFU generated from 1wkOB+MK+

FIG. 4. Hematopoietic enhancing effect of MK and OB. (A) Experimental design of LSK, OB, and MK co-culture. Fresh OB, OB matured in vitro for 1 week (1wkOB), OB+MK co-cultured for 1 week (1wkOB+MK), and fresh MK were co-cultured with fresh LSK for 1 week, LSK were also cultured alone for 1 week, as controls. (B) Progeny were assayed for Lin[−]Sca1⁺ cells or (C) CFU, **P* < 0.05 compared to fresh OB+LSK, N.S., not significantly different from fresh OB+LSK, *n* = 3. CFU, colony-forming unit.

FIG. 5. MK augment OB effect on engraftment and mRNA expression. (A) Percent of donor-derived chimerism from progeny of LSK, 1wkOB+LSK, and 1wkOB+MK+LSK cultures. * $P < 0.05$ versus corresponding values from 1wkOB+MK+LSK cells, mean $\pm$ standard error, $n = 3$, two-way ANOVA, followed by Bonferroni's post hoc t -test. (B) Single-cell gene expression profiles of osteopontin (SPP1), PTHR1, RUNX2, ALPL, and adiponectin (ADIPOQ) of OB cultured with or without MK, $n = 84$ and 56 cells per treatment were analyzed, respectively. ALPL, alkaline phosphatase; mRNA, messenger RNA; PTHR1, parathyroid hormone receptor 1; RUNX2, runt-related transcription factor 2.

LSK cultures were significantly elevated compared to 1wkOB+LSK or LSK alone, again indicating the ability of MK to sustain the HEA of OB matured in vitro for 1 week (Fig. 4C). Note that the number of CFU in co-cultures of LSK+MK were very similar to LSK only, suggesting that MK do not modulate LSK function directly, but through OB instead. These results suggest that LSK progeny from cultures with 1wkOB+MK+LSK may have higher BM repopulating potential. To investigate this, LSK progeny from 1wk OB+MK+LSK, 1wk OB+LSK, and LSK maintained alone in culture were harvested at day 5 of culture and assessed in a competitive repopulation assay in irradiated F1 recipient mice. Chimerism was measured monthly for 4 months. Figure 5A illustrates that 1wk OB+MK+LSK co-cultures sustained the highest level of chimerism over a period of 4 months, confirming that MK sustain the HEA of OB matured in vitro. In addition, MK+LSK results are not different from LSK alone (data not shown). These observations indicate that MK can, in part, regulate HSC function indirectly through their effects on OB proliferation and/or differentiation.

In an effort to identify factors from the OB $\pm$ MK co-cultures that enhance hematopoiesis, we used single-cell expression analysis to analyze 90 genes in fluorescence-activated cell sorting of calvarial OB cells cultured $\pm$ MK for 4 days (Supplementary Table S4). Of these, adiponectin (ADIPOQ) expression was found to have the highest statistically significant percent increase in the OB+MK co-cultures, compared to OB cultures. (Fig. 5B). Consistent with MK-mediated inhibition of OB differentiation [14], the OB expression of differentiation markers osteopontin (SPP1), parathyroid hormone receptor 1 (PTH1R), runt-related transcription factor 2 (RUNX2), and alkaline phosphatase (ALPL) were all downregulated in the OB cultures containing MK compared to the cultures containing OB alone (Fig. 5B and Supplementary Table S3). The violin plots shown in Fig. 4B display relative expression on the y-axis; and the x-axis represents the probability that an individual cell will express a gene at the relative expression value indicated on the y-axis.

Discussion

The long-term goal of this research is to understand the interactions between MK and OB-lineage/osteoprogenitor cells, which result in the regulation of bone formation and

hematopoiesis. To better dissect the interactions between these cells, which increase bone formation, we created mice with an MK-specific deletion of the TPO receptor, *Mpl*^{ff} (*Mpl*^{ff}; *PF4cre*). There are conflicting reports as to the specificity of PF4 expression in MK and platelets, with two studies addressing this issue [16,32]. Ng et al. used lineage tracing with Rosa26 EYFP; *PF4cre* transgenic mice and an IRES-GFP introduced to the *Mpl* locus and found there was no expression of the reporter in the myeloid or lymphoid lineages [16]. Calaminus et al., used lineage tracing with the Rosa26tdtomato; *PF4cre* transgenic mouse and found that PF4 is expressed not only in MK and platelets but also in myeloid and lymphoid lineages, and primitive hematopoietic progenitor cells [32]. This may be due to issues with "leaky" expression of reporters or variability of reporter constructs. As we had generated a similar mouse model and confirmed Ng et al.'s findings, we believe that the PF4 expression is found primarily in the MK lineage cells in our model.

Our approach to the creation of the *Mpl*^{ff} mouse was different compared with Ng et al. [16]. We deleted exon 3 of the *Mpl* gene, whereas they deleted exons 11 and 12 and created a mouse with a virtually identical phenotype. In this study, we not only confirmed their findings but extended them to include the skeletal phenotype.

TPO is considered to be the main MK growth factor, and thus thought to be necessary for normal MK and platelet development/function. In *Mpl*^{ff}; *PF4cre* mice, we had expected to see a reduction in the number of MK, platelets, and HSC, and increased levels of TPO, as observed in mice lacking *Mpl* globally [24,26]. The reason for the discrepancy in platelet and MK numbers between the global knockout and the *Mpl*^{ff}; *PF4cre* mice may be, as postulated by Ng et al., that in the *Mpl*^{ff}; *PF4cre* mice, MK and platelets could not consume TPO, leaving it available for other cells. They speculate that TPO binds to HSC, which in turn increases MK progenitors, resulting in greater numbers of MK and platelets [16]. OB and OC precursors also express TPO receptors; thus, it may be that these cells also bind excess TPO.

As stated previously, several mouse models with high numbers of MK display a high bone mass phenotype. The positive role of estrogen in bone formation and maintenance has been well described [33], and we posit the effect of estrogen accounts for the earlier skeletal phenotype in female mice, possibly owing to the presence of the estrogen receptor in MK and the ability of estrogen to stimulate MK

differentiation [34]. Our data revealed that 100% of female 3-month-old *Mpl^{fl/fl};PF4cre* mice develop a high bone mass phenotype, while in male mice, the skeletal phenotype is delayed by approximately by 4–5 weeks. Thus, there are now five mouse models with increased numbers of MK in BM yielding mice with a strikingly high bone mass phenotype [11,14,18]. These data demonstrate that MK increase osteoblastogenesis, bone formation, and likely a modified BM microenvironment/HSC niche.

In contrast to the distal femur, we observed a striking increase in BV/TV at the femoral midshaft (Fig. 2C–E) in the conditional knockout mice compared to controls. In addition, we measured large increases in MK (Fig. 2A, B) and large increases in Lin[−]CD51⁺PDGFR α ⁺ mesenchymal OB progenitors (Fig. 2G) residing in the marrow space of the mutant mice. This is consistent with other models in which MK stimulate bone formation. Of interest, we found that OB and OC numbers per bone surface were significantly decreased in the epiphysis of the mutants, which is consistent with a low bone turnover phenotype. This does not result in a decrease of BV/TV in this compartment because of the increase in the number of trabeculae and the overall increase in OB-lineage cells. FACS analysis indicated that the expression of CD166⁺ was higher in the OB within the epiphyseal bone in the *Mpl^{fl/fl};PF4cre* mice compared to that observed in control mice. This indicates that the OB in the epiphyses were less mature in mutant mice than that observed in control mice. The greater number of immature (CD166⁺) OB in the epiphyseal region is consistent with the known role that MK play in delaying OB maturation. However, our results indicate that these cells maintain the ability to differentiate into bone forming mature OB cells. This skeletal phenotype confirms that MK play a significant role in bone mass regulation.

Importantly, patients with high numbers of MK in the BM and consequently thrombocytosis may be diagnosed with essential thrombocythemia (ET), a rare chronic myeloproliferative blood disorder, which may be due to mutations in *Mpl* and its downstream signaling of the JAK-STAT pathway. Patients with these conditions may progress to idiopathic myelofibrosis and develop an osteosclerotic phenotype [35,36]. Ng et al. investigated the role of TPO in *Mpl^{fl/fl};PF4cre* mice and postulated that the role of *Mpl* on MK and platelets is to prevent myeloproliferation due to chronic exposure to high levels of TPO [16].

We also hypothesize that in addition to the MK direct effects on HSC (as evidenced by Bruns et al. and Zhao et al.) [3,4], MK signaling can also regulate HSC numbers and function indirectly through their regulation of OB proliferation/differentiation. This is based on the following findings: (1) our previous data demonstrating that MK stimulate OB proliferation in vitro and in vivo, as well as bone formation in vivo [12,37,38]; (2) our data showing that the *Mpl^{fl/fl};PF4cre* mice also exhibit a high bone mass phenotype; (3) data from our group and others demonstrating that OB-lineage cells are known to support hematopoiesis [8–10]; (4) data from Horwitz's group demonstrating that following irradiation, surviving MK migrate to endosteal bone surfaces and stimulate a twofold increase in OB number, which augmented endosteal HSC niches, and this in turn was posited to enhance HSC recovery [39,40]; and (5) our data demonstrating that MK preserve OB HEA, as was shown here in our co-culture experiments.

We have also found that the expression of ADIPOQ in OB-lineage cells is significantly increased when cultured with MK. OB-specific expression of ADIPOQ is regulated by Fra-2/AP-1 and influences the maintenance of bone mass [41]. In addition, ADIPOQ has been observed to increase HSC proliferation, to keep HSC in an immature state, and is reported to be required for long-term hematopoietic reconstitution [42]. Future studies are required to investigate whether OB-derived ADIPOQ plays a functional role in the MK-induced increases in HEA observed in our transplantation studies.

Together, our data support the hypothesis that MK modulate bone formation in vivo and also regulate HSC function indirectly through OB. Furthermore, our findings suggest that the latter may be due to an increased expression of adiponectin. Targeting MK-mediated OB proliferation and hematopoietic supporting pathways may offer novel treatment approaches for bone loss diseases such as osteoporosis and recovery of hematopoiesis in the context of chemotherapy and BM transplantation.

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