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Megakaryocyte–Osteoblast Interaction Revealed in Mice Deficient in Transcription Factors GATA-1 and NF-E2

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ABSTRACT: Mice deficient in GATA-1 or NF-E2 have a 200–300% increase in bone volume and formation parameters. Osteoblasts and osteoclasts generated in vitro from mutant and control animals were similar in number and function. Osteoblast proliferation increased up to 6-fold when cultured with megakaryocytes. A megakaryocyte–osteoblast interaction plays a role in the increased bone formation in these mice.

Introduction: GATA-1 and NF-E2 are transcription factors required for the differentiation of megakaryocytes. Mice deficient in these factors have phenotypes characterized by markedly increased numbers of immature megakaryocytes, a concomitant drastic reduction of platelets, and a striking increased bone mass. The similar bone phenotype in both animal models led us to explore the interaction between osteoblasts and megakaryocytes.
Materials and Methods: Histomorphometry, μCT, and serum and urine biochemistries were used to assess the bone phenotype in these mice. Wildtype and mutant osteoblasts were examined for differences in proliferation, alkaline phosphatase activity, and osteocalcin secretion. In vitro osteoclast numbers and resorption were measured. Because mutant osteoblasts and osteoclasts were similar to control cells, and because of the similar bone phenotype, we explored the interaction between cells of the osteoblast lineage and megakaryocytes.
Results: A marked 2- to 3-fold increase in trabecular bone volume and bone formation indices were observed in these mice. A 20- to 150-fold increase in trabecular bone volume was measured for the entire femoral medullary canal. The increased bone mass phenotype in these animals was not caused by osteoclast defects, because osteoclast number and function were not compromised in vitro or in vivo. In contrast, in vivo osteoblast number and bone formation parameters were significantly elevated. When wildtype or mutant osteoblasts were cultured with megakaryocytes from GATA-1– or NF-E2–deficient mice, osteoblast proliferation increased over 3- to 6-fold by a mechanism that required cell-to-cell contact.
Conclusions: These observations show an interaction between megakaryocytes and osteoblasts, which results in osteoblast proliferation and increased bone mass, and may represent heretofore unrecognized anabolic pathways in bone.
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Key words: bone phenotype, anabolic, proliferation, thrombocytopenia, megakaryocytosis

INTRODUCTION

BONE MARROW IS the source of osteogenic, hematopoietic, and immune cells. Cells from these lineages associate intimately in the marrow compartment and interact with each other. In particular, osteoclastogenesis requires interactions between cells of mesenchymal origin (stromal/osteoblastic) and hematopoietic cells (osteoclast precursors).[1] RANKL expressed on the surface of stromal/osteoblastic cells and macrophage colony-stimulating factor (M-CSF) are known mediators of these interactions.[2,3] Furthermore, $CD4^+$ T-cells or media conditioned by their culture induce osteoclast development, which can be blocked by addition of the decoy receptor osteoprotegerin.[4,5] These results suggest that other hematopoietic cells, like the megakaryocyte (MK), may affect bone formation through interactions with osteoblasts or osteoclasts. Indeed, MKs express mRNA for osteocalcin and secrete the bone matrix proteins osteonectin, bone sialoprotein, and osteopontin.[6–10] These findings are significant in that MKs and blood platelets are the only potential extraosseous sites of osteocalcin expression.[6] These data further imply that

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MKs could, under the appropriate circumstances, contribute to bone formation by secreting these and other factors.

The zinc-finger transcription factor GATA-1 is required for differentiation of MKs and developing erythrocytes, and is also expressed in multipotential hematopoietic progenitors and mast cells.[11] Mice deficient in MK-expressed GATA-1 develop a significant (10- to 100-fold) increase in the number of MKs with a concomitant reduction (85%) in platelet numbers. MKs in GATA-1– and p45 NF-E2–deficient mice are abnormally immature because normal MK differentiation is arrested.[12,13] MKs are morphologically smaller, show evidence of retarded nuclear and cytoplasmic development, and express reduced levels of mRNA encoding markers of cellular maturity.[12] NF-E2 is a heterodimeric leucine zipper transcription factor also largely restricted in expression to erythroid precursors and MKs. Mice lacking the p45 subunit of NF-E2 exhibit profound thrombocytopenia resulting from a maturational arrest of MKs.[13] MK number is greatly increased in the bone marrow and spleen of adult mice, but these cells are unable to differentiate normally or to release blood platelets. These mice also develop myelofibrosis in both the spleen and bone marrow, although this does not occur until the mice are more than 1 year old.[14] A myelofibrotic syndrome with osteosclerosis can be induced in mice by either repeated injections with thrombopoietin (TPO) or by infecting mice with a virus vector carrying the *TPO* gene. Because TPO is the major growth factor required for MK proliferation, like GATA-1– and p45 NF-E2–deficient mice, these mice also have increased numbers of MKs.[15–17] However, unlike the TPO overexpressing mice, GATA-1 and p45 NF-E2 mice have wildtype levels of TPO, suggesting this cannot be the cause of the increased bone mass.[18]

Besides the common features of thrombocytopenia and megakaryocytosis, we report here that loss of GATA-1 or p45 NF-E2 also results in markedly increased trabecular and cortical bone mass. Bone formation parameters, particularly osteoblast number, are significantly increased, and compelling in vitro data indicate that cell-to-cell contact with MKs stimulates osteoblast proliferation resulting in increased bone mass. These observations suggest a novel function for MK–osteoblast interactions in bone homeostasis.

MATERIALS AND METHODS

Mice

Generation and breeding of mutant mice with selective loss of p45 NF-E2 or of MK-expressed GATA-1 were described previously.[19,20] In brief, knockout of GATA-1 in mice by conventional means resulted in an embryonic lethal mutation at the yolk sac stage (E11.5), precluding further analysis. Chimeric mice die early in gestation because of the lack of red cell development.[21] To overcome this problem, a DNase I-hypersensitive region (HS) was identified upstream of the GATA-1 promoter and was subsequently knocked-out by insertion of a neomycin-resistant cassette. This resulted in mice with reduced levels of GATA-1 mRNA and protein (3- to 5-fold reduction in protein), a functional knock-down.[17] These mice have a selective loss of MK expressed GATA-1 but with sufficient expression in the erythroid lineage to preclude lethal anemia.[17] GATA-1–deficient mice are maintained on the C57BL/6 background.

Generation and breeding of p45 NF-E2–deficient mice was also described previously.[13] Briefly, to inactivate the *p45 NF-E2* gene, a PGK-neo cassette (NeoR) was inserted into the unique *Sal*I site upstream of the bZip encoding region.[13] p45 NF-E2–deficient mice are maintained on the inbred 129/Sv genetic background. All animal studies were performed with the approval of the IACUC.

Histomorphometric analysis

To label bone mineralization fronts, mice were injected with 30 mg/kg of calcein 8 days and 24 h before death. Tibias were fixed in 10% neutral buffered formalin, dehydrated, and embedded in methylmethacrylate before being sectioned and stained with toluidine blue.[22,23] Bones were deplastified in acetone and rehydrated before staining for reticulum fibers using Laidlaw's silver stain.[24] Histomorphometric parameters[25] were analyzed by Osteomeasure software (Osteometrics).

Bone morphology and microarchitecture

Femurs were evaluated using a desktop μCT imaging system (μCT20; Scanco Medical AG).[26] The entire femur was scanned using a 34-μm slice increment, requiring $\sim$100–150 μCT slices per specimen. Images were reconstructed and filtered, and appropriate thresholds were applied.[27] Morphometric parameters were computed using a direct 3D approach that does not rely on any assumptions about the underlying bone structure.[28] Histomorphometric and μCT differences were calculated using the unpaired Student's *t*-test. Differences were considered significant at $p < 0.05$.

Cell isolation and culture

Murine calvarial cells were prepared by sequential collagenase digestion (Worthington Biomedical Corp.) as previously described.[29] Cells collected from fractions 3–5 were used as the starting population.

To isolate MKs, livers from 13- to 15-day-old fetal mice were collected, and single cell suspensions were prepared and cultured in DMEM with 10% FCS and 1% conditioned medium (CM) from a murine TPO secreting fibroblast cell line. The MKs were separated from the other cells using a one-step albumin gradient.[30,31] CM from MK cultures was filtered and saved for later use as described below.

To determine the proliferative capacity of osteoblasts isolated from WT and mutant mice, 5000, 2500, and 1250 cells/well were seeded into 96-well tissue culture plates and incubated for up to 10 days at 37°C in α-MEM supplemented with 10% FCS. Proliferation was measured daily by the incorporation of ^{3}H-thymidine (1 μCi/well; 5–8 Ci/mmol) added during the last 16 h of culture.[32]

Osteoblasts from C57BL/6 and 129/Sv (WT) or GATA-1– and p45 NF-E2–deficient (mutant) mice were co-cultured with WT or mutant MKs (e.g., C57BL/6 osteo-

blasts with GATA-1 MKs or 129/Sv osteoblasts with p45 NF-E2 MKs). Optimal numbers of osteoblasts (2500/well, pretested) were cultured with 2500 or 5000 MKs/well in 96-well tissue culture plates. Proliferation was measured as described above with the following modification. Because MKs are nonadherent, they were removed from the plate by washing before harvest to ensure that osteoblast proliferation alone was being measured. Wells containing only MKs served as a control (few if any remained). Microscopic examination as well as cell counts were performed to confirm the lack of MK contamination.

To determine if the MKs activated osteoblasts through a soluble factor(s), osteoblasts were cultured with various concentrations of MK CM, and proliferation was measured. To examine whether cell-to-cell contact was required for osteoblast activation, MKs were co-cultured with osteoblasts and separated by a cell impermeable membrane (pore sizes 0.1 or 0.4 μm, Corning).

In separate experiments, the number of cells entering the S-phase of the cell cycle was determined by the incorporation of 5-bromo-2′-deoxyuridine (BrdU).[33]

Alkaline phosphatase, osteocalcin, and DpD determinations

Alkaline phosphatase (ALP) and osteocalcin productions were measured in osteoblast cell lysates and CM, respectively. ALP activity was determined by substrate conversion in lysates prepared from preconfluent, confluent, and 1- and 2-week post-confluent cultures. Using the same time points, osteocalcin levels in the CM were measured by a standard equilibrium radioimmunoassay (RIA) using specific goat anti-mouse osteocalcin antibody.[34] Both ALP and osteocalcin levels were corrected for variations in cell number by adjusting for protein levels. Deoxypyridinoline (cross-links) was measured by a commercial kit (Pyrilinks, Deoxypyridinoline Immunoassay; Metra Biosystems).

Osteoclast-like cell formation in vitro

Osteoclast-like cells (OCLs) were generated by two previously described methods.[1,35] First, co-cultures containing 2×10^6 spleen cells/ml and 20,000 primary calvarial osteoblasts/ml (from WT and mutant mice) were grown in α-MEM supplemented with 10% FCS and 10^{-8} M vitamin D_3. Second, 2×10^6 spleen cells/ml were cultured in α-MEM supplemented with 10% FCS and 30 ng/ml of both recombinant murine M-CSF and recombinant human RANKL (R&D Systems). Cells were fixed and stained for TRACP using a kit from Sigma. $TRACP^+$ multinucleated (>3) OCLs were counted using light microscopy.

Pit assay for bone resorption

To determine whether the in vitro–generated OCLs were functionally active, their ability to resorb bone was measured. Spleen cells (8×10^4–10^5) were overlayed on bovine cortical bone slices, and M-CSF and RANKL were added to induce osteoclast formation. Cells were cultured for 4–6 days, fixed with 2.5% glutaraldehyde, and stained for TRACP. After counting, the cells were removed by sonication in 0.25 M NH_4OH. The slices were stained with 1% toluidine blue, and the number of pits were counted using light microscopy.[36]

RNA extraction—Northern blot analysis

RNA was isolated from confluent cells using TRIzol Reagent (Life Technologies). Northern analysis using 10 μg of total RNA was done as previously described.[37] GATA-1 and p45 NF-E2 cDNA probes were labeled with ^{32}P-dATP and $^{-32}$P-dCTP by random hexamer extension.

RESULTS

Bone phenotype of GATA-1– and p45 NF-E2–deficient mice

In both GATA-1– and p45 NF-E2–null mutant mice, flushing of the marrow cavity from long bones is progressively more difficult with age and virtually impossible after ~12 weeks. Tibias from 6-week-old, 5-month-old, and 9-month-old mutant mice were processed for histomorphometric analysis and evaluated by light microscopy. Age- and sex-matched C57BL/6 ($GATA\text{-}1^{+/+}$) or 129/Sv ($p45\ NF\text{-}E2^{+/+}$) mice served as controls. While there were no visible differences between 6-week-old mutant and control animals, the bones of both homozygous mutant mice exhibited profound changes at 5 and 9 months of age. The most striking feature was the increase in both cortical (Figs. 1A–1D) and trabecular bone by 2- to 3-fold compared with matched controls (Table 1). Examination by μ-CT of femurs from 5-month-old GATA-1– and p45 NF-E2–deficient mice showed the diaphyseal shafts occluded with bone. In some mice, bone extended the entire length of the femur, whereas in others, bone was observed filling the proximal metaphysis and part of the diaphyseal shaft (Figs. 1E–1H). Trabecular bone volume for the entire medullary canal was increased between 20- and 150-fold, and the rate of bone formation was increased 2.7-fold in the mutant mice.

Because increasing numbers of MKs are associated with the onset of myelofibrosis, the bone marrow of 5-month-old $GATA\text{-}1^{-/-}$ and WT mice were examined for degenerative changes. Reticulum staining of tibias from mutant mice showed the presence of fibers; however, the numbers of fibers was similar to controls on a per bone volume basis (data not shown).

Analysis of osteoclastic cells

Observed increases in bone formation may result, at least in part, from the >3-fold increase in the number of osteoblasts (Table 1). Histomorphometric analysis also indicates a proportional increase in the number of osteoclasts in the mutant mice (Table 1). While these measurements do not guarantee osteoclast functionality, two independent features suggest that they are indeed active. First, the marrow space never closed completely, even in mice observed to 1.5 years of age. Second, the mutant mice show normal tooth eruption, which requires osteoclastic activity. Nevertheless, to further explore the mechanism(s) responsible for increased bone mass, we examined osteoclast numbers and function in more detail. All experiments described in this report were performed using both p45 NF-E2– and GATA-1–deficient

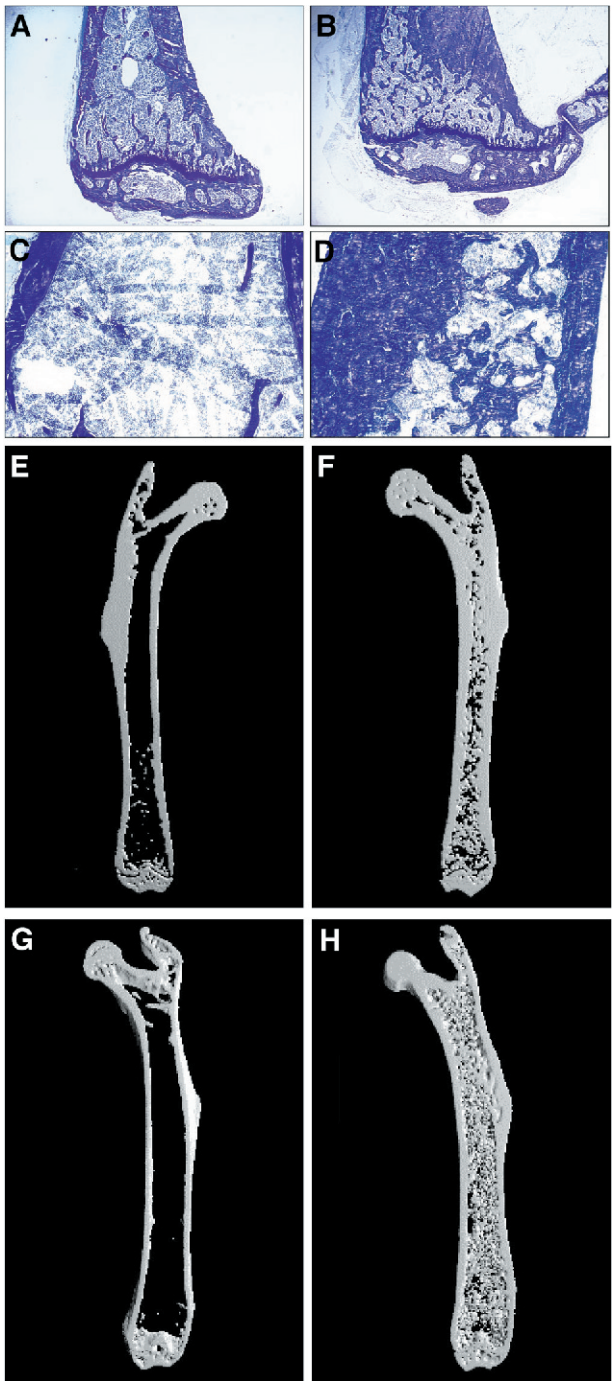

FIG. 1. Histological and μ-CT assessment of mutant and WT long bones. Toluidine blue–stained proximal tibias from 5-month-old NF-E2, 9-month-old GATA-1 mutant, and age-matched WT control mice. (A) 129/Sv and (C) C57BL/6 WT tibias show the typical growth plate compression and otherwise unremarkable histology seen in older mice (25× and 50× original magnification, respectively); (B) NF-E2 and (D) GATA-1 mutant tibias illustrate striking increase in trabecular and cortical bone (magnification same as above). μ-CT analysis of femurs from 5-month-old NF-E2, GATA-1 mutant, and WT mice. (E) 129/Sv and (G) C57BL/6 WT femurs were normal in appearance with respect to the medullary canal, secondary spongiosa, and mid-diaphyseal cortical bone. (F) NF-E2 and (H) GATA-1 mutant femurs show increased trabecular bone volume in the entire medullary canal as well as bone filling the proximal metaphysis and part of the diaphyseal shaft, with cortical thickening.

COLOR

mice, with appropriate controls of the same genetic background in each case. Because results were virtually identical with both mutant strains, we highlight the results from GATA-1–deficient mice for simplicity and to avoid redundancy.

We generated OCLs from co-cultures containing either WT or mutant osteoblasts cultured with WT or mutant spleen cells as a source of osteoclast precursors or by culturing spleen cells with M-CSF plus RANKL. In all cases, the number of OCLs generated from mutant spleen cell cultures was elevated compared with controls (Table 2). To determine if these osteoclasts were functional, spleen cells were cultured on bovine bone slices in the presence of M-CSF and RANKL. OCLs from both mutant and controls resorbed bone equally well, as assessed by the number of resorption pits (data not shown). Finally, urinary cross-link output was the same in mutant and control mice (Table 2), indicating normal bone resorption in vivo. Considered together, these results argue that reduced numbers or function of osteoclasts were not the cause of the bone abnormalities seen in GATA-1– and p45 NF-E2–deficient adult mice.

Assessment of the osteoblast lineage

The logical alternative is that the loss of these transcription factors drive osteoblast proliferation or function, as suggested independently by the large increase in osteoblast numbers in vivo (Table 1). Although expression of GATA-1 and p45 NF-E2 is reported to be largely restricted to hematopoietic cells, the possibility existed that they are also expressed in bone cells and that the mutant phenotypes result from intrinsic osteoblast defects. To address this possibility we isolated RNA from primary WT calvarial osteoblast-like cells at 3, 7, 10, and 14 days of culture and examined GATA-1 and p45 NF-E2 expression by Northern analysis. Figure 2 shows characteristic 1.8-kb bands corresponding to GATA-1 and p45 NF-E2 mRNAs seen in mouse erythroleukemia cells, but the same signals are absent from primary osteoblast-like cells at all time points. The absence of GATA-1 and p45 NF-E2 was confirmed by Taqman analysis (data not shown). Thus, primary osteoblasts do not express GATA-1 or p45 NF-E2.

Next, we compared the proliferative capacity of mutant and WT osteoblasts. We did not observe significant differences in intrinsic proliferation rates between mutant and WT osteoblasts over 5 days (Fig. 3A). Cell-expressed ALP (Fig. 3B) and secretion of osteocalcin (Fig. 3C), corrected for protein levels, also were similar in WT and mutant osteoblast cultures. The sum of these findings argues against intrinsic osteoblast defects in the absence of GATA-1 or NF-E2 function and suggests instead that these defects result from an important cell–cell interaction between osteoblasts and MKs. To address this possibility, we co-cultured MKs from both mutant mouse strains with primary WT osteoblasts and measured cellular proliferation. In more than 10 separate experiments, the MKs consistently induced a significant dose- and time-dependent increase in osteoblast proliferation compared with control cultures (Fig. 4A). Tracking cellular incorporation of BrdU, we also observed that a significantly larger number of osteoblasts entered S-phase in cultures containing both osteoblasts and MKs

TABLE 1. HISTROMORPHOMETRY MEASUREMENTS FROM GATA-1– AND NF-E2–DEFICIENT MICE AND THEIR RESPECTIVE CONTROLS

Mice	*No. of mice*	*BV/TV*	*ObS/BS*	*OcS/BS*	*NOb/TAR*	*NOc/TAR*	*OTh*	*BFR/TV*	*MAR*
C57BL/6	11	4 ± 1	12 ± 2	0.8 ± 0.2	41 ± 7	0.5 ± 0.3	2.4 ± 0.1	53 ± 19	0.96 ± 0.09
GATA-1	6	14 ± 3*	19 ± 3*	2.0 ± 0.1	224 ± 67*	5.2 ± 1.3*	3.2 ± 0.2	143 ± 34	1.33 ± 0.06
129	14	15 ± 1	11 ± 2	1.0 ± 0.3	102 ± 13	2 ± 1	2.4 ± 0.1	176 ± 18	1.2 ± 0.1
NF-E2	9	32 ± 2*	17 ± 2*	2.3 ± 0.8	384 ± 41*	11 ± 4*	3.4 ± 0.2	509 ± 58	1.7 ± 0.2*

BV/TV, bone volume/total volume; ObS/BS, osteoblast surface/bone surface; OcS, osteoclast surface; Nob/TAR, number of osteoblasts/tissue area; NOc, number of osteoclasts; OTh, osteoid thickness; BFR, bone formation rate; MAR, mineral apposition rate.

* Results are presented as mean ± SEM; at least $p < 0.05$ compared with WT.

TABLE 2. IN VITRO OSTEOCLAST GENERATION AND IN VIVO URINARY CROSS-LINKS IN GATA-1–DEFICIENT AND C57BL/6 CONTROL MICE

Source of spleen cells	*Osteoclasts from spleens + MCSF + RANKL*	*Osteoclasts from +/+ osteoblasts and spleens*	*Osteoclasts from −/− osteoblasts and spleens*	*Urine cross-links*
C57BL/6	89 ± 12 ($n = 3$)	255 ± 60 ($n = 4$)	179 ± 46 ($n = 4$)	21 ± 4 ($n = 8$)
GATA-1	522 ± 154 ($n = 3$)*	424 ± 68 ($n = 4$)*	361 ± 59 ($n = 4$)*	15 ± 3 ($n = 15$)

* Results are presented as mean ± SEM; at least $p < 0.05$ compared with WT.

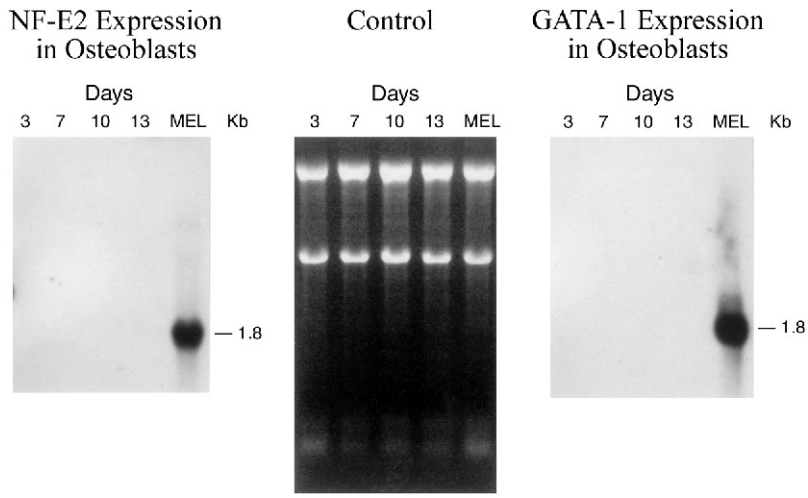

FIG. 2. Lack of NF-E2 and GATA-1 expression in WT calvarial osteoblasts. The mouse erythroleukemia (MEL) cell line was used as a positive control. Northern blot analysis of total RNA (10 μg/lane) shows the characteristic 1.8-kb band for NF-E2 (left) and GATA-1 (right) in the MEL cells, with no expression in the osteoblast-like cells at any time point. (Center) Ethidium bromide loading control.

(Table 3), confirming the data shown in Fig. 4A. WT (C57BL/6) MKs stimulated osteoblast proliferation as well as GATA-1– or p45 NF-E2-deficient MKs (Fig. 4A). Multiple concentration of MKs were used with similar results (data not shown).

Analysis of MK–osteoblast interactions

These findings strongly implicate an important MK interaction in expansion of osteoblasts. In principle, such an interaction could occur through either secreted factors or direct cell–cell contact. To distinguish between these possibilities, we first cultured osteoblasts with or without CM from cultures of mutant MKs. The CM, at all concentrations tested, not only failed to increase the incorporation of tritiated thymidine, but caused a dose-dependent inhibition of osteoblast proliferation (Fig. 4B). CM from WT MKs had a similar effect (data not shown). We next plated osteoblasts in transwell culture dishes and placed cell-impermeable membranes between the osteoblasts and MKs in some samples, whereas in other cultures, osteoblasts were maintained without a barrier from the MKs. The presence of MKs strongly induced osteoblast proliferation, whereas presence of cell-impermeable membrane blocked this induction completely (Fig. 4C), indicating that direct MK-to-osteoblast contact is required to induce osteoblast proliferation. WT MKs had a similar effect (data not shown).

DISCUSSION

Numerous disorders of bone catabolism have been characterized and provide useful insights into normal and pathological bone remodeling. In contrast, disorders of excess bone formation are less common and not as well characterized. Once bone is lost from the skeleton it is difficult, if not impossible, to replace. Therefore, the identification and characterization of anabolic pathways is critical to under-

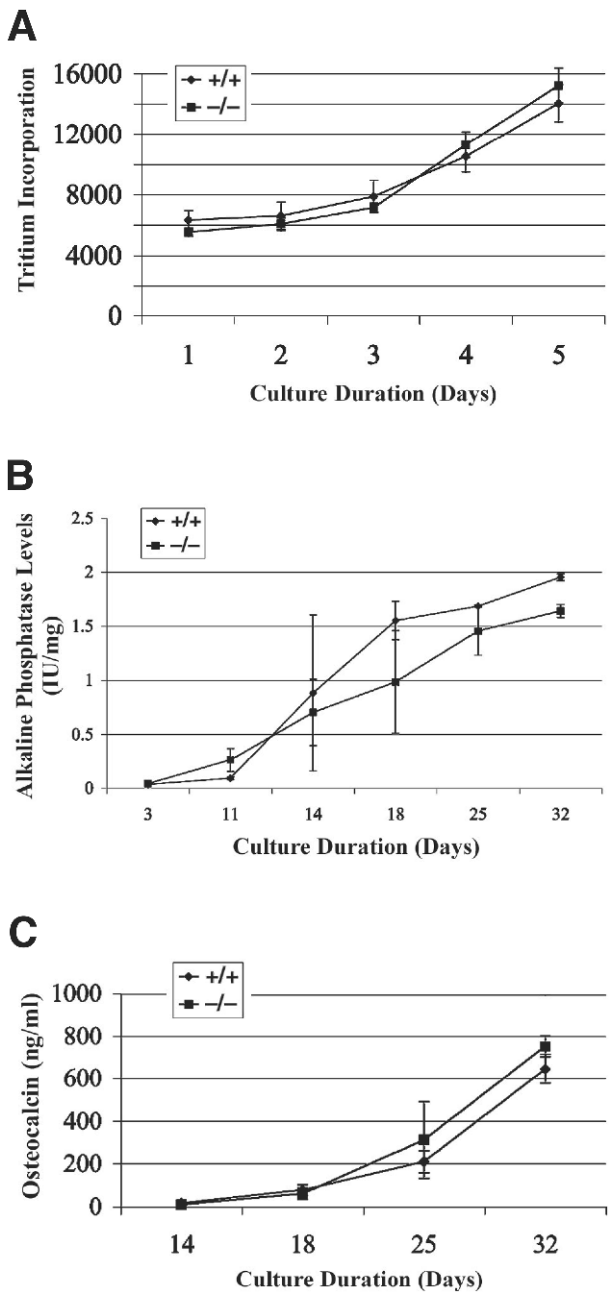

FIG. 3. In vitro analysis of calvarial osteoblasts from GATA-1 mutant and WT mice. Virtually identical results were seen when p45 NF-E2 mutant and 129/Sv WT mice were used (data not shown). (A) Similar proliferative capacity, as assessed by tritium incorporation, between WT and GATA-1 mutant osteoblasts. (B) Cell expressed ALP levels (IU/mg) were identical in WT and GATA-1 mutant osteoblasts. (C) Levels of osteocalcin (ng/ml) secretion were similar among WT and GATA-1 mutant osteoblast cultures. All experiments were repeated at least three times, and individual data points represent the average of triplicate cultures ± SD. There were no significant differences between WT and GATA-1 mutant cultures at any of the time points, as assessed by Student's *t*-test ($p < 0.05$).

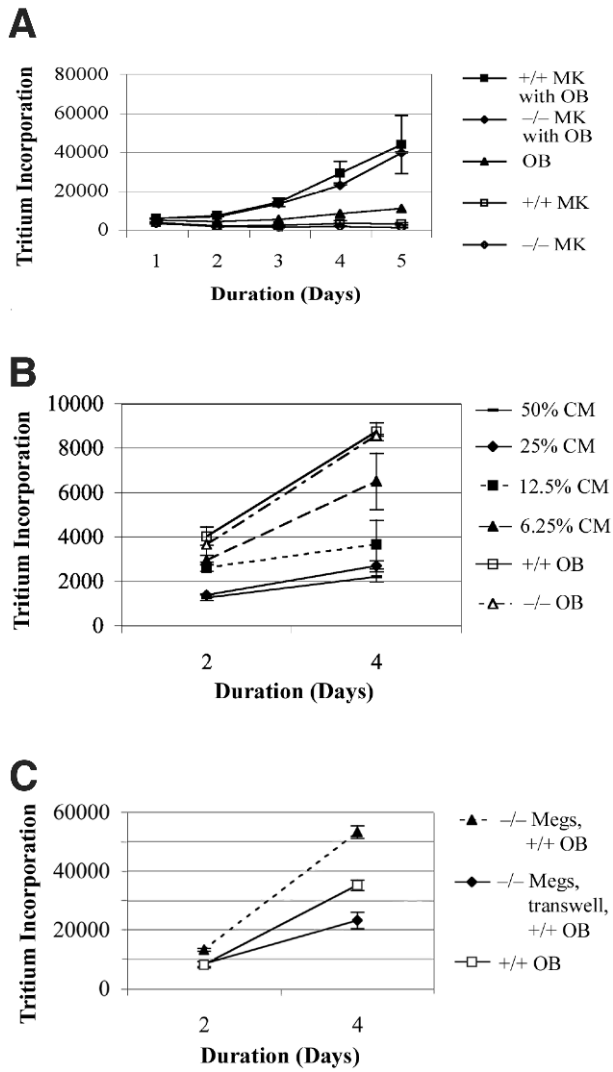

FIG. 4. MK-induced osteoblast proliferation requires cell-to-cell contact. (A) WT osteoblast proliferation was stimulated 5- to 6-fold when cultured with GATA-1 mutant or WT MKs. (B) Osteoblast proliferation was not stimulated when cultured with GATA-1 mutant MK CM. (C) Osteoblast proliferation was not enhanced when separated from MKs by a cell impermeable membrane.

standing skeletal development, to understanding the balance between resorption and formation, and to developing rational treatments for osteopenia. The marked increase in bone and bone matrix seen in GATA-1 and p45 NF-E2 deficiencies provides two genetically defined animal models with the potential to help elucidate these issues.

Our data show that osteoclast formation and function are normal or even elevated in mutant mice and that the medullary canal remains partially open; therefore, the increase in bone is unlikely to result from an osteoclast defect. Instead, the histomorphometric data directly supports increased bone formation as being responsible for this phenotype, because all the bone formation parameters were significantly increased. In particular, the increase in the number of osteoblasts could account for the increase in bone formation, provided there is no simultaneous decline in their function. Indeed, osteoblasts from WT and GATA-1– or p45 NF-E2–deficient mice are entirely normal in their abil-

Table 3. Numbers of Osteoblasts Entering S-Phase, As Determined by BrdU Incorporation

Culture	*Number of S-phase osteoblasts*
C57BL/6 osteoblasts alone	14 ± 2
C57BL/6 osteoblasts cultured with +/+ MKs	76 ± 18*
C57BL/6 osteoblasts cultured with −/− MKs	93 ± 12*

* Results are presented as mean ± SEM; at least $p < 0.05$ compared with osteoblasts alone.

ity to secrete osteocalcin, to produce ALP, and to proliferate. These data suggest that increases in the number of osteoblasts cause the increased bone mass in these animals. The increase in the number of osteoblasts could also account for the parallel increase in osteoclasts through the added expression of RANKL.

Our results also indicate that the increased bone mass is not cell autonomous for the osteoblasts in the mutant mice. This conclusion is supported by the lack of GATA-1 and p45 NF-E2 expression in osteoblasts or other cells in the osteoblast lineage (data not shown). Rather, it seems likely that the mutant bone marrow microenvironment provides some element(s) that allows for the large increase in osteoblast number. The increased bone mass in the GATA-1– and p45 NF-E2–deficient mice is distinct from the high bone mass phenotype seen in transgenic mice overexpressing ΔFosB or Fra-1, two members of the AP-1 family of transcription factors. Increased bone mass in both these transgenic mice results from accelerated osteoblast differentiation, and in the case of ΔFosB, from inhibition of adipogenesis.[38,39] Osteoblasts from these transgenic mice strongly express their respective transcription factors and function in a cell-autonomous manner. Other reported increases in bone mass in vivo are in osteocalcin-deficient mice, in mice following repeated treatment with parathyroid hormone, in the leptin-deficient *ob/ob* or leptin receptor–deficient *db/db* mice, and *Lrp5* mutations.[40–44] The increase in bone mass of these models is substantially less than that seen in the ΔFosB, Fra-1, and GATA-1– or p45 NF-E2–deficient mice. Marked increases in bone density have also been seen in mice overexpressing leukemia inhibitory factor (LIF) and oncostatin M.[45,46] Increase bone mass can be induced by treating mice or rats in vivo with 17β estradiol, fibroblast growth factor (FGF)-1, platelet-derived growth factor (PDGF), or TGFβ1.[47–50] Nevertheless, the mechanism responsible for the increased bone mass in GATA-1– and p45 NF-E2–deficient mice is likely distinct from these other models.

Our data showing the normal secretion and expression of osteoblast proteins, coupled with the lack of GATA-1 or p45 NF-E2 expression in osteoblasts, suggests that loss of these transcription factors is not directly responsible for the increased bone formation. Rather, we believe that the increased numbers of mutant MKs in the bone marrow of GATA-1– and p45 NF-E2–deficient mice plays a central role in the induction of new bone formation. Because the GATA-1– and p45 NF-E2–deficient mice have defects in MK maturation and platelet release in common, the proposed relationship between osteoblasts and MKs constitutes the most plausible and likely scenario. In support of this hypothesis, we show that direct contact between mutant MKs and osteoblasts is required to induce osteoblast proliferation. Direct contact is also required by WT MKs to induce osteoblast proliferation, suggesting a physiologic role for MKs in regulating osteoblast function. Moreover, regulation of bone remodeling by cell–cell interactions is well established.

Opposing the osteoinductive effect of MK–osteoblast interactions is the apparent inhibitory effect of MK-derived CM on osteoblast proliferation. These data suggest that MK have a contact positive effect while simultaneously having a released or secreted negative effect. In the mutant mice, the positive effect predominates, implying a more complex regulation by the MK. These data further support the idea that the maintenance of skeletal mass may depend, at least in part, on normal megakaryopoiesis.

Nevertheless, it seems unlikely that the increased bone mass can be attributed solely to the megakaryocytosis. In vivo overexpression of TPO, either by daily injection or by infecting normal mice with a retroviral vector expressing TPO cDNA, resulted in a marked increase in MK number as well as bone, but the bone was sclerotic and associated with extensive myelofibrosis.[16,51] It has been recently reported the osteosclerosis in TPO overexpressing models is primarily a result of upregulated OPG levels, which in turn inhibit bone resorption.[52] To the contrary, circulating levels of TPO are similar in GATA-1–deficient, p45 NF-E2–deficient, and WT mice.[18] The lack of increased TPO levels in the mutant mice along with the increase in osteoclast number and function reported here argue that there is a distinct difference between the mechanism of the increased bone phenotypes of mice with increased MK number caused by TPO overexpression versus mice with increased MK number because of genetic alterations in MK differentiation.

Moreover, extensive myelofibrosis with increased bone was recently reported in GATA-1–deficient mice that are similar to ours.[14] However, in these mice, the first signs of fibrosis were not evident until 12 months of age, and myleofibrosis appeared at 15 months. Our GATA-1 and p45 NF-E2 mice have a high bone mass phenotype with extensive remodeling by 3–5 months of age, with fibrosis comparable with controls. The equivalent levels of fibrosis in animals younger than 1 year, documented by both our laboratory and Vannucchi et al.,[14] indicate that the increased bone formation occurs well in advance of the myelofibrosis. Purified MKs from GATA-1 mutant mice expressed lower levels of mRNA encoding growth factors TGF-β1, PDGF, and vascular endothelial growth factor (VEGF) than did WT cells.[14] These growth factors are known to contribute to the induction of myelofibrosis, especially TGF-β1.[53–57] In contrast, TPO overexpressing mice exhibited 2- and 5-fold increases in TGF-β1 and PDGF levels, respectively.[17] MKs from p45 NF-E2–deficient mice have significantly reduced numbers of granules, whereas the MKs from GATA-1–deficient mice are so immature as to have few if any specific granules inhibiting if not precluding their ability to secrete growth factors like

WT megakaryocytes.[13,21] These data further show the intrinsic differences between mutant and WT cells that can be attributed to their stage of differentiation.

It may be that MKs can carry out two independent functions. The first is the induction of bone formation, which is cell number dependent. The second is the induction of myelofibrosis, which is dependent on both cell number and state of differentiation. Because MK–osteoblast contact is a prerequisite for the enhanced bone formation, the massive number of MKs in the mutant mice causes the early induction of new bone formation. Although there are a large number of cells, because the mutant MKs are immature and poor producers of the growth factors required to induce myelofibrosis, no myelofibrosis is seen. However, over time, sufficient growth factors are secreted and myelofibrosis appears, explaining the late (>12–15 months of age) appearance of the disease. Neither increased bone formation nor the fibrosis is observed in WT animals because the number of cells is insufficient. This would also explain the early onset of myelofibrosis and sclerotic bone formation observed in mice overexpressing TPO.

Thus, loss of the transcription factors GATA-1 and p45 NF-E2 results in a high bone turnover phenotype with a net massive increase in bone. The data suggest that the new bone is undergoing appropriate remodeling and histologically appears normal up to 9 months of age. The onset of the bone phenotype in the mutant mice is not evident at birth, but begins early in life and progresses steadily thereafter, perhaps as the number of immature mutant MKs increases.[21] The increased bone formation is caused by the large increase in activated osteoblasts rather than a loss or inactivation of osteoclasts. Therefore, these studies describe an anabolic pathway of the skeleton that requires direct contact between immature MKs and osteoblast-like cells, resulting in increased bone formation and overall bone mass.

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