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# c-Fms and the $\alpha_v\beta_3$ integrin collaborate during osteoclast differentiation

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## Introduction

All forms of osteoporosis represent enhanced bone-resorptive activity of osteoclasts (OCs), relative to the bone forming capacity of osteoblasts. Thus, curing this family of diseases will depend upon understanding how these cells are regulated.

Two molecules, MCSF and receptor activator of NF- $\kappa$ B ligand (RANKL), are essential and sufficient to promote osteoclastogenesis. MCSF, which is obligatory for macrophage maturation, binds its receptor, c-Fms, which dimerizes and autophosphorylates several tyrosine residues, thereby providing signals required for survival and proliferation of early OC precursors (1). Hence, MCSF deficiency, because of a virtual absence of OCs, prompts an osteopetrotic phenotype (2, 3).

While the role of MCSF in macrophage proliferation is established, less is known about its direct effect on OC differentiation. To explore this issue, our laboratory

developed a chimeric strategy to identify the structural components of c-Fms that mediate signaling in macrophages and OCs (4). Tyrosine (Y) 559 and Y807 in the c-Fms cytoplasmic domain are essential for OC proliferation and differentiation, while tyrosine Y697, Y706, and Y721, which are dispensable for macrophage proliferation, exert no impact on wild-type OCs (4).

Integrins are transmembrane proteins that comprise  $\alpha$  and  $\beta$  subunits. These heterodimers mediate cell-cell and cell-matrix interactions and generate intracellular signals when occupied by ligands (5), or upon treatment of cells with growth factors or cytokines (6). The integrin  $\alpha_v\beta_3$  is expressed by OCs, and binding of this complex to bone is pivotal to the resorptive process (7). Since pharmacological blockade of  $\alpha_v\beta_3$  prevents experimental postmenopausal osteoporosis (8), the integrin is currently a therapeutic target (9, 10). Despite the critical role played by  $\alpha_v\beta_3$  in skeletal resorption, little is known about its involvement in osteoclastogenesis. However, the fact that commitment of macrophages to the OC phenotype requires matrix recognition is consistent with the concept that integrins are involved in OC differentiation (11).

The likelihood that both MCSF and the  $\alpha_v\beta_3$  integrin regulate osteoclastogenesis raises the possibility that they induce similar intracellular signals that eventuate in OC differentiation. In fact, cross-talk between integrin and growth factor receptor signaling pathways regulates cell proliferation, differentiation, and survival (12). Potentiation of growth factor receptor signaling by integrins contributes to cell cycle progression, as growth

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**Conflict of interest:** The authors have declared that no conflict of interest exists.

**Nonstandard abbreviations used:** osteoclast (OC); receptor activator of NF- $\kappa$ B ligand (RANKL); extracellular signal-regulated kinase (ERK); erythropoietin (Epo); erythropoietin receptor (EpoR); tartrate-resistant acid phosphatase (TRAP); osteopontin (OPN); microphthalmia transcription factor (MITF).

factor-stimulated mitogenic signaling is transient in the absence of integrin-mediated cell adhesion. For example, growth factors rapidly provoke the extracellular signal-regulated kinase (ERK) pathway, but prolongation of such activation requires cell adhesion (12–14).

ERK activation is central to survival of hematopoietic cells (15) and mature OCs (16). Moreover, induction of this MAPK cascade is indispensable for proliferation and differentiation of selected cells in response to growth factors. In some circumstances, transient stimulation of the ERK cascade leads to cell proliferation, whereas sustained activation prompts differentiation (17). Because prolonged ERK activation results in nuclear translocation (18–20), the enhanced cellular maturation attending this event may reflect modulated gene expression via transcription factor phosphorylation by the kinase. For example, prolonged ERK activation is required for stable expression of c-Fos (21), a transcription factor essential for osteoclastogenesis whose gene is transactivated, in macrophages, by MCSF (22–24).

Because little is known about the impact of  $\alpha_v\beta_3$  on osteoclastogenesis, we turned to this issue. We find that bone marrow macrophages (BMMs) derived from  $\beta_3^{-/-}$  mice, when plated in standard osteoclastogenic conditions, fail to normally differentiate into OCs. Interestingly, while increased RANKL levels are ineffective, enhanced MCSF completely rescues the differentiation defect in  $\beta_3^{-/-}$  OC precursors, an event mediated by c-Fms Y697. MCSF normalization of  $\beta_3^{-/-}$  osteoclastogenesis requires prolonged ERK signaling and enhanced c-Fos expression, but the integrin, per se, is needed for matrix degradation.

## Methods

**Construction and expression of retroviral vector expressing EpoR/c-Fms chimera and c-Fos.** We constructed erythropoietin receptor/c-Fms chimeras that consisted of the external domain of the erythropoietin receptor (EpoR) linked to the transmembrane and intracellular domains of c-Fms. Individual chimeras contained single tyrosine-to-phenylalanine point mutation in the cytoplasmic tail. We used the pMX-puro retroviral vector to express these chimeras, as described previously (4). pBabe-puro/c-Fos vector was kindly provided by Erwin Wagner (Research Institute of Molecular Pathology, Vienna, Austria) (25). The plasmids were transiently transfected into Phoenix-E cells using LipofectAmine Plus (Life Technologies Inc., Rockville, Maryland, USA).

**Infection of BMMs.** We isolated BMMs from bone marrow of 4- to 8-week-old mice, cultured them overnight in  $\alpha$ -MEM containing 10% heat-inactivated FBS, and subjected them to Ficoll-Hypaque gradient purification as described previously (26). We then collected  $5 \times 10^6$  cells at the gradient interface and plated them in a p100 suspension culture dish (Corning Inc. Costar, New York, New York, USA) in the presence of 1:10 CMG14-12 culture supernatant (CMG) (27), which contained the equivalent of 240  $\mu\text{g}/\text{ml}$  of recombinant

MCSF. After 2 days, cells were transduced with virus for 24 hours in the presence of 1:10 CMG supernatant and 4  $\mu\text{g}/\text{ml}$  polybrene (Sigma-Aldrich, St. Louis, Missouri, USA). Transduced cells were selected for an additional 3–5 days in puromycin-containing medium prior to analysis of osteoclastogenesis.

**In vitro generation of OCs and bone resorption assay.** BMMs from  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice were cultured in  $\alpha$ -MEM containing 10% heat-inactivated FBS in the presence of 100 ng/ml RANKL and increasing concentrations of MCSF (from 10 to 100 ng/ml) in 96-well tissue-culture plates.  $\beta_3^{-/-}$  BMMs transduced with pBabe/c-Fos were cultured in the presence of 100 ng/ml RANKL and 10 ng/ml MCSF. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity after 3, 5, 7, or 10 days in culture, using a commercial kit (Sigma-Aldrich 387-A). BMMs transfected with the different erythropoietin/c-Fms mutants were cultured in the presence of 100 ng/ml RANKL and 25 U/ml recombinant human erythropoietin (Epo). Bone resorption was performed using  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  pre-OCs retrovirally transduced with pBabe vector alone or with pBabe/c-Fos cultured for 4 additional days under the indicated osteoclastogenic condition. Cells were fixed and stained with TRAP or removed by brief treatment with 2N NaOH. The resorptive pits were visualized by hematoxylin staining.

**Proliferation assay.** BMMs from  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice were cultured in the presence of different concentrations of MCSF (from 6.25 to 100 ng/ml) with or without 100 ng/ml RANKL. After 3 days, the number of viable cells was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) method. Briefly, 10  $\mu\text{l}$  MTT (5 mg/ml) was added to 100  $\mu\text{l}$  culture medium in each well and incubated at 37°C for 4 hours. One hundred fifty microliters 0.04N HCl in isopropanol was added to each well to stop the reaction, and MTT absorbance was determined at an OD of 570 nm. Six wells were used for each treatment, and experiments were repeated twice.

**Apoptosis assay.**  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs were cultured with 10 ng/ml or 100 ng/ml MCSF alone or in combination with 100 ng/ml RANKL, the latter added at initiation of culture (day 4) or after 2 days (day 2). As positive control for cell death, the cytokines were removed from half the wells during the last 24 hours of the culture period. The magnitude of apoptosis was determined by ELISA (Cell Death Detection ELISA; Roche Molecular Biochemicals, Mannheim, Germany). Briefly, culture medium was carefully removed and the provided lysis buffer was added to each well for 30 minutes at room temperature. Twenty microliters of the supernatant was immediately analyzed according to the manufacturer's directions. Data represent mean  $\pm$  SE from three independent experiments.

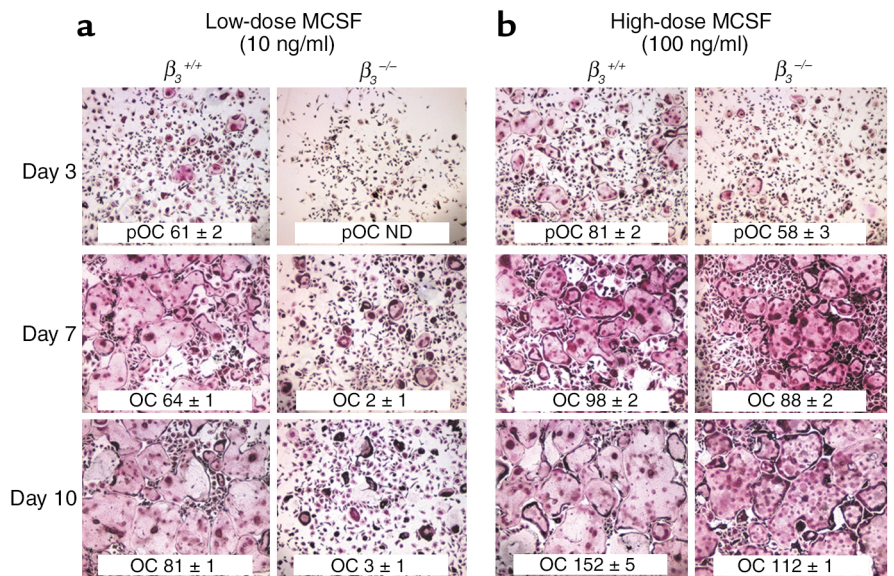
**ELISA and Western blot for MCSF.** Circulating levels of MCSF were detected in the serum of three different  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice (mouse MCSF Quantikine ELISA; R&D Systems Inc., Minneapolis, Minnesota, USA) according to the manufacturer's directions. Membrane-bound

MCSF was detected in bone marrow cells, flushed from tibia and femur with a minimal amount of PBS, and immediately lysed in 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 1% NP 40, 1 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitor cocktail (Sigma-Aldrich). Fifty micrograms of proteins were loaded under nonreducing conditions in a 12% SDS gel, transferred to nitrocellulose membranes using a semidry blotter (Bio-Rad Laboratories Inc., Richmond, California, USA), and incubated in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween-20) for 1 hour, to reduce nonspecific binding. Western analysis was performed using the anti-MCSF mAb 5A1, kindly provided by H.-S. Lin (Washington University) (28), and showed a 22-kDa band corresponding to membrane-bound MCSF. Equal loading was confirmed by  $\beta$ -actin (Sigma-Aldrich) blot.

**Western analysis.** BMMs from  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice were cultured for 3 days in the presence of 100 ng/ml RANKL and 10 ng/ml purified MCSF, starved for 2 hours, and stimulated for the indicated times with 10 or 100 ng/ml purified MCSF. BMMs transfected with EpoR/c-Fms chimeras were grown in the presence of RANKL and MCSF, starved of serum and growth factors, and stimulated with 25 U/ml Epo before lysis. A different set of experiments was performed using day 3  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  pre-OCs. Cells were lifted with trypsin/EDTA solution, resuspended in a serum-free medium, and plated onto dishes coated with 5  $\mu$ g/ml osteopontin (OPN) for the indicated times. Cells were then washed twice with ice-cold PBS and lysed in the buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 1% NP 40, 1 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 $\times$  protease inhibitor cocktail (Sigma-Aldrich). Forty micrograms per milliliter of cell lysates were boiled in the presence of SDS sample buffer for 5 minutes and subjected to electrophoresis on 8% SDS-PAGE. For ERK-MAPK Western analysis, a polyclonal anti-ERK1/2 or an anti-phospho-p42/p44 MAPK polyclonal antibody was used (Cell Signaling Technology Inc., Beverly, Massachusetts, USA). For RSK analysis, the anti-phospho-p90RSK antibody was purchased from Cell Signaling Technology Inc., and the monoclonal anti-RSK antibody was from BD Transduction Laboratories Inc. (San

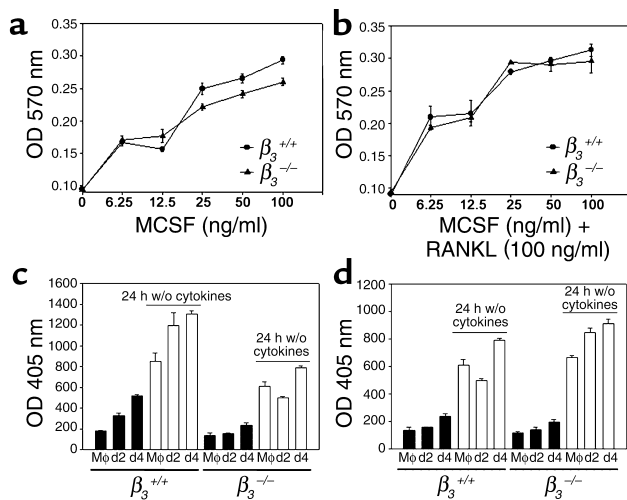
Diego, California, USA). Anti-c-Fos and anti-c-Fms were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA).

**RNA preparation and RT-PCR analyses.** Total RNA from cultured cells was isolated by the guanidine/phenol method. For RT-PCR analysis, cDNAs were synthesized from 1  $\mu$ g of total RNA using reverse transcriptase and oligo-dT primers in a volume of 20  $\mu$ l, and the reaction mixture was finally adjusted to 100  $\mu$ l with TE buffer for PCR analysis. PCR was performed with 1  $\mu$ l of cDNA reaction mixture using Platinum Pfx polymerase (Invitrogen Corp., Carlsbad, California, USA) and appropriate primers in a volume of 50  $\mu$ l. The following primers were used: for cathepsin K, 5'-GGAAGAAGACTCACCAGAAGC-3' and 3'-GCTATATAGC-GCCTCCACAG-5'; for MMP-9, 5'-CCTGTGTGTTCC-GTTCATCT-3' and 3'-CGCTGGAATGATCTAAGCCCA; for TRAP, 5'-ACAGCCCCCTCCACCCT-3' and 3'-TCAGGGTCTGGTCTCCTTGG-5'; for microphthalmia transcription factor (MITF), 5'-ACCATCAGCAACTCT-GTCC-3' and 3'-GAATCGGATCATCAAGCAAGAA-5'; for calcitonin receptor, 5'-CATTCTGTACTTGGTTGGC-3' and 3'-AGCAATCGACAAGGAGTGAC; and for GAPDH, 5'-ACTTTGTCAAGCTCATTTC-3' and 3'-TGCAGCGAAC-TTTATTGATG-5'. The samples were transferred to a programmable thermal cycler (Hybaid US, Franklin, Massachusetts, USA) that had been preheated to 95°C, and incubated for 21–40 PCR cycles. Each cycle consisted of a denaturation step at 95°C for 1 minute, an



**Figure 1**

High-dose MCSF rescues  $\beta_3^{-/-}$  osteoclastogenesis. BMMs derived from  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  mice were cultured in RANKL and either 10 ng/ml (a) or 100 ng/ml (b) MCSF for 3, 7, or 10 days, fixed in 4% paraformaldehyde, and stained for TRAP activity. Within 3 days,  $\beta_3^{+/+}$  BMMs in low-dose MCSF developed into mononuclear and binuclear TRAP-expressing cells (pOCs). Characteristic multinucleated OCs appeared within 7–10 days. In contrast,  $\beta_3^{-/-}$  BMMs failed to normally form pre-OCs and to differentiate into typical OCs within this time frame. When  $\beta_3^{-/-}$  and  $\beta_3^{+/+}$  BMMs were cultured in high-dose (100 ng/ml) MCSF, their differentiation into mature OCs was similar at days 7 and 10. Indicated are the numbers of binucleated pre-OCs (pOCs) (day 3) or multinucleated OCs (days 7 and 10) per well, from three independent experiments.  $\times 4$ . ND, not determined.



**Figure 2**

The capacity of high-dose MCSF to increase  $\beta_3^{-/-}$  OC number is not due to accelerated proliferation or arrested apoptosis.  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs were cultured with increasing concentrations of MCSF in the absence (a) or presence (b) of 100 ng/ml RANKL. After 3 days, proliferation was assessed by MTT assay. Regardless of genotype, the proliferative rates of the cells were similar at each concentration of cytokine.  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs were maintained in culture in low-dose (10 ng/ml, c) or high-dose (100 ng/ml, d) MCSF, alone (M $\phi$ ) or with 100 ng/ml RANKL, the latter added at initiation of culture (d4) or after 2 days (d2). Cytokines were present during the entire 4-day culture period in half the wells (black bars) and were withdrawn from the other half during the last 24 hours to induce apoptosis (white bars), the magnitude of which was determined by ELISA. The apoptotic rate of  $\beta_3^{-/-}$  cells was diminished in low-dose MCSF but indistinguishable from WT in high concentrations of the cytokine.

annealing step at 60°C for 1 minute, and an extension step at 72°C for 1 minute. Ten-microliter aliquots of PCR products were separated by electrophoresis on a 1.5% agarose gel.

*Statistics.* All data are presented as mean  $\pm$  SEM.

## Results

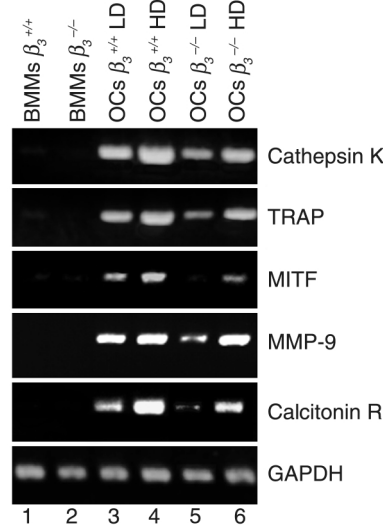
*Osteoclastogenesis by  $\beta_3^{-/-}$  BMMs is rescued by high-dose MCSF.* To assess the role of  $\alpha_v\beta_3$  in osteoclastogenesis, we measured the capacity of  $\beta_3^{-/-}$  BMMs to differentiate into mature OCs when cultured in standard osteoclastogenic conditions.  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  BMMs were maintained in 10 ng/ml MCSF and 100 ng/ml RANKL for 3, 5, 7, or 10 days, and differentiated OCs were detected as a function of multinucleation and TRAP expression. TRAP-expressing  $\beta_3^{+/+}$  mononuclear and binuclear pre-OCs and a few small multinuclear OCs appeared by day 3 and became fully differentiated by day 7–10 (Figure 1a). In contrast, the capacity of  $\beta_3^{-/-}$  BMMs to differentiate into pre-OCs by day 3 or assume the fully OC phenotype by day 10 was arrested (Figure 1a). Increasing RANKL levels to 500 ng/ml did not impact the osteo-

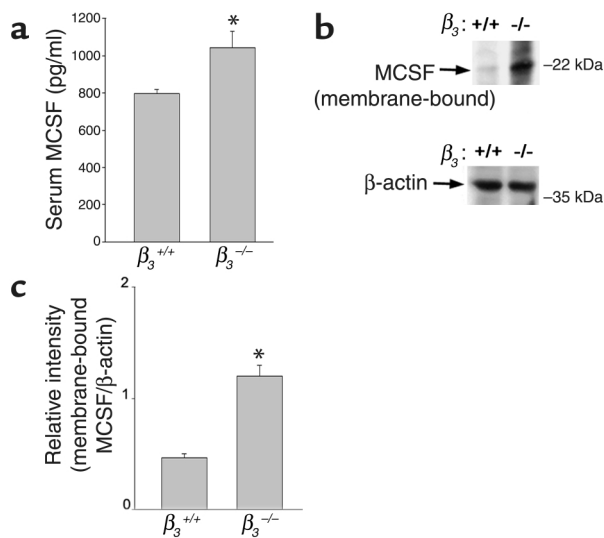
clastogenic defect (not shown). On the other hand, OC formation by  $\beta_3^{-/-}$  BMMs normalized when MCSF concentration was increased from 10 to 100 ng/ml without RANKL concentration being altered (Figure 1b). While characteristic  $\beta_3^{+/+}$  OCs appeared earlier (i.e., by day 3) under the latter conditions, the impact of increased MCSF on  $\beta_3^{-/-}$  BMMs was striking. High-dose MCSF promoted differentiation of these mutant macrophages into TRAP-expressing pre-OCs, and into OCs indistinguishable from WT, by day 7–10.

We next asked whether the increase in  $\beta_3^{-/-}$  OC number induced by high-dose MCSF reflects enhanced proliferation and/or attenuated apoptosis. Proliferation of  $\beta_3^{-/-}$  BMMs in increasing concentrations of MCSF, with (Figure 2b) or without (Figure 2a) RANKL, mirrored that of WT cells. Interestingly, along with their failure to differentiate into OCs, the rate of constitutive and stimulated (by 24-hour cytokine withdrawal) apoptosis of  $\beta_3^{-/-}$  BMMs was reduced in low-dose MCSF plus RANKL (Figure 2c). Programmed death of these mutant cells was, however, indistinguishable from that of their WT counterparts when ambient MCSF was

**Figure 3**

High-dose MCSF promotes expression of osteoclastogenic markers in  $\beta_3^{-/-}$  pre-OCs. BMMs derived from  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  mice were cultured with RANKL and either low-dose (10 ng/ml; LD) or high-dose (100 ng/ml; HD) MCSF. After 3 days, RNA was extracted, and expression of the mRNA of osteoclastogenic markers cathepsin K, TRAP, MITF, MMP-9, and calcitonin receptor was measured by RT-PCR. Untreated  $\beta_3^{+/+}$  (lane 1) and  $\beta_3^{-/-}$  (lane 2) BMMs, serving as negative control, failed to express these mRNA species, whereas they were abundant in  $\beta_3^{+/+}$  OCs regardless of ambient MCSF (lanes 3 and 4). Compared with those of their WT counterparts, TRAP mRNA levels were decreased twofold, cathepsin K mRNA threefold, MMP-9 mRNA threefold, MITF mRNA fivefold, and calcitonin receptor (R) mRNA fivefold in  $\beta_3^{-/-}$  cells treated with low-dose MCSF (lane 5). Reflecting its capacity to rescue  $\beta_3^{-/-}$  osteoclastogenesis, high-dose MCSF substantially enhanced these mRNAs in mutant cells (lane 6).





**Figure 4** M-CSF is increased in  $\beta_3^{-/-}$  mice in vivo. Circulating and bone-microenvironment M-CSF levels from three  $\beta_3^{-/-}$  and three  $\beta_3^{+/+}$  mice were measured by ELISA (a) or Western blot (b and c). (a) Serum M-CSF levels analyzed by ELISA in  $\beta_3^{-/-}$  mice were significantly increased relative to those of  $\beta_3^{+/+}$  counterparts. \* $P < 0.05$ . (b) Marrow cells isolated from the same  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice were lysed and subjected to immunoblot using an anti-M-CSF mAb. Representative gels demonstrate a 22-kDa band, corresponding to membrane-residing M-CSF, in both circumstances but was three times higher in  $\beta_3^{-/-}$  cells. Actin served as loading control. (c) Densitometric analysis of ratio of membrane-residing M-CSF relative to actin in bone marrow cells derived from three mice of each genotype. \* $P < 0.005$ .

enhanced (Figure 2d). Thus, the increase in the number of  $\beta_3^{-/-}$  OCs that was induced by high-dose M-CSF reflects stimulated differentiation and neither accelerated division nor arrested apoptosis of OC precursors.

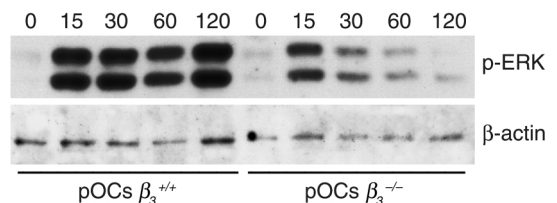
To determine whether the profound morphological changes in cultured  $\beta_3^{-/-}$  BMMs that were prompted by high-dose M-CSF represented bona fide OC differentiation, we assessed expression of various markers of mature OCs.  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  cells were cultured for 3 days in RANKL and low-dose (10 ng/ml) or high-dose (100 ng/ml) M-CSF. RNA was extracted, and TRAP, MMP-9, and calcitonin receptor mRNA levels were determined by RT-PCR (Figure 3). We also assessed mRNA expression of cathepsin K (CK), a lysosomal cysteine protease involved in dissolution of bone organic matrix (29), and microphthalmia transcription factor (MITF), known to regulate transcription of TRAP and CK (30). Confirming arrest of OC differentiation,  $\beta_3^{-/-}$  BMMs cultured in RANKL plus low-dose M-CSF contained twofold less TRAP, threefold less MMP-9, and threefold less CK mRNA than did  $\beta_3^{+/+}$  cells. MITF and calcitonin receptor were particularly dampened in  $\beta_3^{-/-}$  cells (fivefold). Consistent with its morphological effects, high-dose M-CSF enhanced levels of each of the indicated OC differentiation markers in  $\beta_3$ -null cells to approximately those found in their WT counterparts exposed to the standard concentration of the cytokine.

Reflecting induction of WT osteoclastogenesis, high-dose cytokine also stimulated OC marker expression by  $\beta_3^{+/+}$  pre-OCs. As expected, none of the indicated species of mRNA was substantially expressed by  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs cultured only with M-CSF. Thus, failed osteoclastogenesis of  $\beta_3^{-/-}$  cells cultured in low-dose M-CSF reflects arrested differentiation that is corrected by increased concentrations of the cytokine.

*M-CSF is increased in  $\beta_3^{-/-}$  mice in vivo.* The data presented thus far are consistent with the hypothesis that the increased OC number extant in  $\alpha_v\beta_3$  deficient mice reflects the impact of abundant M-CSF. In fact, circulating levels of the cytokine were increased about 30% in the mutant animals (Figure 4a). In order to measure the M-CSF level in the bone microenvironment, freshly isolated bone marrow cells were lysed and subjected to Western blot analysis. A 22-kDa band, corresponding to the membrane-residing form of M-CSF (31), was detected in the lysates derived from both genotypes (Figure 4b). Importantly, membrane M-CSF expression was enhanced threefold in  $\beta_3^{-/-}$  mice (Figure 4c).

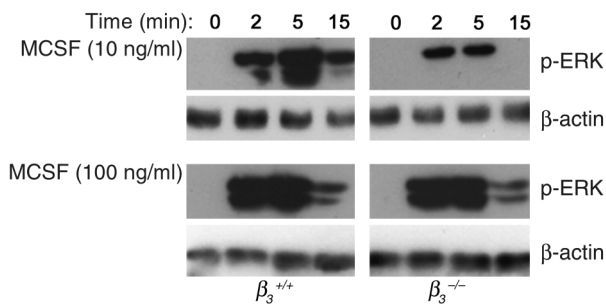
*Defective ERK signaling in  $\beta_3^{-/-}$  pre-OCs is rescued by high-dose M-CSF.* The fact that high-dose M-CSF compensates for lack of  $\alpha_v\beta_3$  suggests that a common signaling pathway emanates from the cytokine receptor and the integrin. Because c-Fms or  $\beta_3$  integrin engagement activates ERKs (32, 33), these MAPKs are possible downstream candidates. Furthermore, ERK phosphorylation correlates with proliferation, survival, and differentiation of various cells (20, 34). Hence, we determined the ability of  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  cells to induce ERK phosphorylation upon adhesion to the  $\alpha_v\beta_3$  substrate OPN.

Day 3 pre-OCs, generated from  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs, were lifted and replated on OPN in the absence of serum. After 15, 30, 60, or 120 minutes, adherent cells were lysed and ERK activation, as manifest by its phosphorylation, was assessed by immunoblot. Fifteen minutes of attachment of  $\beta_3^{+/+}$  pre-OCs to the  $\alpha_v\beta_3$  ligand and prompted ERK1/2 phosphorylation, the magni-



**Figure 5** Adhesion-induced ERK activation is defective in  $\beta_3^{-/-}$  pre-OCs.  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs were maintained for 3 days in low-dose M-CSF and 100 ng/ml RANKL. The cells were detached and replated in OPN-coated dishes. Nonadherent cells were removed, and attached pre-OCs were lysed at the indicated times. Equal amounts of total protein were immunoblotted with an antibody to phospho-ERK (p-ERK) or  $\beta$ -actin. ERK phosphorylation maximized within 15 minutes of attachment of  $\beta_3^{+/+}$  pre-OCs and was sustained for at least 120 minutes. While ERK phosphorylation also maximized within 15 minutes of attachment, the signal rapidly dissipated in the absence of the integrin.





**Figure 6**  
High-dose MCSF rescues ERK activation in  $\beta_3^{-/-}$  pre-OCs. Pre-OCs derived from BMMs treated with RANKL and low-dose MCSF for 3 days were starved for 2 hours in serum-free medium and exposed to 10 or 100 ng/ml MCSF, with time. ERK activation was detected in total cell lysates using an anti-phospho-ERK antibody.  $\beta$ -Actin served as loading control. In the presence of low-dose MCSF, ERK activation was more pronounced and sustained longer in  $\beta_3^{+/+}$  than in  $\beta_3^{-/-}$  pre-OCs. In the presence of high-dose MCSF, however, ERK phosphorylation is indistinguishable in both cell types.

tude of which remained unaltered for at least 120 minutes (Figure 5). In contrast, activation of MAPK in  $\beta_3^{-/-}$  cells attached to OPN was greatly reduced and short-lived. Therefore,  $\alpha_v\beta_3$  is required for OPN-induced ERK activation in pre-OCs.

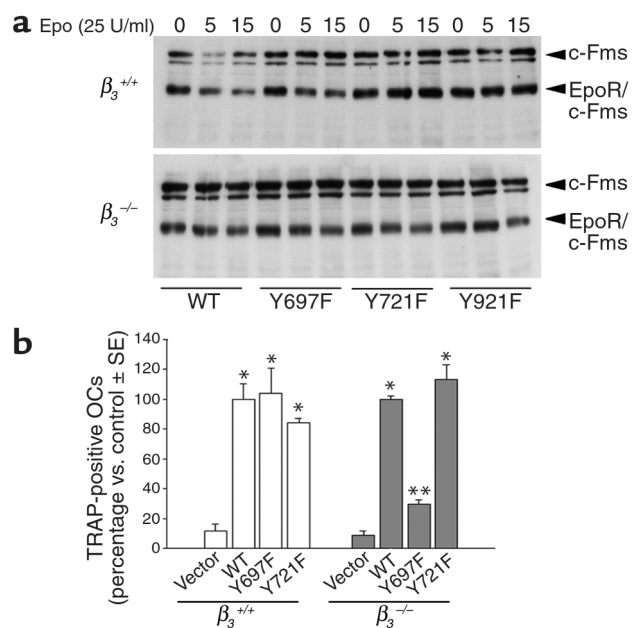
We next asked whether, like osteoclastogenesis, ERK activation in response to MCSF requires a high dose of the cytokine to compensate for lack of the integrin. Thus, pre-OCs derived from BMMs exposed to RANKL and low-dose MCSF for 3 days were stimulated with low- or high-dose MCSF for 2, 5, or 15 minutes. As seen in Figure 6, the magnitude and duration of ERK phosphorylation was dampened in  $\beta_3^{-/-}$  cells exposed to low-dose MCSF. In response to high-dose MCSF, however, ERK phosphorylation in WT and  $\beta_3$ -null pre-OCs was indistinguishable. Thus,  $\alpha_v\beta_3$  and c-Fms share a common intracellular signaling pathway, a component of which involves ERK activation.

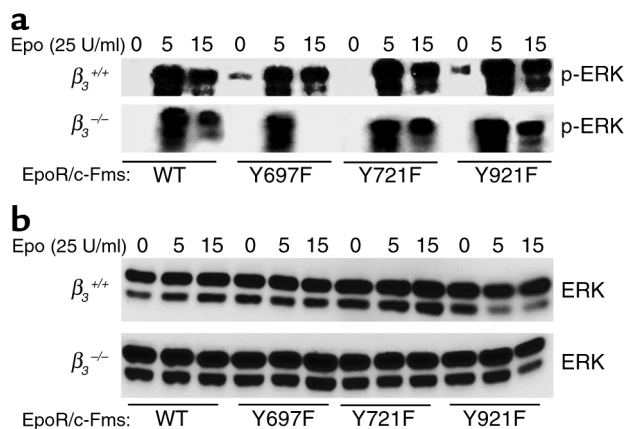
**Figure 7**  
*c-Fms*<sup>Y697</sup> is specifically required for osteoclastogenesis in the absence of  $\alpha_v\beta_3$ . (a) Equal numbers of  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  BMMs were retrovirally transduced with vector alone, nonmutated EpoR/c-Fms (WT), or EpoR/c-Fms carrying individual tyrosine-to-phenylalanine point mutation. Cells were selected in puromycin for 3 days and exposed for 3 days to RANKL and low-dose MCSF. The cells were then placed in serum-free medium for 2 hours, exposed to 25 U/ml Epo for varying times, and lysed. Equivalent expression of c-Fms and mutated forms of EpoR/c-Fms was established by immunoblot using a C-terminal anti-c-Fms antibody. (b) Puromycin-selected cells were cultured for 7 days in the optimal osteoclastogenic concentration of Epo (25 ng/ml) and RANKL (100 ng/ml). Osteoclastogenesis was measured by TRAP assay and expressed as a percentage of the value obtained with control (WT) EpoR/c-Fms transductants. While EpoR/c-Fms<sup>Y697F</sup> was as effective as control in inducing  $\beta_3^{+/+}$  osteoclastogenesis, the same mutant dampened the process 3.5-fold in  $\beta_3^{-/-}$  cells. In contrast, EpoR/c-Fms<sup>Y721F</sup> did not impact the osteoclastogenic process. \**P* < 0.001 vs. vector; \*\**P* < 0.05 vs. vector.

*c-Fms*<sup>Y697</sup> is specifically required for osteoclastogenesis in the absence of  $\alpha_v\beta_3$ . The cytoplasmic domain of c-Fms contains seven tyrosine residues, which are potentially phosphorylated upon receptor occupancy (35–37). To determine which, if any, such residues mediate the differentiating influence of high-dose MCSF on  $\beta_3^{-/-}$  pre-OCs, we retrovirally expressed a chimeric protein comprising the external domain of the EpoR linked to the transmembrane and cytoplasmic domains of c-Fms. BMMs expressing the EpoR/c-Fms chimera differentiate into bone-resorbing OCs when treated with RANKL and MCSF, which binds endogenous c-Fms receptor. Importantly, these same transductants, exposed to RANKL and Epo, which recognizes the chimeric receptor, also undergo osteoclastogenesis (4).

The same number of puromycin-selected  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  BMMs, transfected with either WT EpoR/c-Fms receptor or an EpoR/c-Fms construct carrying a Y697F to phenylalanine (F) (Y697F), Y721F, or Y921F mutation, were cultured for 7 days in the presence of RANKL and Epo. Expression of all forms of the chimeric receptor was equivalent (Figure 7a). Since BMMs carrying EpoR/c-Fms<sup>Y559F</sup> or EpoR/c-Fms<sup>Y807F</sup> are incapable of OC differentiation (4), these mutants were not studied.

Exposure to Epo and RANKL of either  $\beta_3^{+/+}$  or  $\beta_3^{-/-}$  BMMs expressing WT EpoR/c-Fms resulted in generation of numerous OCs (Figure 7b). This observation indicates that the nonmutated chimeric receptor, like endogenous c-Fms, rescues the defect in  $\beta_3^{-/-}$  OC differentiation. In contrast, while ligand-occupied EpoR/c-Fms<sup>Y697F</sup> failed to impact osteoclastogenesis in  $\beta_3^{+/+}$  cells, the same mutant resulted in 3.5-fold fewer  $\beta_3^{-/-}$  OCs. The chimeric receptor bearing the mutation Y721F (Figure 7b), Y706F (not shown), or Y921F (not shown) exerted no effect on Epo-induced





**Figure 8**  
 c-Fms<sup>Y697</sup> is specifically required for sustained ERK phosphorylation in  $\beta_3^{-/-}$  pre-OCs. (a) BMMs from  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  mice, transfected with the control EpoR/c-Fms (WT) or with the indicated EpoR/c-Fms mutants and selected in puromycin for 3 days, were exposed for 3 days to RANKL and low-dose MCSF. Pre-OCs were maintained for 2 hours in serum-free medium and exposed to 25 U/ml Epo, for the indicated times. ERK activation in response to Epo was detected in total cell lysates using an anti-phospho-ERK antibody. Only one EpoR/c-Fms mutant, Y697F, differentially affected the ERK signal in  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  cells. Specifically, while it did not impact  $\beta_3^{+/+}$  cells, this mutation completely abrogated the prolonged ERK activation in  $\beta_3^{-/-}$  OCs. (b) Samples used in a were immunoblotted with an anti-ERK mAb as loading control.

osteoclastogenesis. Thus, while it is not necessary in BMMs expressing  $\alpha_v\beta_3$ , c-Fms<sup>Y697F</sup> mediates osteoclastogenesis in cells lacking the integrin.

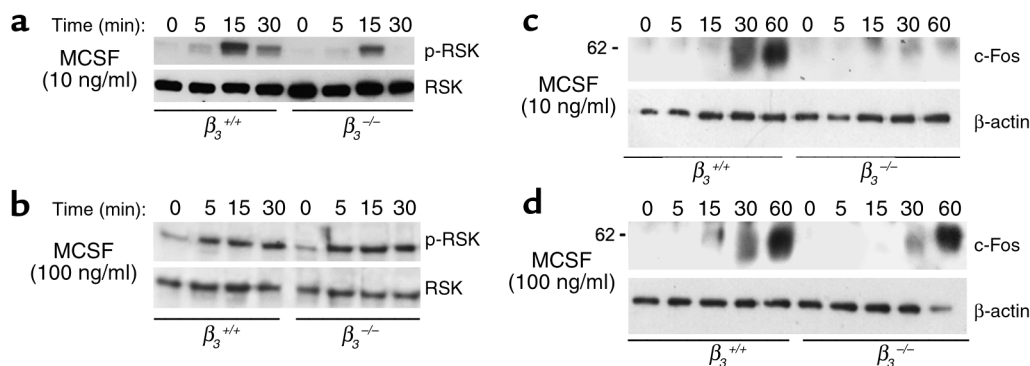
*c-Fms<sup>Y697</sup> is required for sustained ERK phosphorylation in pre-OCs lacking  $\alpha_v\beta_3$ .* Having established a correlation between arrested OC differentiation and defective ERK phosphorylation, we asked whether the anti-osteoclastogenic effect of c-Fms<sup>Y697F</sup>, in the absence of  $\alpha_v\beta_3$ , is mirrored by failure to activate this signaling pathway.  $\beta_3^{-/-}$  or  $\beta_3^{+/+}$  pre-OCs, retrovirally transduced with different

EpoR/c-Fms mutants, were cultured for 3 days in the presence of MCSF and RANKL, serum-starved, and stimulated with Epo for 0, 5, or 15 minutes (Figure 8a). Total ERK immunoblot established equal loading (Figure 8b). EpoR/c-Fms bearing Y721F or Y921F mutations phosphorylated ERKs in  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  cells in a manner indistinguishable from that of the WT chimeric receptor. However, while it did not impact  $\beta_3^{+/+}$  cells, EpoR/c-Fms<sup>Y697F</sup> failed to sustain ERK activation in  $\beta_3^{-/-}$  OCs, in all other transductants, which persisted for at least 15 minutes after Epo exposure. Thus, in the absence of  $\alpha_v\beta_3$ , ERK phosphorylation is maintained via c-Fms occupancy, a process requiring Y697 in the cytoplasmic domain of this receptor tyrosine kinase.

*High-dose MCSF rescues  $\beta_3^{-/-}$  osteoclastogenesis, but not resorptive function, via c-Fos expression.* Turning to candidate downstream molecules, we found that activation of the ERK1/2 substrate RSK2 was impaired in  $\beta_3^{-/-}$  pre-OCs stimulated with low-dose MCSF (Figure 9a), but not in those stimulated with high-dose MCSF (Figure 9b). Mirroring the results for ERKs, high-dose MCSF rescued RSK activation in  $\beta_3^{-/-}$  pre-OCs.

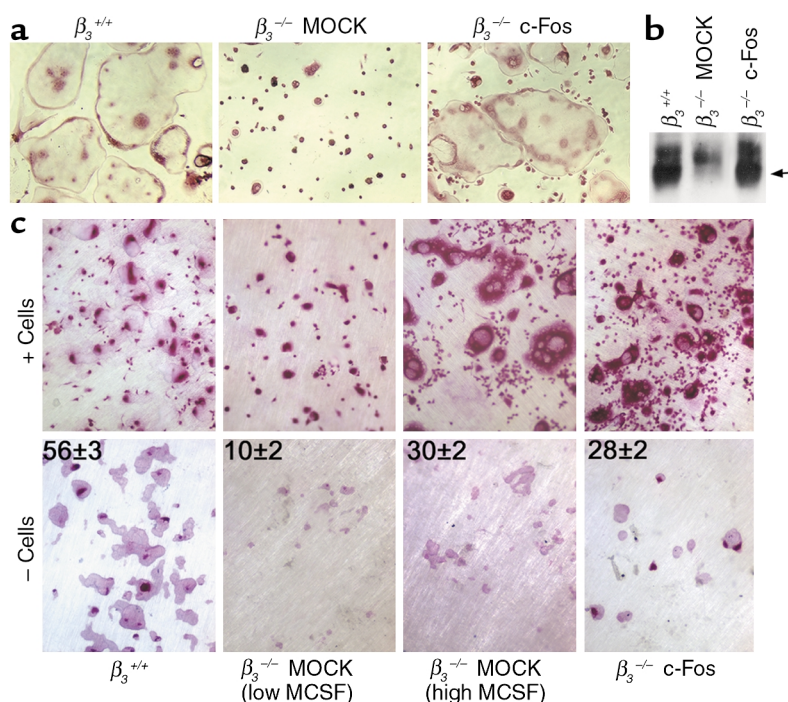
ERK induced c-Fos expression and its phosphorylation. In fact, c-Fos expression was enhanced in  $\beta_3^{+/+}$  pre-OCs treated for 30 or 60 minutes with low-dose (Figure 9c) or high-dose (Figure 9d) MCSF. In contrast, and mirroring morphological rescue by the cytokine, c-Fos was induced in  $\beta_3^{-/-}$  cells only when they were treated with high-dose MCSF. Sixty minutes of exposure to high-dose cytokine, in all circumstances, promoted appearance of c-Fos. These data suggest that high-dose MCSF compensates for the lack of  $\beta_3$  by inducing c-Fos expression.

The fact that c-Fos is a target of ERK and is essential for osteoclastogenesis raised the possibility that its blunted expression, in  $\beta_3^{-/-}$  cells, is responsible for arrested OC differentiation. To test this hypothesis,  $\beta_3^{-/-}$  BMMs were retrovirally transduced with pBabe/c-Fos or vector alone, selected with puromycin for 5 days, and plated in the presence of RANKL and low-dose MCSF.



**Figure 9**  
 High-dose MCSF is required to activate RSK and c-Fos in  $\beta_3^{-/-}$  pre-OCs.  $\beta_3^{+/+}$  and  $\beta_3^{-/-}$  BMMs exposed to RANKL and low-dose MCSF for 3 days were placed in serum-free medium for 2 hours and then exposed to 10 or 100 ng/ml MCSF, for the indicated times. (a and b) RSK activation was assessed by immunoblot of its phosphorylated species (p-RSK). Total RSK served as loading control. RSK phosphorylation was attenuated in  $\beta_3^{-/-}$  pre-OCs cultured in low-dose MCSF (a), but not in those cultured in high-dose MCSF (b). (c and d) c-Fos expression was detected by immunoblot in pre-OCs treated for 30 or 60 minutes with low- or high-dose MCSF. In  $\beta_3^{-/-}$  pre-OCs, c-Fos expression was induced only when cells were treated with high-dose MCSF.  $\beta$ -Actin served as loading control.





**Figure 10**

c-Fos overexpression by  $\beta_3^{-/-}$  cells rescues osteoclastogenesis but not matrix resorption.  $\beta_3^{+/+}$  BMMs and  $\beta_3^{-/-}$  BMMs retrovirally transduced with pBabe vector (MOCK) or pBabe/c-Fos were selected in puromycin for 5 days, and resistant cells were used in the indicated experiments. (a) An equal number of BMMs were plated in 96-well plates and cultured in the presence of RANKL and low-dose MCSF. After 7 days, cells were stained for TRAP activity. While osteoclastogenesis remained arrested in MOCK-transduced  $\beta_3^{-/-}$  cells, those overexpressing c-Fos generated OCs indistinguishable from WT. (b) Equal amounts of protein were loaded in each lane, and c-Fos content was assessed by immunoblot.  $\beta_3^{-/-}$  OCs retrovirally transduced with pBabe/c-Fos vector expressed the same level of c-Fos protein as did  $\beta_3^{+/+}$  cells (arrow). (c)  $\beta_3^{+/+}$ , MOCK  $\beta_3^{-/-}$ , and c-Fos  $\beta_3^{-/-}$  pre-OCs were plated on dentin slices with RANKL and low-dose MCSF. MOCK  $\beta_3^{-/-}$  cells were also cultured with RANKL and high-dose MCSF. After four days, dentin slices were stained for TRAP activity (+ Cells), or the cells were removed to visualize resorptive pits (- Cells).  $\beta_3^{+/+}$  cells differentiated into OCs with a characteristic resorptive phenotype and excavated many large, well-demarcated lacunae. MOCK-transduced  $\beta_3^{-/-}$  cells formed few OCs in the presence of low-dose MCSF and generated poorly defined, small pits. High-dose MCSF and c-Fos overexpression yielded numerous multinucleated TRAP-expressing OCs that exhibited a nonresorbing phenotype and also generated poorly defined, small pits. Indicated are the mean numbers of pits ± SEM from three different fields per variable.  $\times 10$ .

While vector-transduced  $\beta_3^{-/-}$  cells remained unaltered, those overexpressing c-Fos became fully differentiated OCs, morphologically indistinguishable from their  $\beta_3^{+/+}$  counterparts (Figure 10a). Immunoblot established that c-Fos expression by WT and overexpressing cells was equivalent (Figure 10b).

To determine whether high-dose MCSF or c-Fos overexpression also rescues OC function, we plated equal numbers of committed pre-OCs on dentin. Four days later, we analyzed OC morphology and resorptive-pit formation.  $\beta_3^{+/+}$  OCs, on dentin, assumed the typical “round” appearance of resorptive cells and excavated numerous, well-demarcated large pits (Figure 10c). In contrast,  $\beta_3^{-/-}$  cells in low-dose MCSF, while expressing TRAP, were largely mononuclear,

generating only a few small resorptive lacunae. Interestingly, those mutant cells exposed to high-dose MCSF, or overexpressing c-Fos, formed large, spread polykaryons on dentin, a characteristic of nonresorbing OCs. These cells demonstrated a limited capacity to generate lacunae, providing further evidence that they were nonresorbing OCs. Thus, high-dose MCSF rescues the ability of  $\beta_3^{-/-}$  BMMs to differentiate into OCs, but  $\alpha_v\beta_3$  is essential for these cells to express their full resorptive capacity.

### Discussion

Mice lacking  $\alpha_v\beta_3$  contain increased numbers of histologically normal, albeit dysfunctional, OCs and thus develop osteosclerosis (7, 26). Curiously, OC differentiation of  $\beta_3^{-/-}$  BMMs in vitro is blunted, and the OCs generated appear distinctly abnormal. This paradox suggests that the osteoclastogenic milieu in which  $\beta_3^{-/-}$  BMMs find themselves in vivo and in vitro differs. Importantly, the  $\beta_3^{-/-}$  mouse is markedly hypocalcemic (7), and thus almost certainly hyperparathyroid. This observation raises the possibility that the relative normalization of OC differentiation in vivo reflects the influence of excess parathyroid hormone, which mediates its osteoclastogenic effect by prompting expression of RANKL and MCSF (38–41). Our data suggest that increased levels of the latter cytokine, in the bone microenvironment, may account for the observed in vitro versus in vivo differences. The fact that  $\beta_3^{-/-}$  mice are exposed to increased soluble MCSF and, particularly, enhanced membrane-residing MCSF in bone marrow cells provides compelling support of this posture.

Several groups have shown a correlation between increased OC number and elevated levels of MCSF. Kimble et al. highlighted a role for MCSF in estrogen deficiency-induced bone loss (42), and Lea et al. provided intriguing evidence that membrane MCSF is selectively upregulated following sex hormone withdrawal (31). Thus, our data are consistent with the conclusion of Yao et al. that membrane MCSF plays an important role in osteoblast-mediated osteoclastogenesis within the bone microenvironment (43).

The salutary impact of MCSF on  $\beta_3^{-/-}$  OC number reflects stimulated differentiation and not accelerated proliferation or arrested apoptosis. In fact, the magnitude of programmed cell death, while diminished in low-dose MCSF and RANKL, is normalized when MCSF is

increased. These data are consistent with the fact that OCs are terminally differentiated, nonreplicating cells and hence are more likely to be susceptible to apoptosis than BMMs are. Thus, failure of low-dose MCSF to stimulate osteoclastogenesis of  $\beta_3^{-/-}$  BMMs likely prolongs the lifespan of these mononuclear precursors.

The ERK family of MAPKs is induced by many agonists, but the biological consequences of such induction in a given cell (i.e., proliferation or differentiation) are determined by quantitative differences in amplitude and duration of activation (44). Having shown that  $\beta_3^{-/-}$  osteoclastogenesis is dampened along with ERK activation, we asked whether high-dose MCSF, which rescues OC formation by  $\alpha_v\beta_3$  deficient cells, also restores ERK signaling. In fact, when  $\beta_3^{-/-}$  pre-OCs are stimulated with high-dose MCSF, ERK is phosphorylated independent of the integrin. These data suggest that physiological osteoclastogenesis reflects cooperative ERK activation by both  $\alpha_v\beta_3$  and MCSF. On the other hand, loss of the integrin obviates one means of ERK stimulation, which is compensated by high-dose MCSF.

The capacity of high-dose MCSF to rescue  $\beta_3^{-/-}$  OCs called our attention to the components of the cytokine's receptor that regulate this event. To identify the c-Fms tyrosine residues that mediate  $\beta_3^{-/-}$  OC rescue and ERK activation in the context of primary precursor cells, we turned to a chimeric receptor consisting of an external component of EpoR and the transmembrane and cytoplasmic domains of the c-FMS. BMMs transduced with this chimera differentiate into OCs when stimulated with Epo, acting on the chimeric receptor, or with MCSF, through endogenous c-Fms.

We find that Y697 in the c-Fms cytoplasmic domain is essential for rescue of osteoclastogenesis by BMMs lacking  $\alpha_v\beta_3$ . Specifically,  $\beta_3^{-/-}$  BMMs transduced with EpoR/c-Fms<sup>Y697F</sup> fail to differentiate into multinucleated OCs or to normally activate ERK. This observation is in keeping with the fact that, upon stimulation with MCSF, c-Fms<sup>Y697</sup> binds Grb-2 (37), which is an ERK-inducing molecule. Interestingly, the same EpoR/c-Fms mutant, namely Y697F, expressed in  $\alpha_v\beta_3$ -bearing cells is as effective as its WT counterpart in promoting osteoclastogenesis. Thus, a specific residue in the c-Fms cytoplasmic domain participates in the osteoclastogenic process only in the absence of an integrin also capable of activating ERK.

Our model proposes that  $\alpha_v\beta_3$  and c-Fms share a common signaling pathway during OC differentiation. Specifically, expression of early osteoclastogenic markers is dependent on the integrin when pre-OCs are maintained in a low concentration of MCSF. Increasing levels of the cytokine override the differentiation defect in  $\beta_3^{-/-}$  cells, permitting normal OC formation. Thus, in the presence of limited concentrations of MCSF, signals derived from the  $\alpha_v\beta_3$  integrin are likely to be indispensable for transcriptional activation of immediate-early-response genes. While MCSF is known to regulate cell proliferation and survival, these data

establish that, in the context of the OC precursors that lack  $\alpha_v\beta_3$ , the cytokine also promotes differentiation.

c-Fos is crucial to osteoclastogenesis (22, 23). Since expression of the transcription factor, in other circumstances, is stimulated by activated ERK (21), this MAPK is a potential upstream inducer of c-Fos in OCs. In fact, decreased ERK phosphorylation in  $\beta_3^{-/-}$  pre-OCs, attended by deficient RSK activation, arrests c-Fos expression in response to low-dose MCSF. High-dose MCSF increases the intensity and duration of the ERK signal in these mutant cells, sustains activation of RSK, and, importantly, upregulates c-Fos.

Murphy et al. recently reported that prolonged ERK and RSK activation eventuates in enhanced c-Fos expression via phosphorylation-mediated stabilization of the protein (21). Their studies were performed, however, in transformed cells and are thus of unknown physiological relevance. Taken with the persistent ERK and RSK activation that appears in  $\beta_3^{-/-}$  pre-OCs exposed to high-dose MCSF, the increase in c-Fos, which is likely to be phosphorylated, may provide physiological validation of Murphy's observations.

Our results indicate that MCSF promotes OC differentiation by upregulating c-Fos. Confirmation of this posture comes from the fact that  $\beta_3^{-/-}$  OCs overexpressing c-Fos are morphologically indistinguishable from those exposed to high-dose MCSF. Thus,  $\alpha_v\beta_3$  and c-Fms mediate OC differentiation via the ERK/c-Fos pathway. Mirroring the in vivo situation, however,  $\beta_3^{-/-}$  OCs in which this pathway is activated, while well spread and multinucleated, fail to adequately resorb bone. This observation is in keeping with the fact that  $\beta_3^{-/-}$  OCs in vivo, when examined by electron microscopy, have abnormal ruffled membranes (7). Thus, while c-Fos expression is sufficient to promote OC formation in the absence of  $\alpha_v\beta_3$ , the integrin is pivotal to the matrix-degradative capacity of the cell.

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