

# Involvement of Integrins $\alpha_3\beta_1$ and $\alpha_5\beta_1$ and Glycoprotein IIb in Megakaryocyte-Induced Osteoblast Proliferation

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## 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 (MKs) can induce osteoblast (OB) 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 MKs with another cell type of mesenchymal origin—the fibroblast (FB). 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 (gp) IIb (CD41), all of which are known to be expressed on MK membranes. Furthermore, we demonstrate that interleukin (IL)-3 can enhance MK-induced OB activation *in vitro*, as demonstrated in the MK–FB 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 in several ways. *J. Cell. Biochem.* 109: 927–932, 2010. © 2010 Wiley-Liss, Inc.

**KEY WORDS:** MEGAKARYOCYTES; OSTEOLASTS; INTEGRINS; CD41; IL-3

Skeletal fragility has emerged as a major limitation to quality of life as we age. 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 1-year post-hip fracture mortality of 24% (National Osteoporosis Foundation). 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.

Over the past decade, a new paradigm has emerged wherein megakaryocytes (MKs) have been found to play an important role in skeletal homeostasis. In brief, data demonstrate that MKs may act to stimulate bone formation by expressing/secreting bone-related proteins, and by directly enhancing osteoblast (OB) proliferation and differentiation [Breton-Gorius et al., 1992; Chenu and Delmas, 1992; Kelm et al., 1992; Frank et al., 1993; Thiede et al., 1994; Kacena et al., 2004; Miao et al., 2004; Sipe et al., 2004; Bord et al., 2005; Ciovacco et al., 2009, *in press*]. Simultaneously, MKs may regulate bone resorption by expressing/secreting several factors known to be involved in osteoclastogenesis, and recent studies demonstrate that MKs can inhibit osteoclast (OC) formation *in vitro* [Jiang et al., 1994; Wickenhauser et al., 1995a,b; Soslau et al., 1997; Kartsogiannis et al., 1999; Pearse et al., 2001; Bord et al., 2003, 2004; Chagraoui et al., 2003; Beeton et al., 2006; Kacena et al., 2006; Ciovacco et al., *in press*]. Specifically, our laboratory has demonstrated that MKs

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induce OB activation *in vitro* via a mechanism(s) requiring direct physical contact between the two cell types [Kacena et al., 2004], whereas MKs inhibit OC development *in vitro* via the elaboration of an as-yet unidentified soluble factor(s) [Kacena et al., 2006]. The net result, as demonstrated *in vivo*, is that increases in MK number can lead to concomitant increases in bone mass [Yan et al., 1995, 1996; Villeval et al., 1997; Frey et al., 1998a,b; Kacena et al., 2004, 2005; Suva et al., 2008].

In the present study, we have focused our efforts on characterization of the contact-dependent mechanism(s) by which MKs induce OB proliferation/differentiation. To this end, we have effectively neutralized several adhesion molecules known to function in the analogous interaction of MKs with another cell type of mesenchymal origin—the fibroblast (FB) [Schmitz et al., 1998; Wickenhauser et al., 2000]. Furthermore, we have explored the effect of interleukin (IL)-3 on our MK–OB model system, as this cytokine has been shown to enhance MK-induced FB activation *in vitro* [Schmitz et al., 1995, 1999]. Here we examine these new data which may offer insight as to the mechanism(s) of this interaction.

## MATERIALS AND METHODS

### PREPARATION OF NEONATAL CALVARIAL CELLS (OB) AND FETAL LIVER-DERIVED MKS AND EXPERIMENTAL CONDITIONS

C57BL/6 murine calvarial cells of the OB lineage were prepared by sequential collagenase digestion as previously described [Wong and Cohn, 1975; Horowitz et al., 1994]. Cells collected from fractions 3–5 were used as the starting population for OB/osteoprogenitor culture. To isolate MKs, livers from 13- to 15-day-old embryos (C57BL/6 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. MKs were then separated from other cell types (90–95% pure MK population) using a one-step albumin gradient [Drachman et al., 1997]. Two thousand five hundred OB/well were co-cultured with 5,000 MKs/well in 96-well tissue culture plates (optimal, pre-tested).

Ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, 0.0125 and 0.125 mM) and soluble tetrapeptide Arg-Gly-Asp-Ser (RGDS; Sigma, 0.0125 and 0.625 mM) were titrated into MK–OB co-cultures and OB control cultures. The following neutralizing antibodies were purchased: integrin  $\alpha_3$  chain/CD49c (10  $\mu$ g/ml; polyclonal; R&D Systems, Minneapolis, MN), integrin  $\alpha_5$  chain/CD49e (20  $\mu$ g/ml; clone: 5H10-27 MFR5; BD Biosciences Pharmingen, San Diego, CA), and gp IIb/integrin  $\alpha_{IIb}$  chain/CD41 (10 ng/ml; clone: MWReg30; BD Biosciences Pharmingen). Goat IgG fraction was added to separate controls at equivalent concentrations to assure that blocking was antigen-specific. Finally, recombinant murine IL-3 (R&D Systems, 10 and 30 ng/ml) was also titrated into co-cultures and controls.

### PROLIFERATION ANALYSIS

Proliferation was measured by  $\beta$ -scintillation count following tritiated ( $^3\text{H}$ )-thymidine incorporation (1  $\mu$ Ci/well; 5–8 Ci/mmol; added 16 h prior to freezing of culture at day 4). Because MKs are non-adherent, they were removed by washing ( $4\times$ ) prior to freezing

and subsequent harvesting to ensure that OB proliferation alone was measured [Kacena et al., 2004].

### STATISTICS

Unless otherwise stated, all data are presented as the mean  $\pm$  SD. For all studies one-way ANOVA was used to determine significant differences ( $P < 0.05$ ). All analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS, Inc., Chicago, IL) software and were two-tailed with a level of significance set at 0.05. Experiments are always repeated, in some cases multiple times. Within individual experiments, data points are based on a minimum of triplicate samples.

## RESULTS

### EFFECT OF EDTA ON MK–OB CULTURES

The bivalent cation chelator EDTA was titrated into MK–OB co-cultures and OB controls to examine the effect of non-selective integrin inhibition on MK-induced OB activation. While the addition of EDTA at 12.5  $\mu$ M failed to inhibit MK-induced activation, 125  $\mu$ M EDTA significantly reduced tritium incorporation in co-cultures by 18% without affecting OB controls (Fig. 1A). At higher concentrations tested, EDTA began to inhibit OB monoculture proliferation (data not shown).

### EFFECT OF RGDS ON MK–OB CULTURES

To elucidate the involvement of Arg-Gly-Asp (RGD)-binding receptors in our model system, the soluble tetrapeptide Arg-Gly-Asp-Ser (RGDS; Sigma) was titrated into co-cultures and OB controls. RGDS caused dramatic, dose-dependent inhibition of proliferation in co-cultures, with 12.5 and 62.5  $\mu$ M, respectively, diminishing MK-induced activation by 26% and 50%, respectively, without affecting OB controls (Fig. 1B). Further increases in RGDS concentration did not result in greater inhibition of proliferation in co-cultures.

### EFFECT OF ANTI- $\alpha_3$ , ANTI- $\alpha_5$ , AND ANTI-CD41 ON MK–OB CULTURES

Neutralizing antibodies directed against the following specific adhesion molecules were applied to co-cultures and OB controls: integrin  $\alpha_3$  chain/CD49c, integrin  $\alpha_5$  chain/CD49e, and gp IIb/integrin  $\alpha_{IIb}$  chain/CD41. Each of the neutralizing antibodies tested yielded virtually identical results, causing moderate, yet significant, reductions in co-culture proliferation without affecting OB monocultures. Specifically, maximal suppression of 21% by anti- $\alpha_3$  was seen at 10  $\mu$ g/ml, 20% by anti- $\alpha_5$  at 20  $\mu$ g/ml, and 20% by anti-CD41 at 10 ng/ml (Fig. 2). Non-specific IgG did not affect tritium incorporation in co-cultures or OB monocultures when added at respective control concentrations (data not shown).

### EFFECT OF IL-3 ON MK–OB CULTURES

Lastly, recombinant murine IL-3 was titrated into co-cultures and controls to explore the effect of this cytokine on MK-induced OB proliferation. Ten and 30 ng/ml IL-3 enhanced MK-induced OB proliferation by 41% and 37%, respectively, while OB monocultures

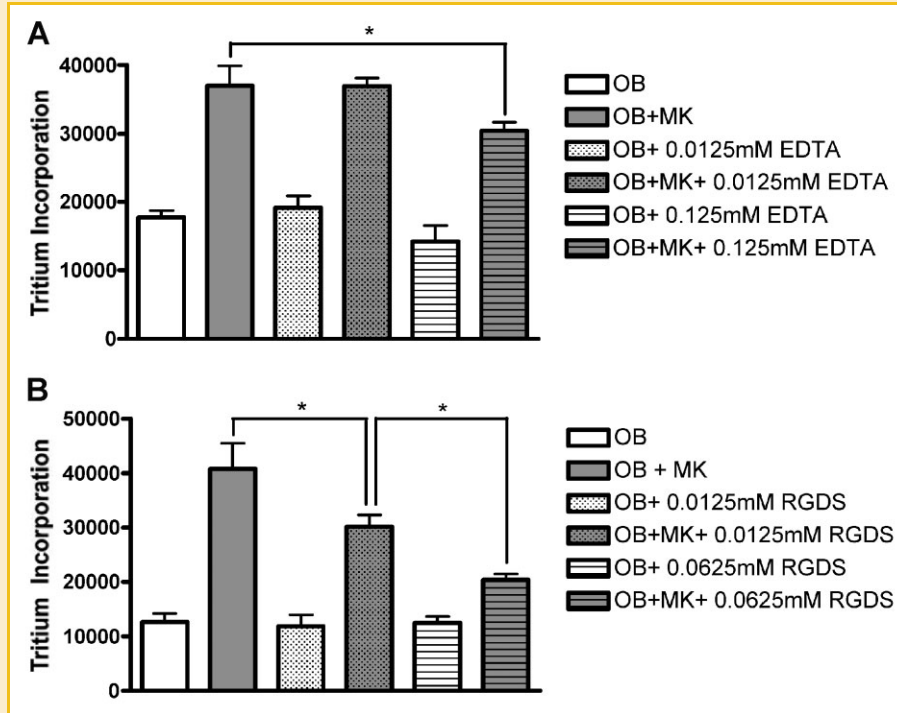


Fig. 1. Integrin involvement in MK-induced OB activation. A: 12.5  $\mu$ M EDTA was not sufficient to disrupt MK-induced OB proliferation; however, 125  $\mu$ M EDTA diminished MK-induced proliferation by 18%. Tritium incorporation by OB monocultures was not affected at either concentration reported. B: Soluble tetrapeptide RGDS caused a dose-dependent inhibition of MK-induced proliferation with 12.5 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. Asterisk (\*) denotes a significant difference between groups examined ( $P < 0.05$ ).

remained unaffected (Fig. 3). Further increasing IL-3 concentration did not yield additional enhancement of MK-induced activation.

## DISCUSSION

Our laboratory has previously demonstrated that MKs can induce OB proliferation, but do so only when direct cell-to-cell contact is permitted [Kacena et al., 2004]. To further investigate the nature of this interaction, we have systematically disrupted known mechanisms of MK-FB adhesion/signaling in our own MK-OB 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 [Ruoslahti, 1991; Hynes, 1992] and has been shown to inhibit MK-FB adherence and signaling in vitro [Schmitz et al., 1998]. The ability of EDTA to diminish MK-induced OB proliferation without affecting OB monocultures thus implicates integrin involvement in MK-OB adherence/signaling as well. Refining the scope of our investigation, we next examined the role of RGD receptors in our model system. The conformation of the RGD sequence of fibronectin is approximated in soluble form by the tetrapeptide RGDS [Pierschbacher and Ruoslahti, 1987]. Therefore, our finding that the addition of this tetrapeptide inhibits MK-induced proliferation without affecting OB monocultures implicates specifically, although not

exclusively, RGD-binding integrins. These data are again consistent with those pertaining to MK-FB adherence/signaling [Schmitz et al., 1998]. The respective roles of MK-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 MK-induced proliferation. As OB monocultures were not affected by these antibodies, and non-specific IgG affected neither co-cultures nor OB controls, we conclude that these specific integrins directly contribute to MK-OB adhesion/activation, as demonstrated in the MK-FB model system [Schmitz et al., 1998]. Employing the same technique and reasoning, we next elucidated the involvement of the MK-expressed gp 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 MK-FB interaction findings [Wickenhauser et al., 2000], we conclude that this gp plays an important role in MK-induced adherence/activation.

Lastly, our data demonstrate that IL-3 significantly enhances MK-induced OB proliferation without affecting OB monocultures. Although we have not demonstrated that this enhancement is contingent upon direct cell-cell contact, Schmitz et al. [1995] showed that IL-3 could not enhance FB proliferation when MK-FB

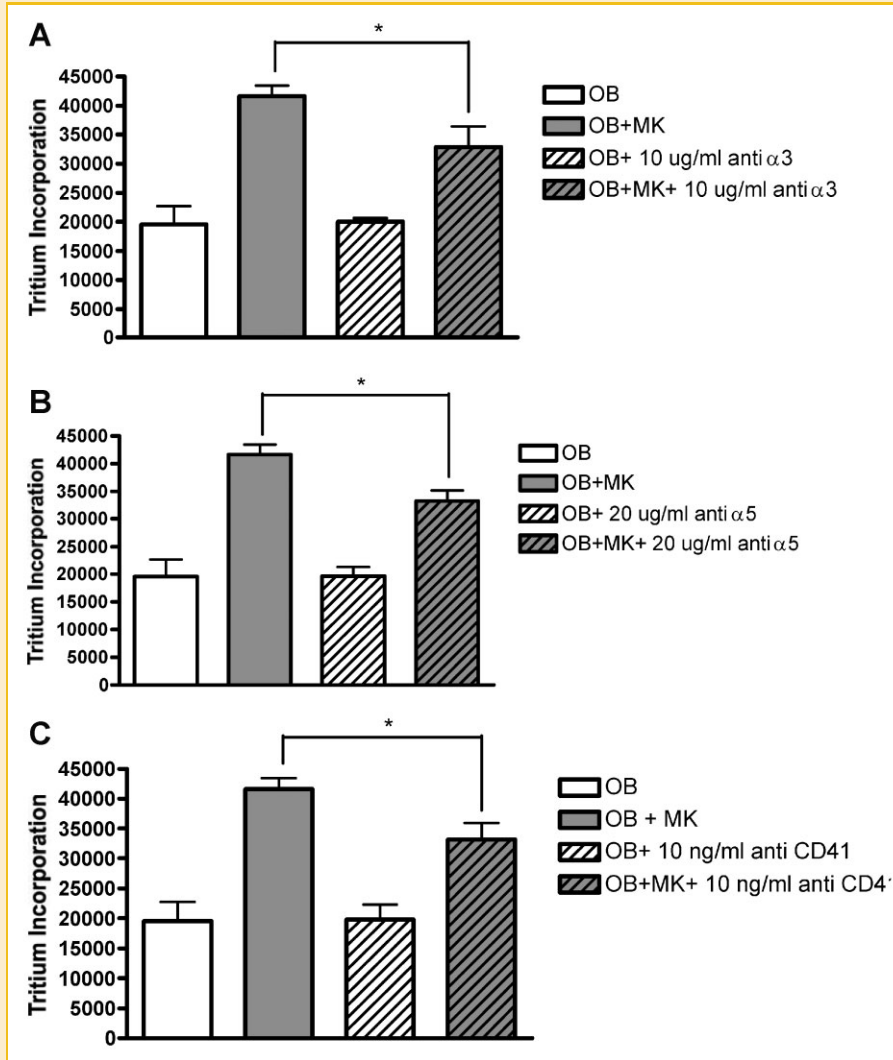


Fig. 2. Involvement of specific adhesion molecules in MK-induced OB activation. Application of neutralizing antibodies against integrin  $\alpha_3$  chain (A), integrin  $\alpha_5$  chain (B), and CD41 (C) each diminished MK-induced OB proliferation by approximately 20%, without affecting tritium incorporation in OB monocultures. Asterisk (\*) denotes a significant difference between groups examined ( $P < 0.05$ ).

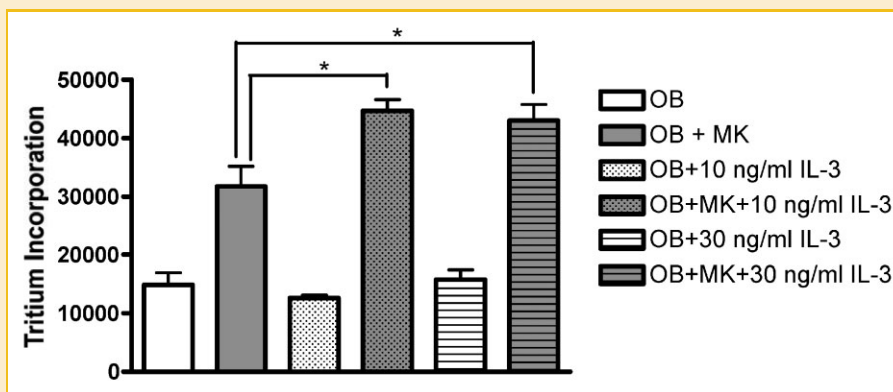


Fig. 3. IL-3 enhances MK-induced OB activation. At 10 and 30 ng/ml, IL-3 enhanced MK-induced OB proliferation by 41% and 37%, respectively. Tritium incorporation by OB monocultures remained unaffected at both concentrations reported. Asterisk (\*) denotes a significant difference between groups examined ( $P < 0.05$ ).

co-cultures were divided by cell-impermeable membranes. Furthermore, the prerequisite of direct cell–cell contact in no way excludes signaling via soluble factors as a mechanism for MK-induced mesenchymal cell activation. To the contrary, Schmitz et al. [1995] speculate that adhesion may serve principally to expose FBs to supra-threshold levels of MK-derived growth factors such as PDGF and TGF $\beta$ . While many such details of our own model—and skeletal homeostasis in general—remain obscure, we remain optimistic that our efforts will contribute to identification of pathways which when engaged will function as an anabolic stimulator of bone formation for the treatment of osteoporosis and other bone loss diseases.

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