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# Mechanisms of Hematopoietic-Mesenchymal Cell Activation

Justin Lemieux

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**Mechanisms of  
Hematopoietic-Mesenchymal Cell Activation**

A Thesis Submitted to the  
Yale University School of Medicine  
In Partial Fulfillment of the Requirements for the  
Degree of Doctor of Medicine

by  
Justin M. Lemieux  
Class of 2009

**Abstract**

As the prevalence of osteoporosis is expected to increase over the next few decades, the development of novel therapeutic strategies to combat this disorder becomes clinically imperative. These efforts draw extensively from an expanding body of knowledge pertaining to the physiologic mechanisms of skeletal homeostasis. To this body of knowledge, we contribute that cells of hematopoietic lineage may play a crucial role in balancing osteoblastic bone formation against osteoclastic resorption. Specifically, our laboratory has previously demonstrated that megakaryocytes can induce osteoblast proliferation *in vitro*, but do so only when direct cell-to-cell contact is permitted. To further investigate the nature of this interaction, we have effectively neutralized several adhesion molecules known to function in the analogous interaction of megakaryocytes with another cell-type of mesenchymal origin - the fibroblast. Our findings implicate the involvement of fibronectin/RGD-binding integrins including  $\alpha_3\beta_1$  (VLA-3) and  $\alpha_5\beta_1$  (VLA-5) as well as glycoprotein IIb (CD41), all of which are known to be expressed on megakaryocyte membranes. Furthermore, we demonstrate that IL-3 can enhance megakaryocyte-induced osteoblast activation *in vitro*, as demonstrated in the megakaryocyte-fibroblast model system. Taken together, these results suggest that although their physiologic and clinical implications are very different, these two models of hematopoietic-mesenchymal cell activation are mechanistically analogous.

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## **Introduction**

Skeletal fragility has emerged as a major limitation to quality of life as we age. Osteoporosis currently affects approximately 10 million people in the United States alone; another 34 million Americans have low bone density, placing them at increased risk of the disease. Together, more than 55 percent of the population aged 50 years and older are affected or at risk of osteoporosis. The disease carries a significant financial burden. In 2005, osteoporosis-related fractures were responsible for approximately 19 billion dollars in health care costs; this number is expected to increase to more than 25 billion dollars by 2025. More importantly, osteoporosis and the ensuing hip, wrist, and vertebral fractures are significant sources of morbidity and pain among the elderly: such a fracture can be the sentinel event that transforms a relatively healthy, independent senior citizen into a person requiring significant assistance for daily living. This downward spiral is evidenced by a one-year post-hip fracture mortality of 24 percent. Further, as many as 20 percent of individuals who were previously ambulatory require long-term care after a hip fracture (1).

As the prevalence of osteoporosis is expected to increase over the next few decades, the development of novel therapeutic strategies to combat this development of this disorder becomes clinically imperative. These efforts draw extensively from an expanding body of knowledge pertaining to the physiologic mechanisms of skeletal homeostasis. To this body of knowledge, we contribute that cells of the hematopoietic lineage may play a crucial role in balancing osteoblastic bone formation against osteoclastic resorption.

Over the past decade, a new paradigm has emerged wherein megakaryocytes have been found to play a regulatory role in skeletal homeostasis. Cumulatively, the data demonstrate that megakaryocytes may act to stimulate bone formation by expressing and secreting bone-related proteins to enhance osteoblastic proliferation and differentiation directly (2-10)

Several bone-related proteins are produced or secreted by megakaryocytes to influence bone mineralization and catabolism. Osteocalcin is a bone matrix protein considered to be a marker of bone turnover. Previously, this protein was thought to be synthesized exclusively by osteoblasts at mineralizing surfaces; however, osteocalcin mRNA transcripts have been found to be enriched in platelet-producing megakaryocytes within peripheral blood. The levels of megakaryocyte-associated osteocalcin in blood increased during periods of rat growth, suggesting that megakaryocytes function in the regulation of bone remodeling and turnover. (2)

Osteonectin, a secreted calcium-binding glycoprotein, is a component of bone extracellular matrix. This protein binds extracellular matrix, via protein-protein interactions with type I collagen, to inhibit the growth of hydroxyapatite crystals, implicating osteonectin in the control of bone anabolism. While this glycoprotein is produced by both osteoblasts and megakaryocytes, the two forms of the protein differ in both complex glycosylation structure (3) and immunogenicity (4). Within platelets, osteonectin forms a calcium-dependent complex with the platelet-specific protein thrombospondin (4). While the implications of these differences are not clear, the

regulated production of this protein by megakaryocytes, its inclusion into platelets, and its unique associations within those cells again implicates a unique role for megakaryocytes and megakaryocyte-derived osteonectin in the regulation of bone growth (3).

Bone sialoprotein is major component of bone and other mineralized tissues. While the function of this protein remains to be clarified, it has been hypothesized that this protein may function to nucleate apatite crystals during bone formation. This protein is also found within megakaryocytes and platelets (5), although it does not appear to be synthesized by these cells; instead it appears to be acquired by endocytosis, proteolytically processed, and stored in the alpha granules of platelets to be released upon platelet activation (11). It is unclear if release of this protein by megakaryocytes or platelets plays a role in bone homeostasis, but megakaryocytes are again implicated in the overall regulation of bone mass.

Osteopontin, also known as secreted phosphoprotein 1 (SPP1), was originally isolated as a structural component of bone synthesized by osteoblasts. Synthesis of this protein is enhanced by activated vitamin D. Osteopontin is thought to act as a “bridge” anchoring osteoclasts to bone. This protein has since been found to be expressed by multiple cell types, including bone marrow megakaryocytes (6). It has been hypothesized that megakaryocytes secrete factors that influence osteoblast activity, but that close proximity to bone stromal cells is required for this effect. Expression of osteopontin by megakaryocytes may serve to localize these cells to regions of bone formation,



facilitating megakaryocyte function in bone mineralization. megakaryocytes express osteocalcin and secrete the bone matrix proteins osteonectin, osteopontin, and bone sialoprotein.

megakaryocytes also produce bone morphogenic proteins (BMPs); proteins that induce ectopic osteogenesis. BMP mRNA and proteins can be detected within both megakaryocytes and platelets by in situ hybridization and immunoblotting.(7) GATA-1- and NF-E2-deficient mice, which both display increased numbers of immature marrow megakaryocytes, exhibit increased BMP production and excessive bone formation with a 200-300% increase in bone volume and growth parameters (8). GATA-1 and NF-E2 are transcription factors required for the normal differentiation of megakaryocytes. Deficient animals exhibited normal osteoblast and osteoclast numbers and function. However, the proliferation of osteoblasts wild-type and mutant, increased approximately six-fold upon co-culture with megakaryocytes from deficient mice (8). These results indicate that an interaction, either direct or indirect, between osteoblasts and megakaryocytes results in osteoblast proliferation and increased bone formation. Cultures of osteoblasts with conditioned medium from mutant megakaryocytes failed to produce this increase in osteoblast proliferation; direct contact between megakaryocytes and osteoblasts was required for this effect. Thus, direct contact between megakaryocytes and osteoblasts results in an increase in osteoblast proliferation, leading to increased bone mass. This finding may help explain the osteosclerosis observed in human patients with forms of myelogenous leukemias associated with increased numbers of marrow megakaryocytes (12).

Such an interaction between osteoblasts and megakaryocytes is also supported by work from Miao *et al.* (9) examining osteoblast colony formation and alkaline phosphatase expression. Aggregates of bone marrow stromal cells, which contain megakaryocytes, formed large colonies expressing abundant alkaline phosphatase. When these aggregates were dissociated, allowing osteoblast precursors to grow as single-cell suspensions, colonies still formed, but were smaller and expressed only minimal amounts of alkaline phosphatase. These data suggest that removing the supportive influence of megakaryocytes reduces the osteoblastic differentiation of bone marrow stromal cells. Again, these results point to an interaction of osteoblasts with megakaryocytes to achieve maximal osteoblast differentiation and activity.

Our own laboratory has recently examined the role of gap junction intracellular communication (GJIC) between osteoblasts and megakaryocytes (45). This work has demonstrated that megakaryocytes and osteoblasts can communicate by GJIC and that GJIC may serve to inhibit megakaryocyte-mediated enhancement of osteoblast proliferation, but does not appear to alter megakaryocyte-mediated reductions in osteoblast differentiation. Additionally, megakaryocytes were observed to inhibit osteoblast differentiation when co-cultured for extended durations.

In addition to increasing osteoblast proliferation, megakaryocytes appear to influence osteoblastic activity. Osteoblast activity was assessed by measuring the expression of type-I collagen, and osteoprotegerin, proteins associated with increased bone

formation activity, and RANKL, which is upregulated during bone remodeling and resorption. osteoblasts cultured with CD61-positive megakaryocytes exhibited increased intensity of type-I collagen expression over that seen by osteoblasts cultured alone or with control non-megakaryocytic cells. Co-culture with megakaryocytes also increased both the proportion of osteoblasts producing osteoprotegerin and the intensity of OPG expression by those cells. In addition, RANKL expression by osteoblasts was suppressed by co-culture with megakaryocytes.

Megakaryocytes can also produce OPG to influence osteoblastic and osteoclastic activity directly (13). High levels of OPG mRNA are seen in both platelets and megakaryocytes. Strong immunostaining for OPG could be seen in mature megakaryocytes. OPG staining in megakaryocytes was enhanced by treatment with thrombopoietin, a megakaryocyte-specific growth factor, indicating that megakaryocyte maturation correlates with increased OPG expression. Thus, in addition to indirectly affecting osteoclast and osteoblast function, megakaryocytes can directly stimulate osteoblast differentiation and inhibit osteoclast function through the production of osteoprotegerin.

Estrogen deficiency is well known to play a role in the pathogenesis of osteoporosis, resulting in increased bone resorption and bone loss. Estrogen participates in the maintenance of bone mass and inhibits bone resorption. Estrogen therapy, although associated with other risks, is effective at increasing bone mass or at least stopping bone loss in post-menopausal women (14). Recent studies have begun to elucidate some of the

underlying mechanisms of these results. The effect of estrogen on human osteoblast cultures was assessed by measuring factors involved in bone homeostasis. These data revealed that osteoblasts treated with estradiol exhibit a sustained increase in OPG expression at both 24 and 48 hours after stimulation. A significant increase in RANKL expression at 24 hours was also observed; however, this increase was not sustained at 48 hours. These effects could be specifically inhibited with an estrogen antagonist. Thus, estrogen may exert an anti-resorptive effect on bone by stimulating the production of factors involved in bone anabolism in excess of catabolic factors (15). The molecular mechanism underlying this effect is unclear, but may be mediated at least in part by interactions with megakaryocytes. The addition of estrogen to cultures of hematopoietic stem cells stimulates the colony-forming potential to a megakaryocytic phenotype, implicating megakaryocytes in estrogen-induced bone formation (16). In addition to encouraging megakaryocyte differentiation, estrogen significantly increased OPG expression and suppressed RANKL expression by megakaryocytes (17). Thus, the function of estrogen in the maintenance of bone mass may be mediated, at least in part, by the stimulation of megakaryocytopoiesis and the upregulation of factors involved in bone formation and the downregulation of osteoclastogenesis.

Simultaneously, megakaryocytes may regulate bone resorption by expressing/secreting several factors known to be involved in osteoclastogenesis, and recent studies demonstrate that megakaryocytes can inhibit osteoclast formation *in vitro* (13, 15-24). Co-cultures of megakaryocytes with spleen cells from wild-type C57BL/6 mice resulted in a 10-fold reduction in osteoclastogenesis under conditions that produce

ample osteoclasts in the absence of megakaryocytes (24). This effect could be reproduced by the addition of conditioned media from megakaryocytes to spleen cell cultures, indicating that the inhibition of osteoclast development by megakaryocytes was mediated by a soluble factor. While osteoprotegerin, which is known to inhibit osteoclast proliferation, was present in megakaryocyte-conditioned media, this factor was not responsible for megakaryocyte-mediated growth inhibition, as conditioned media from OPG-deficient mice and conditioned media containing OPG-neutralizing antibodies were still able to inhibit osteoclastogenesis *in vitro*. Similarly, neutralization of TGF- $\beta$ , which was also present in conditioned media, was also unable to abrogate the growth inhibitory effect of megakaryocytes on osteoclasts (24).

The role of megakaryocytes in bone growth and catabolism was confirmed by studies in mice deficient in p45 NF-E2, a key transcription factor required for megakaryocyte differentiation (25). These mice are characterized by increased numbers of immature megakaryocytes and the absence of functional platelets. As mentioned above, these animals display an increased bone mass, and increased bone formation rate. Increased bone formation, however, was only observed in sites of hematopoiesis, not in flat bones, such as calvariae, again confirming the role of megakaryocytes in bone homeostasis. This phenotype of increased bone mass and growth could be adoptively transferred into recipient irradiated wild-type mice with the hematopoietic cell population, again implicating megakaryocytes as the responsible cell lineage. The net result, as demonstrated *in vivo*, is that increases in megakaryocyte number lead to concomitant increases in bone mass (8, 25)

In attempting to identify molecules involved in the megakaryocyte-osteoblast interactions that regulate bone homeostasis, we look to the interactions of megakaryocytes with fibroblasts, another cell of mesenchymal origin. Through examination of the pathogenesis of myelofibrosis, the molecular mechanisms underlying both the paracrine and direct cell-cell interactions of megakaryocytes with fibroblasts have been elucidated. These mechanisms may represent a generalized mechanism by which megakaryocytes influence the function and proliferation of neighboring cells.

Myelofibrosis, characterized by bone marrow fibrosis, extramedullary hematopoiesis, splenomegaly, and increased peripheral blood immature hematopoietic progenitors, is a secondary phenomenon that accompanies the clonal transformation of hematopoietic lineage cells in chronic myeloproliferative disorders. In this disease, excessive fibroblast cell proliferation and increased secretion of matrix proteins, such as collagen and reticulin, reduces the ability of the bone marrow to produce functional cells, leading to bone marrow failure and multiple cytopenias. The correlation of fiber density and megakaryocyte number first linked the reactive proliferation of fibroblasts in this condition to interactions with megakaryocytes (26). In addition, mice given bone marrow grafts of cells infected with a retrovirus expressing exogenous thrombopoietin, the primary stimulator of megakaryocytopoiesis, develop a lethal myeloproliferative disorder resembling human idiopathic myelofibrosis (27). Subsequent studies demonstrated that megakaryocytes could stimulate fibroblast proliferation (28). Homogenates of megakaryocytes were found to support fibroblast growth, which was initially attributed to

cytokines and growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (22, 28). Megakaryocyte-dependent growth promotion was effectively abrogated by neutralizing antibodies directed against these proteins, demonstrating the critical role of these growth factors in fibroblast proliferation (29).

To determine additional cytokines that may function in the megakaryocyte-dependent enhancement of fibroblast proliferation, Schmitz et al. examined the effect of a barrage of cytokines on fibroblasts and fibroblast-megakaryocyte co-cultures. While many of these cytokines, such as IL-1, did not have any effect on fibroblast proliferation, recombinant human IL-3 (rhIL-3) significantly increased the number of fibroblasts in co-cultures. No enhancement of fibroblast proliferation could be detected with the addition of rhIL-3 in the absence of megakaryocytes, suggesting that IL-3 induced megakaryocytes to undergo changes that promoted fibroblast growth. Interestingly, the effect of IL-3 could be inhibited by the addition of neutralizing antibodies against PDGF and TGF- $\beta$ , suggesting that IL-3 stimulation of megakaryocytes induced the secretion of growth factors that enhanced fibroblast proliferation.

For years, lymphocytes have been thought to be the major sources of cytokines within the bloodstream and the bone marrow. Substantial evidence, as detailed above, is now accumulating that megakaryocytes may produce a barrage of cytokines that influence both hematopoietic cells and surrounding stromal cells in autocrine and paracrine mechanisms. While the role of IL-3 in megakaryocyte-osteoblast interactions is unclear, it is clear that this cytokine plays a key role in controlling the production of

soluble growth factors by megakaryocytes. Wickenhauser *et al.* (23) examined the effect of rhIL-3 on megakaryocyte activity by looking at the resulting stimulation of cytokine production. Following stimulation with IL-3, megakaryocytes demonstrated significantly increased production of IL-3 itself, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-6. Likewise, TGF- $\beta$ , whose production is also stimulated by IL-3 treatment (22), upregulated megakaryocyte production of IL-6, IL-1, and GM-CSF (20). A megakaryocyte-like cell line, HU3, was shown to produce IL-1 $\beta$ , IL-6, IL-10, and TNF- $\alpha$ , while a bipotential progenitor-type cell line, MB02, did not. This secretion could be enhanced following treatment with thrombopoietin, indicating the association between cytokine secretion and megakaryocyte differentiation (21). Also, these factors also act in a positive feedback mechanism in concert with thrombopoietin to enhance megakaryocyte differentiation (30). Estrogens have previously been shown to control the production of cytokines, such as IL-1 and IL-6, that modulate bone homeostasis (31). These cytokines appear to act in both an autocrine fashion, acting as a positive feedback loop encouraging additional cytokine secretion by megakaryocytes, and a paracrine fashion, possibly acting on osteoblasts and osteoclasts to influence their proliferation and function.

Despite substantial evidence implicating soluble factors such as cytokines in fibroblast proliferation, the addition of soluble factors alone to fibroblast cultures was unable to produce the substantial enhancement of fibroblast proliferation seen with intact megakaryocytes (32). These results and the close proximity of fibroblasts and megakaryocytes within the bone marrow, however, suggested a more intimate associate



might be required for the enhancement of proliferation. Indeed, in the cytokine studies detailed above, inhibition of cell-cell contact in both unstimulated and IL-3-stimulated cultures significantly impaired megakaryocyte-induced fibroblast proliferation (32). Using a transwell assay system, cell-cell contacts were inhibited via tissue culture inserts, generating a substantial impairment in fibroblast growth, even in the presence of IL-3 (33). Identification of the cell surface receptors functioning in fibroblast- megakaryocyte interactions may identify candidate molecules that may control the interactions of megakaryocytes with osteoblasts.

Integrins are abundant cell-surface proteins that interact with both other cell surface receptors and components of the extracellular matrix. These proteins are also well-known to initiate multiple conserved signal transduction pathways that trigger cell activation and proliferation. The  $\alpha_3\beta_1$  and  $\alpha_5\beta_1$  integrins were implicated in direct cell-cell interactions utilizing blocking studies with specific anti- $\alpha_3$  and anti- $\alpha_5$  antibodies. Such antibodies not only inhibited the megakaryocyte-mediated enhancement of fibroblast proliferation, but significantly disrupted megakaryocyte attachment to the adherent fibroblast monolayer (34).  $\beta_1$ -containing integrins bind to fibronectin through recognition of a conserved peptide sequence of Asp-Gly-Asp (RGD). Addition of a soluble oligomer, Asp-Gly-Asp-Ser (RGDS), interferes with this interaction, blocking the interaction of  $\beta_1$  integrins with their substrates. Megakaryocyte-associated fibroblast proliferation, although not adhesion, was significantly inhibited by the addition of RGDS to megakaryocyte-fibroblast co-cultures (34). Wickenhauser *et al.* also confirmed the role of the glycoprotein CD41 (glycoprotein IIb) in megakaryocyte-fibroblast

interactions, as specific anti-CD41 monoclonal antibodies completely blocked fibroblast proliferation in megakaryocyte-fibroblast co-cultures (35).

While all of these studies were performed in *in vitro* cultures, in total, these experiments provide substantial evidence that megakaryocytes play an important role in bone remodeling, directly affecting osteoblastic proliferation and function, osteoclast growth and development, and the expression of factors involved in both bone formation and resorption.

While substantial evidence points to an overall anabolic effect of megakaryocytes, there remains conflicting evidence for a pro-catabolic influence of megakaryocytes on bone homeostasis as well. While p45 NF-E2-deficient animals exhibit a clear increase in both bone mass osteoblast numbers, the quantity of osteoclasts is also increased. These catabolic cells appear to exhibit normal functioning *in vitro*, which suggests that megakaryocytes also encourage osteoclast growth (7). Megakaryocytes have been demonstrated to produce RANKL, which exerts a positive influence on osteoclast growth and function (19). In addition, while one might expect that animals lacking normal functioning megakaryocyte would exhibit increased or more rapid-onset osteoporosis, mice deficient in platelet activating factor receptor develop only mild osteoporosis. Osteoclast survival in such mice is diminished; osteoclastic bone resorption is impaired (reviewed in (36)). In addition, the production of cytokines, such as IL-1, IL-6 and TNF $\alpha$ , also stimulates the production of RANKL by osteoblasts (37), which promotes osteoclast development and function. In multiple myeloma, neoplastic cells induce bone

destruction through the upregulation of factors such as RANKL also known as tumor necrosis factor-related activation-induced cytokine (TRANCE), the production of which, as the name suggests, can be upregulated by cytokines such as TNF $\alpha$  (18). Overall, there is likely to be a highly complex interplay between megakaryocytes and the network of cells regulating bone formation and turnover.

In this study, we have focused our efforts on the characterization of the contact-dependant mechanism(s) by which megakaryocytes induce osteoblast proliferation/differentiation. To this end, we have effectively neutralized several adhesion molecules known to function in the analogous interaction of megakaryocytes with fibroblasts. Furthermore, we have explored the effect of interleukin (IL)-3 on our megakaryocyte-osteoblast model system. These new data provide a new outlook on the mechanism(s) of megakaryocyte-osteoblast interactions and the role of this interplay in bone disease, such as in osteoporosis.

**Statement of Purpose**

This study attempts to characterize the specific contact-dependent mechanisms by which megakaryocytes might induce osteoblast activation. To this end, we have selectively disrupted the function of several candidate cell surface proteins and examined the effect of said disruptions on megakaryocyte-induced enhancement of osteoblast proliferation *in vitro* as assayed by incorporation of a radio-labeled nucleotide. Furthermore, we examine the response of this model system to IL-3 as a potential mechanism of paracrine influence.

*Specific Aim 1: Assay megakaryocyte-induced osteoblast proliferation in the presence of EDTA, soluble RGDS tetrapeptide, and neutralizing antibodies against integrin  $\alpha_3$  chain/CD49c, integrin  $\alpha_5$  chain/CD49e, and glycoprotein (gp) IIb/integrin  $\alpha_{IIb}$  chain/CD41, respectively.* This is intended to demonstrate the involvement of Ca-dependent adhesion/signaling in general, RGD-binding receptors, and the relevant specific integrins, respectively.

*Specific Aim 2: Assay megakaryocyte-induced osteoblast proliferation in the presence of IL-3.* This is intended to examine this cytokine as a potential mechanism of paracrine regulation of megakaryocyte-induced osteoblast proliferation.

## **Materials and Methods**

### **Animals**

C57BL/6 mice were obtained from Jackson Labs (Bar Harbor, ME) and housed under standard conditions under a 12/12 hour light-dark cycle. Animals were allowed access to standard chow and water *ad libitum*.

### **Primary osteoblast cultures**

C57BL/6 murine calvarial cells were prepared by sequential collagenase digestion (Worthington Biomedical) as previously described (38). Three- to five-day-old mice were anesthetized by hypothermia and sacrificed by cervical decapitation. Calvariae isolated from these 3- to 5-day old C57BL/6 mice were treated with 4 mM ethyl disodium acetate (EDTA) in PBS for 10 minutes. After repeating this treatment two additional times, samples were then subjected to sequential digestion with 200 U/ml CLS-2 bacterial collagenase (Worthington Biomedical Corp, Freehold, NJ) in PBS for 75 minutes. After the second 15 minutes period, the supernatant was harvested and designated fraction 1. The solution was then replaced; after an additional 15 minutes, cells released during that time frame were designated fraction 2. This was repeated three additional times, for a total of five fractions. Fractions 1 and 2 were discarded; cells collected from fractions 3-5 were used as the starting population for OB/osteoprogenitor cultures. After washing extensively in culture medium [ $\alpha$ -Minimum Essential Media ( $\alpha$ MEM, Sigma Chemical Co., St. Louis, MI) supplemented with 10% newborn calf

serum (HyClone, Logan, UT)], cells were plated at low density (approximately  $2 \times 10^4$  cells per ml;  $2 \times 10^3$  per well in 96-well plates) and grown to confluence (typically 5-7 days).

### **Primary megakaryocyte cultures**

C57BL/6 breeding pairs were set-up by placing four females and one male C57BL/6 mouse in a cage together; the timing of pregnancies were determined by observing the presence of a mucus plug on female mice. At this point, the male mouse was removed, pregnant C57BL/6 mice were housed in individual cages, then sacrificed between embryonic days 13-15. Whole livers recovered from mouse fetuses at embryonic days 13 and 15 were homogenized by successive passages through a 25-gauge needle to obtain a single cell suspension. The resulting cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% FCS (HyClone) and 1% conditioned medium (CM) from a murine thrombopoietin-secreting fibroblast cell line. After five days in culture, megakaryocytes were harvested by low-speed centrifugation at 400 rpm for 10 minutes, then overlaid on a discontinuous bovine serum albumin (BSA, Sigma) density gradient with consecutive layers of 0%, 1.5%, and 3.0% BSA dissolved in PBS. Megakaryocytes were highly enriched in the cells that settled to the bottom within 40 minutes at 1 x gravity, making up more than 90% of the cells as determined by visual inspection and, in prior studies by FACS analysis. Final cultures of megakaryocytes were collected by centrifugation and resuspended in  $\alpha$ -Minimum Essential Medium ( $\alpha$ MEM, Life Technologies Inc.) supplemented with 0.5% BSA prior to experimentation (39, 40).

**Proliferation Assays**

$2 \times 10^3$  osteoblasts/well were co-cultured in the presence or absence of  $5 \times 10^3$  megakaryocytes/well in 96-well tissue culture plates. As negative controls,  $5 \times 10^3$  megakaryocytes/well were cultured alone. After four days in culture, 5-8 Ci/mmol tritiated ( $^3\text{H}$ )-thymidine was added to each well to a final target concentration of 1  $\mu\text{Ci/well}$ . After an additional 16 hours in culture, megakaryocytes, which are non-adherent, were removed by extensive washing to ensure measurement of osteoblast proliferation alone (8). Osteoblast cultures were then frozen at  $-20^\circ\text{C}$  until measurement, at which time cultures were thawed to  $37^\circ\text{C}$  and incorporated radioactivity was determined by scintigraphy. All experiments were performed in triplicate and averaged. Significant differences in tritium incorporation were determined by one-tailed Student's T-test ( $p < 0.05$ ).

**OB proliferation time course**

$2 \times 10^3$  osteoblasts/well were cultured in the presence or absence of  $5 \times 10^3$  megakaryocytes/well in 96-well tissue culture plates. As negative controls,  $5 \times 10^3$  megakaryocytes/well were cultured alone.  $^3\text{H}$ -thymidine was added to cultures at 1, 2, 3, 4, and 5 days of culture, then tritium incorporation was assessed as described above.

**Transwell Assays**

To determine if direct cell-cell contact was a requisite to megakaryocyte induction of osteoblast proliferation, megakaryocytes and osteoblasts were cocultured while separated by a cell-impermeable membrane (pore sizes 0.1 or 0.4  $\mu\text{m}$ , Corning)  $^3\text{H}$ -thymidine was added to the osteoblast cultures after 2 and 4 days. Tritium incorporation was assessed as described above.

### **Integrin Inhibition**

The bivalent cation chelator ethylenediaminetetraacetic acid (EDTA, Sigma) was added to megakaryocyte-osteoblast co-cultures and osteoblast control cultures to examine the effect of non-selective integrin inhibition on megakaryocyte-induced osteoblast activation. EDTA was added at low-dose (0.0125 mM) and high-dose (0.125 mM) quantities at the beginning of the four day culture period. Control cultures were treated with vehicle alone (PBS). EDTA remained in the culture medium throughout the 16-hour tritium incorporation under the culture conditions described above.

As a subset of integrins bind to a conserved peptide sequence of Arg-Gly-Asp (RGD), we examined the effect of blockade of such receptors under the culture conditions described above. To elucidate the involvement of RGD-binding receptors in our model system, the soluble tetrapeptide Arg-Gly-Asp-Ser (RGDS; Sigma) was titrated into co-cultures and osteoblast controls at a high dose (0.0625 mM) or low dose (0.0125 mM) at the beginning of a four-day culture period. Controls were treated with vehicle alone (PBS).  $^3\text{H}$ -thymidine was added as described above on the fourth day of culture; samples were cultured for an additional 16 hours before harvesting.



### **Neutralizing Antibody Studies**

To determine the identity of those integrins functioning in the osteoblast- megakaryocyte interaction, we tested the effect of several neutralizing antibodies directed against specific adhesion molecules. The following antibodies were applied to co-cultures and osteoblast controls: integrin  $\alpha_3$  chain/CD49c (10  $\mu\text{g}/\text{mL}$ ; polyclonal; R and D Systems), integrin  $\alpha_5$  chain/CD49e (20  $\mu\text{g}/\text{mL}$ ; clone: 5H10-27 MFR5; BD Pharmingen), and glycoprotein (gp) IIb/integrin  $\alpha_{\text{IIb}}$  chain/CD41 (10  $\text{ng}/\text{mL}$ ; clone: MWReg30; BD Pharmingen). Goat IgG fraction was added to control cultures at equivalent concentrations to assure that blocking was antigen-specific. After four days in culture, 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine was added to each well; then cultures were incubated at  $37^\circ\text{C}$  for an additional 16 hours, and tritium incorporation was measured by scintigraphy.

### **IL-3-mediated proliferation**

To explore the effect of the cytokine IL-3 on megakaryocyte-induced osteoblast activation, recombinant murine IL-3 (R&D Systems) was titrated into co-cultures and controls at 0, 10, and 30  $\text{ng}/\text{mL}$ . After a four-day incubation,  $^3\text{H}$ -thymidine was added to cultures for 16 hours prior to harvesting and quantitation of incorporated radioactivity.

## **Results**

### **Enhancement of osteoblast proliferation by co-culture with megakaryocytes**

First, we confirmed the enhancement of osteoblast proliferation following co-culture with megakaryocytes. Osteoblast proliferation was measured by tritium incorporation after 1, 2, 3, 4, and 5 days at 37°C. In comparison to osteoblast cultured alone, co-culture with megakaryocytes enhanced osteoblast proliferation by 335% at five days ( $p=0.012$ ; Figure 1A). Minimal proliferation, which became detectable only after the five-day culture period, could be seen in the absence of megakaryocytes. As megakaryocytes are not adherent, the substantial washing performed prior to measurement of incorporated radioactivity should remove any megakaryocytes from cultures, ensuring that the detected signal was derived entirely from the contained osteoblasts. The absence of any significant tritium incorporation in cultures of megakaryocytes alone confirmed the specificity of this assay, demonstrating that the increased radioactivity seen in osteoblast-megakaryocyte co-cultures was due to increased osteoblast proliferation in the presence of megakaryocytes.

### **Requirement for cell-cell contact for MK enhancement of osteoblast growth**

The enhancement of osteoblast proliferation by megakaryocytes was dependent on direct cell-cell contact as the separation of these two cell types by a membrane abrogated the enhancement of osteoblast growth. By measuring cell proliferation via tritium incorporation after two and four days in culture, we again confirmed the

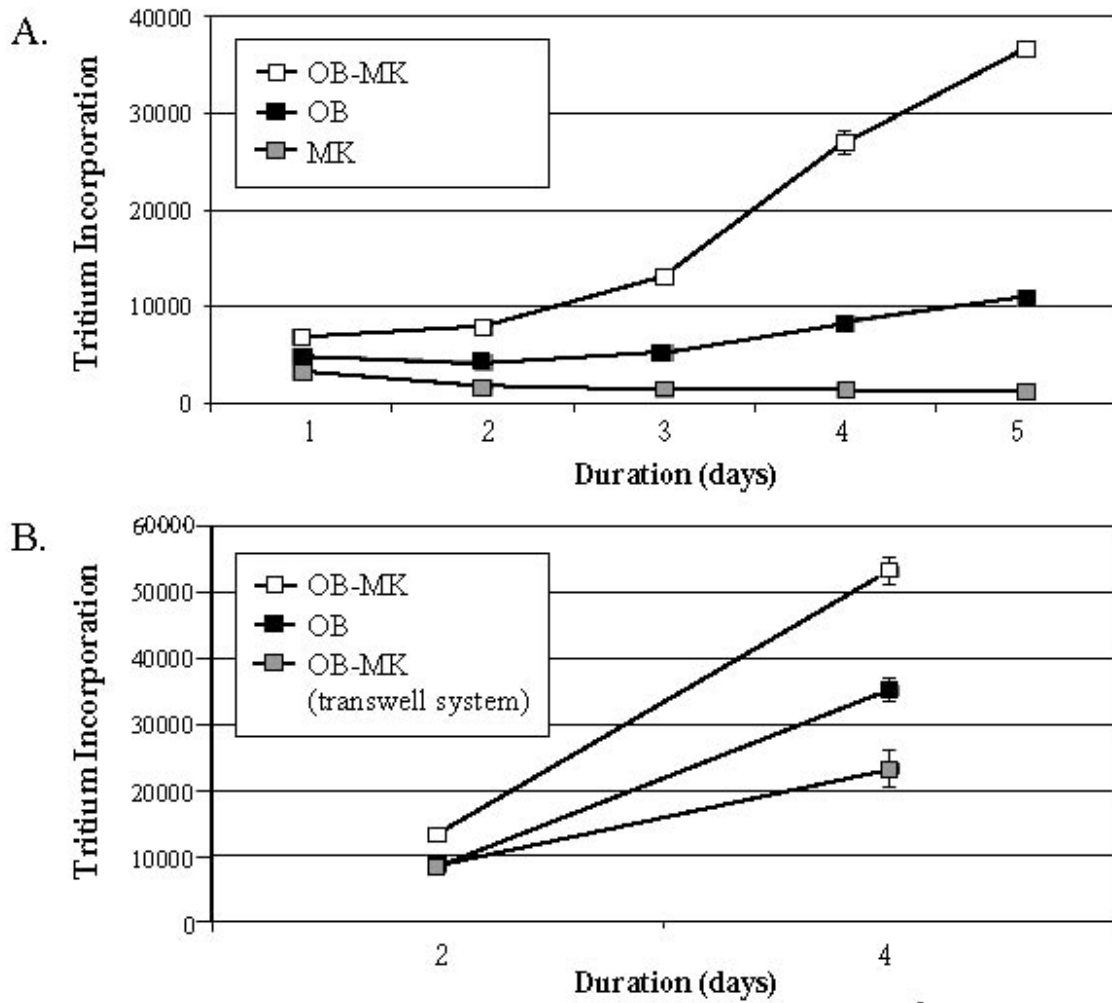


Figure 1. Megakaryocytes induce osteoblast activation via contact-dependent mechanism(s). (A) Osteoblast proliferation increased more than three-fold by co-culture with megakaryocytes for five days. (B) Proliferation was not enhanced, however, by co-culture with megakaryocytes across a cell-impermeable membrane.

enhancement of osteoblast proliferation by co-culture with megakaryocytes over the levels seen by osteoblasts cultured alone. Separation of these two cell types in a transwell system, which allows free exchange of soluble factors, but prevents direct cell-cell contact, abrogated any enhancement of osteoblast growth. Tritium incorporation in these cultures was not significantly different from osteoblasts cultured alone, again confirmed the requirement for a direct interaction between megakaryocytes and osteoblasts to stimulate osteoblast growth and function.

### **Implication of Cell Adhesion in megakaryocyte-mediated osteoblast proliferation**

Integrins are cell surface adhesion molecules that have been implicated as candidate molecules involved in the contact-dependent enhancement of osteoblast proliferation and activity by megakaryocytes. To interfere with the calcium-dependent interactions of integrins and their substrates, we utilized the bivalent cation chelator EDTA as a mechanism of inducing non-selective integrin inhibition in megakaryocyte-osteoblast co-cultures. EDTA was titrated into megakaryocyte-osteoblast co-cultures and osteoblast controls during a four-day culture period. As measured by tritium incorporation during an additional 16-hour culture period, the addition of 12.5 $\mu$ M EDTA failed to inhibit megakaryocyte-induced activation. Incubation with 125 $\mu$ M EDTA significantly ( $p=0.048$ ) reduced tritium incorporation in co-cultures by 18% without affecting osteoblast controls (Fig. 2A), suggesting that disruption of calcium-dependent protein-protein interactions abrogated the effect of megakaryocytes on osteoblast proliferation. At higher concentrations tested, EDTA began to inhibit osteoblast proliferation in mono-culture.

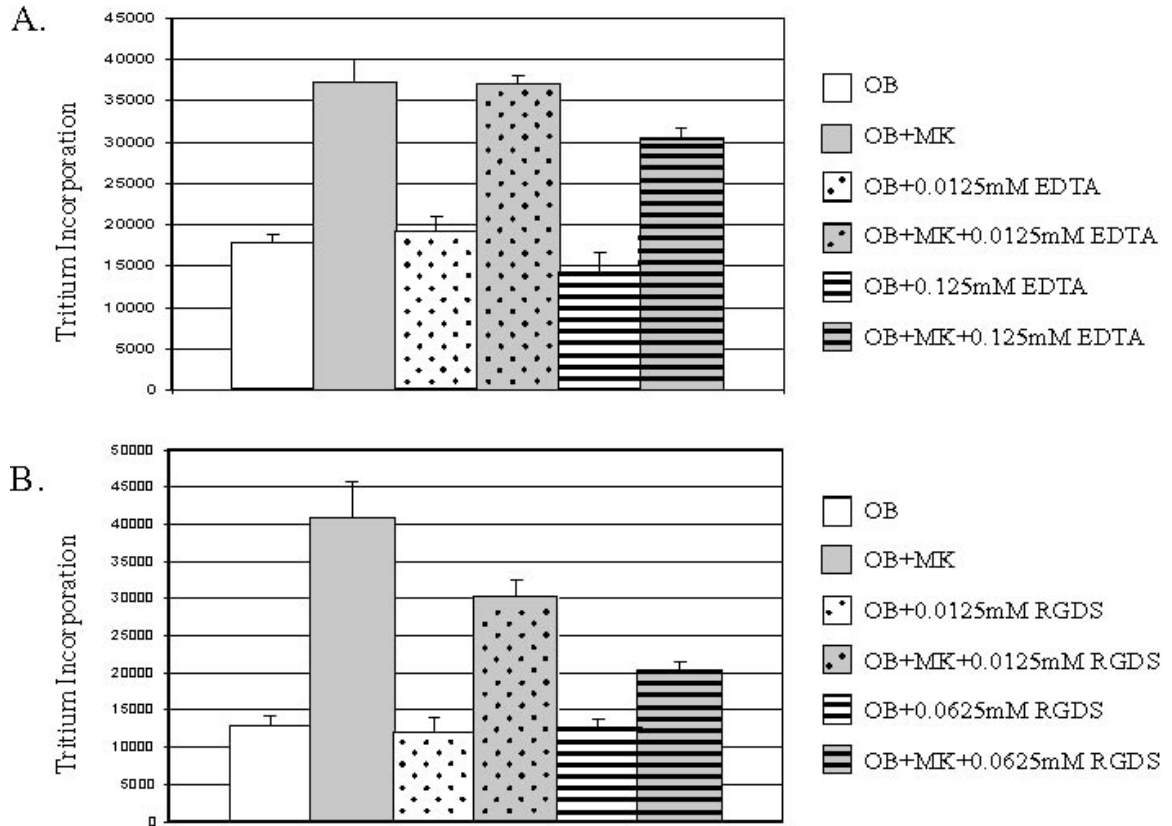


Figure 2. Integrin involvement in MK-induced OB activation. (A) 12.5 $\mu$ M EDTA was not sufficient to disrupt MK-induced OB proliferation, however 125 $\mu$ M EDTA diminished MK-induced proliferation by 18%. Tritium incorporation by OB monocultures was not affected at either concentration reported. (B) Soluble tetrapeptide RGDS caused a dose-dependant inhibition of MK-induced proliferation with 12.5 $\mu$ M and 62.5 $\mu$ M decreasing tritium incorporation in co-cultures by 26% and 50%, respectively. Again, OB monoculture proliferation remained unaffected at both concentrations reported.

proliferation (data not shown), however, at the lower dose tested, the inhibition was specific to osteoblast-megakaryocyte co-cultures.

### **Involvement of integrins in megakaryocyte-enhanced osteoblast growth**

A subset of integrins bind to a conserved sequence composed of a Arg-Gly-Asp (RGD) binding sequence, including integrins  $\alpha_3$  (CD49c),  $\alpha_5$  (CD49e), and glycoprotein (gp) IIb/integrin  $\alpha_{IIb}$  chain (CD41). To elucidate the involvement of RGD-binding receptors in our model system, we titrated the soluble tetrapeptide Arg-Gly-Asp-Ser (RGDS; Sigma) into co-cultures and osteoblast controls as a competitive inhibitor of these interactions. RGDS caused a dramatic, dose-dependant inhibition of proliferation in co-cultures. Addition of 12.5 $\mu$ M and 62.5 $\mu$ M decreased megakaryocyte-induced activation in a dose-dependent manner, resulting in 26% (p=0.032) and 50% (p=0.014) reduction in osteoblast proliferation. RGDS had no effect on osteoblast control monocultures (Fig. 2B). Further increases in RGDS concentration did not result in additional inhibition of proliferation in co-cultures. These results support the hypothesis that RGD-binding integrins mediate the megakaryocyte-dependent enhancement of osteoblast proliferation.

### **Neutralization of specific adhesion molecules abrogated megakaryocyte enhancement of osteoblast growth**

To achieve more specific inhibition of cell adhesion molecules, we added neutralizing antibodies directed against the following specific adhesion molecules to co-

cultures and osteoblast controls: integrin  $\alpha_3$  chain/CD49c (10 $\mu$ g/mL), integrin  $\alpha_5$  chain/CD49e (20 $\mu$ g/mL), and glycoptotein (gp) IIb/integrin  $\alpha_{IIb}$  chain/CD41 (10ng/mL).

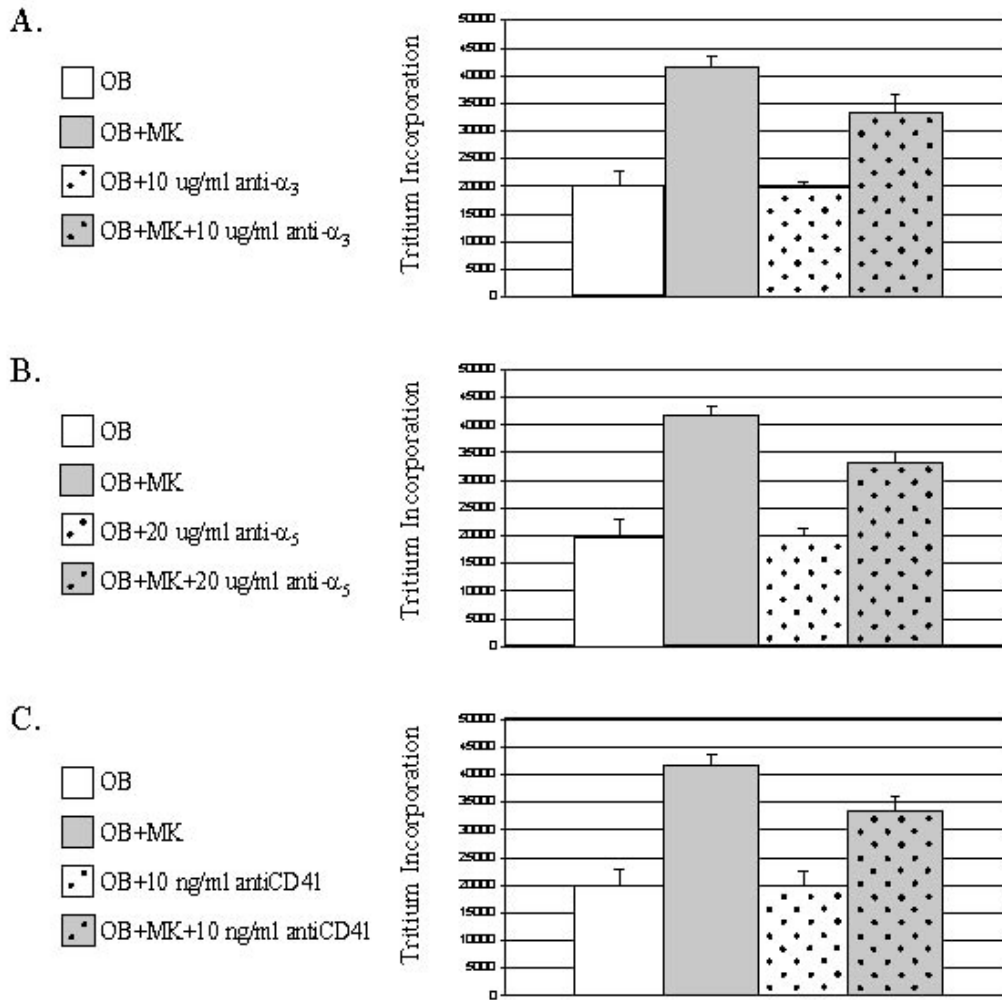


Figure 3. Involvement of specific adhesion molecules in MK-induced OB activation. Application of neutralizing antibodies against integrin  $\alpha_3$  chain (A), integrin  $\alpha_5$  chain (B), and CD41 (C) each diminished MK-induced OB proliferation by approximately 20%, without affecting tritium incorporation in OB monocultures.



Goat IgG fraction was added to separate controls at equivalent concentrations to assure that blocking was antigen-specific. Each of the neutralizing antibodies tested yielded virtually identical results, causing moderate reductions in co-culture proliferation without affecting osteoblast monocultures. A maximal suppression of 21% ( $p=0.045$ ) was seen with the addition of anti- $\alpha_3$  antibodies at 10 $\mu$ g/mL, 20% ( $p=0.023$ ) with anti- $\alpha_5$  antibodies at 20 $\mu$ g/mL, and 20% ( $p=0.036$ ) by anti-CD41 antibodies at 10ng/mL (Fig. 3). Non-specific IgG did not affect tritium incorporation in co-cultures or osteoblast monocultures when added at respective control concentrations (data not shown). These results implicated the involvement of fibronectin/RGD-binding integrins, including  $\alpha_3\beta_1$  (VLA-3) and  $\alpha_5\beta_1$  (VLA-5) as well as glycoprotein IIb (CD41), all of which are known to be expressed on megakaryocyte membranes, in the cell contact-dependent interactions required for the megakaryocyte-mediated enhancement of osteoblast proliferation and function.

### **IL-3 enhances megakaryocyte-mediated osteoblast proliferation**

Lastly, recombinant murine IL-3 (R and D Systems) was titrated into co-cultures and controls to explore the effect of this cytokine on megakaryocyte-induced osteoblast activation. Finally, 10 ng/mL and 30 ng/mL IL-3 enhanced megakaryocyte-induced osteoblast proliferation by 41% ( $p=0.025$ ) and 37% ( $p=0.034$ ) respectively, while osteoblast monocultures remained unaffected (Fig. 4). Further increasing IL-3 concentration did not yield additional enhancement of megakaryocyte-induced activation.

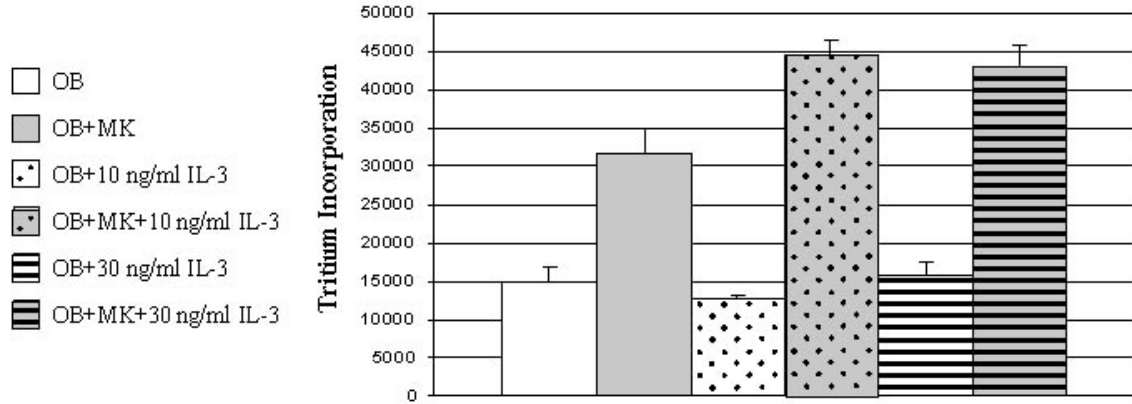


Figure 4. IL-3 enhances MK-induced OB activation. At 10ng/mL and 30ng/mL, IL-3 enhanced MK-induced OB proliferation by 41% and 37%, respectively. Tritium incorporation by OB monocultures remained unaffected at both concentrations reported.

## Discussion

Our laboratory has previously demonstrated that megakaryocytes can induce osteoblast proliferation, but do so only when direct cell-to-cell contact is permitted (8). To further investigate the nature of this interaction, we have systematically disrupted known mechanisms of megakaryocyte-fibroblast adhesion/signaling in our megakaryocyte-osteoblast co-culture model system. We began our investigation with the addition of EDTA to co-cultures and controls. This chelating agent reduces the availability of bivalent cations necessary for proper dimerization and ligand-binding of integrin heterodimers (41, 42) and has been shown to inhibit megakaryocyte-fibroblast adherence and signaling *in vitro*.(32). The ability of EDTA to diminish megakaryocyte-induced osteoblast proliferation without affecting osteoblast monocultures thus implicates integrin involvement in megakaryocyte-osteoblast adherence/signaling. Refining the scope of our investigation, we next examined the role of RGD-binding receptors in our model system. The conformation of the RGD sequence of fibronectin is approximated in a soluble form by the tetrapeptide RGDS (43), which serves to inhibit the interaction of fibronectin-binding integrins with their substrates. Therefore, our finding that the addition of this tetrapeptide inhibits megakaryocyte-induced proliferation without affecting osteoblast monocultures implicates specifically, although not exclusively, RGD-binding integrins. These data are again consistent with those pertaining to megakaryocyte-fibroblast adherence/signaling (34). The respective roles of megakaryocyte-expressed, fibronectin-binding integrins  $\alpha_3\beta_1$  (VLA-3) and  $\alpha_5\beta_1$  (VLA-5; an RGD receptor integrin) were then examined by application of neutralizing antibodies to co-cultures and controls. This resulted in significant reductions in megakaryocyte-induced proliferation. As osteoblast

mono-cultures were not affected by these antibodies, and non-specific IgG affected neither co-cultures nor osteoblast controls, we conclude that these specific integrins directly contribute to megakaryocyte-osteoblast adhesion/activation, as demonstrated in the megakaryocyte-fibroblast model system (34). Employing the same technique and reasoning, we next elucidated the involvement of the megakaryocyte-expressed glycoprotein CD41. This molecule, also known as gpIIb, is retained on the surfaces of mature platelets where it complexes with CD61 (gpIIIa) forming a heterodimeric receptor capable of recognizing a host of extracellular proteins (fibrinogen, fibronectin, von Willebrand factor, vitronectin, etc.) with affinities modulated by the state of platelet activation. In agreement with megakaryocyte-fibroblast interaction findings (35) we conclude that this glycoprotein also plays an important role in megakaryocyte-induced adherence/activation.

Lastly, our data demonstrate that IL-3 significantly enhances megakaryocyte-induced osteoblast proliferation without affecting osteoblast monocultures. Although we have not demonstrated that this enhancement is contingent upon direct cell-cell contact, Schmitz *et al.* (32) showed that IL-3 could not enhance fibroblast proliferation when megakaryocyte-fibroblast co-cultures were divided by cell-impermeable membranes. Furthermore, the prerequisite of direct cell-cell contact in no way excludes an additive effect of signaling via soluble factors as a mechanism for megakaryocyte-induced mesenchymal cell activation. To the contrary, Schmitz *et al.* (32) speculate that adhesion may serve principally to expose fibroblasts to supra-threshold levels of megakaryocyte-derived growth factors such as PDGF and TGF $\beta$ . Our laboratory is currently performing

a series of real-time PCR studies on IL-3-treated versus untreated megakaryocytes to explore these and other possibilities. While many such details of our own model - and skeletal homeostasis in general - remain obscure, we propose the following model of skeletal homeostasis, in which a complex network of both direct and indirect interactions between megakaryocytes and osteoblasts control bone homeostasis (Figure 5). Megakaryocytes induce osteoblast proliferation/differentiation via a contact-dependant mechanism(s), characterization of which is the subject of this investigation. While additional factors may contribute, the fibronectin-binding integrins  $\alpha_3\beta_1$  (VLA-3) and  $\alpha_5\beta_1$  (VLA-5) and the gpIIb (CD41)/gpIIIa (CD61) heterodimer function to increase osteoblast proliferation and differentiation through direct cell-cell interactions. Cytokines produced by megakaryocytes act indirectly on osteoblasts to promote bone growth, both by enhancing osteoblast proliferation and by increasing the deposition of matrix proteins, such as collagen and osteocalcin. Megakaryocytes also elaborate an unidentified soluble factor(s) of 10-50kDa (red) which inhibits osteoclast formation. Megakaryocytopoiesis is promoted by thrombopoietin, which is produced within the marrow space by osteoblast/stromal cells. The stimulation of megakaryocytopoiesis by thrombopoietin enhances the production of cytokines by megakaryocytes, which act in a positive feedback manner to enhance megakaryocyte differentiation additively. Lastly, osteoblasts induce osteoclast formation via RANK/RANK-ligand interaction. Megakaryocyte may also contribute to increased osteoclast differentiation and function through elaboration of proteins such as RANKL. Estrogen, deficiency in which produces bone loss and fragility, has been shown to enhance megakaryocyte differentiation and

increase the production of osteoblast growth factors by megakaryocytes, again implicating a central role for megakaryocytes in the regulation of bone density.

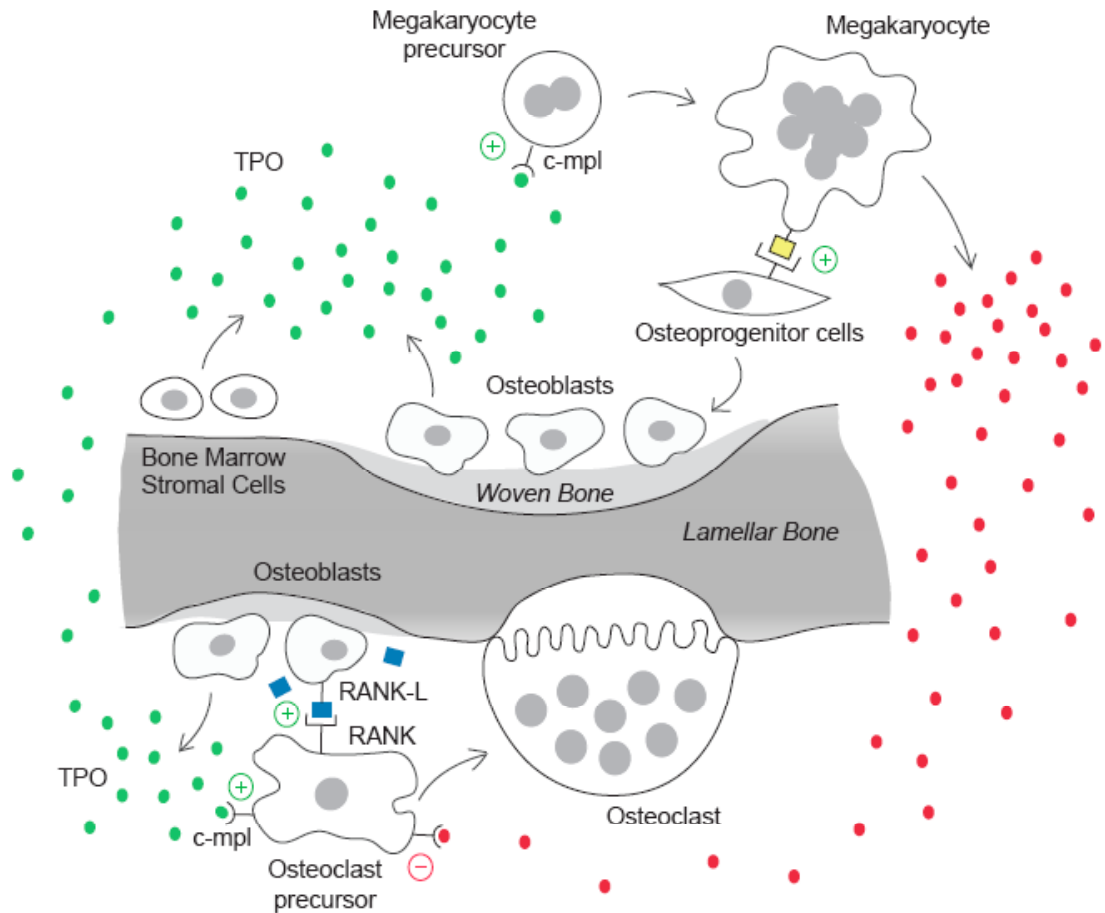


Figure 5. The evolving role of MKs in skeletal homeostasis. MKs induce OB proliferation/differentiation via a contact-dependant mechanism(s) (yellow), characterization of which is the subject of this investigation. MKs also elaborate an unidentified soluble factor(s) of 10-50kDa (red) which inhibits OC formation. Megakaryopoiesis is promoted by thrombopoietin (TPO; green) elaborated within the marrow space by OB/stromal cells. Lastly, OBs induce OC formation via RANK/RANK-ligand (blue) interaction.

Multiple studies in vitro and in mutant mice demonstrate that the bone density correlates well with the overall numbers of megakaryocytes and the amount of megakaryocytopoiesis. GATA-1- and NF-E2-deficient mice possess increased numbers of megakaryocytes, which correlates with excessive bone formation (8). Thrombopoietin, a megakaryocyte-specific growth factor that is the primary stimulator of megakaryocytopoiesis in vivo, exerts a substantial effect on bone homeostasis through direct modification of megakaryocyte growth and activity. The receptor for thrombopoietin, c-Mpl, is constitutively expressed by megakaryocytes at all stages of development and can be upregulated by thrombopoietin stimulation. The receptor then undergoes a conformational change to initiate signal transduction through via Janus kinase (JAK) family kinases that are constitutively bound to the cytoplasmic tail of c-Mpl. Activated JAK in turn phosphorylates the receptor and other signaling molecules, such as signal transducers and activators of transcription (STATs), phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs), eventually resulting in the activation of the transcriptional regulators GATA-1 and NF-E2 that are key controllers of megakaryocyte-specific gene expression. These signaling pathways lead to cell survival and proliferation as well as the stimulation of cytokine production. Eventually, kinase activity activates the SHP1 and SHIP1 phosphatases as well as suppressors of cytokine signaling (SOCSs) to terminate cell signaling (reviewed in reviewed in 44.). While the signal transduction molecules functioning downstream of thrombopoietin are common, highly-conserved proteins that function downstream of

multiple important cell surface receptors, both the downstream transcriptional regulators (GATA-1 and NF-E2) and initiators of transcription (thrombopoietin, c-Mpl) appear to function relatively specifically in megakaryocytopoiesis, making these proteins good potential sites for therapeutic manipulation. While inhibition or enhancement of megakaryocytopoiesis might have the desired effect on bone homeostasis, it would also have undesirable effects on hemostasis, leading to either thrombocytopenia and resultant bleeding or thrombocytosis and possible myelofibrosis, significant complications with profound sequelae.

Megakaryocytes appear to exert their effects on osteoblasts via both direct intracellular interactions and indirect paracrine manipulation. Our studies implicate the megakaryocyte-expressed, fibronectin-binding integrins  $\alpha_3\beta_1$  (VLA-3) and  $\alpha_5\beta_1$  (VLA-5; an RGD receptor integrin) and the gpIIb (CD41)/gpIIIa (CD61) heterodimer as critical cell surface molecules on megakaryocytes mediating the cell-cell contacts functioning to enhance osteoblast growth and function. Although these cell adhesion molecules also function in multiple cellular systems throughout the body, it has been proposed that cell adhesion in the interactions of megakaryocytes with osteoblasts may serve only to facilitate localized secretion of soluble mediators, resulting in an increased local concentration of paracrine factors that exceeds the cellular threshold required for activation (25) If this hypothesis is true, it may be possible to facilitate cell-cell interactions with the rational design of bivalent agents that interact with cell surface proteins on both cells, increasing cell adhesion and the downstream bone anabolism that



follows. Such an approach is as yet hypothetical, but could be attempted with minimal effects on other organ systems.

Megakaryocytes also influence osteoblast activity through the elaboration of multiple soluble factors, both autocrine, such as IL-3 and IL-6, and paracrine factors, including osteoprotegerin, osteocalcin, and osteonectin. We have specifically demonstrated the importance of IL-3 as a key upstream regulator in this process. Given the role of interleukins in multiple tissues, as in the immune system, treatment with exogenous cytokines could have substantial undesirable systemic side effects. Thus, it would be more desirable to attempt manipulation of more bone-specific factors. Osteoprotegerin is a central regulator of bone homeostasis, maintaining the careful balance between bone anabolism and catabolism. Manipulation of the production of some of these soluble, bone-specific proteins produced by megakaryocytes might be sufficient to increase bone density without interfering with platelet production or function, allowing for the selective treatment of osteoporosis and other diseases of bone loss.

Estrogens have also been shown to influence megakaryocytes and the regulation of bone homeostasis by megakaryocytes. While estrogen replacement is an effective treatment for osteoporosis associated with reductions in endogenous hormone levels, this treatment is administered with caution due to the unfortunately side effects that include an increased incidence of certain cancers and increased heart disease in certain individuals. A better understanding of the mechanisms by which estrogens influence megakaryocyte function and in turn affect bone density may allow us to activate only

these pathways selectively, resulting in effective treatment for osteoporosis and reduced systemic side effects.

While the fine details of the molecular mechanisms governing the regulation of bone homeostasis by megakaryocytes remain unclear, these studies could have a significant impact on the treatment of disease of decreased bone mass, such as osteoporosis. The identification of the pathways and substrates on bone stromal cells functioning in the regulation of osteoclast and osteoblast function by megakaryocytes has identified new potential targets that may be manipulated pharmacologically in the treatment of bone diseases. In addition, better understanding of the soluble molecules produced by megakaryocytes to regulate bone homeostasis provides us with starting points for the rational design of drugs that could be used to manipulate any abnormalities of bone, from osteopenia and the lytic bone destruction of multiple myeloma to the excessive bone production seen in diseases such as Paget's disease. While significant research still remains before we can manipulate these pathways with ease, we remain ambitious that our efforts will contribute to a higher standard of clinical care for osteoporotic patients in the coming years.

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