



# Glucocorticoids suppress bone formation via the osteoclast

Hyun-Ju Kim,<sup>1</sup> Haibo Zhao,<sup>1</sup> Hideki Kitaura,<sup>1</sup> Sandip Bhattacharyya,<sup>2</sup> Judson A. Brewer,<sup>2</sup> Louis J. Muglia,<sup>2</sup> F. Patrick Ross,<sup>1</sup> and Steven L. Teitelbaum<sup>1</sup>

<sup>1</sup>Department of Pathology and Immunology and <sup>2</sup>Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA.

**The pathogenesis of glucocorticoid-induced (GC-induced) bone loss is unclear. For example, osteoblast apoptosis is enhanced by GCs in vivo, but they stimulate bone formation in vitro. This conundrum suggests that an intermediary cell transmits a component of the bone-suppressive effects of GCs to osteoblasts in the intact animal. Bone remodeling is characterized by tethering of the activities of osteoclasts and osteoblasts. Hence, the osteoclast is a potential modulator of the effect of GCs on osteoblasts. To define the direct impact of GCs on bone-resorptive cells, we compared the effects of dexamethasone (DEX) on WT osteoclasts with those derived from mice with disruption of the GC receptor in osteoclast lineage cells (*GR<sup>oc</sup>/-* mice). While the steroid prolonged longevity of osteoclasts, their bone-degrading capacity was suppressed. The inhibitory effect of DEX on bone resorption reflects failure of osteoclasts to organize their cytoskeleton in response to M-CSF. DEX specifically arrested M-CSF activation of RhoA, Rac, and Vav3, each of which regulate the osteoclast cytoskeleton. In all circumstances *GR<sup>oc</sup>/-* mice were spared the impact of DEX on osteoclasts and their precursors. Consistent with osteoclasts modulating the osteoblast-suppressive effect of DEX, *GR<sup>oc</sup>/-* mice are protected from the steroid's inhibition of bone formation.**

## Introduction

Glucocorticoids (GCs) are central to the treatment of inflammatory and immune disorders. These steroids, however, profoundly impact the skeleton, particularly when administered for prolonged periods. In fact, high-dose GC therapy is almost universally associated with bone loss, causing one of the most crippling forms of osteoporosis. Despite the frequency and severity of GC-induced osteoporosis, its treatment is less than satisfactory, suggesting that its pathogenesis is incompletely understood.

Net bone mass represents the relative activities of osteoblasts and osteoclasts, and there is little question that GCs suppress bone-forming cells, in vivo, via a process involving accelerated apoptosis (1, 2). In keeping with these in vivo observations, GCs blunt expression of molecules such as Runx2 and collagen I (3), which mediate osteoblast differentiation and function, respectively. Similarly, these steroids prompt marrow mesenchymal cells to express adipocytic genes (4). Surprisingly, however, addition of GCs to cultures of osteoprogenitor cells actually increases their capacity to form mineralized bone nodules (5, 6). This paradox raises the possibility that GC suppression of bone formation in vivo reflects, at least in part, targeting of the steroid to intermediary cells, which in turn inhibit the osteoblast.

Bone remodeling is an ever-occurring event characterized by sequential tethering of the activities of osteoclasts and osteoblasts. In fact, the majority of acquired, systemic diseases of the skeleton reflect imbalance between osteoclast and osteoblast activity in the remodeling process. Remodeling units are initiated by the appearance of osteoclasts that degrade a packet of bone approximately 50  $\mu\text{m}$

deep. These resorptive cells are replaced, at the same location, by osteoblast precursors, which synthesize new bone. The osteoporosis attending menopause or GC therapy reflects failure of osteoblasts to fully restore bone previously resorbed in remodeling sites. This process establishes that osteoclastic bone resorption, in some manner, promotes osteoblastic bone formation at the same location. Similarly, pathologically or pharmacologically inhibited resorption eventuates in arrested osteoblast activity (7, 8).

Bone resorption reflects the sum of osteoclast recruitment and death and the rate at which the average cell degrades matrix, each of which is controversial regarding GC therapy, as the drugs are variously perceived to suppress (9) or enhance (10) generation of these cells. In particular, there is little evidence that the steroids impact the resorptive machinery of the mature polykaryon.

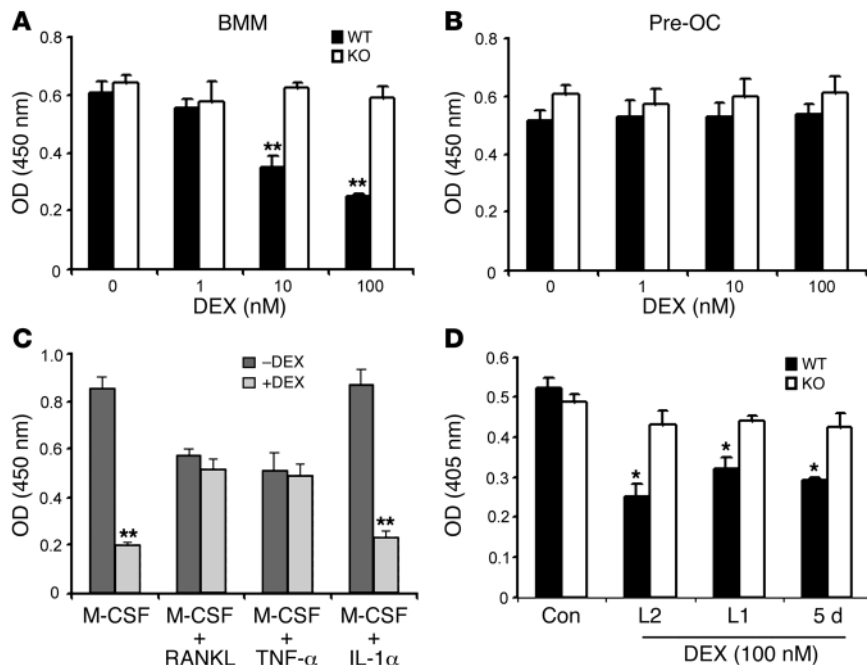
Osteoclasts are members of the monocyte/macrophage family that differentiate under the influence of 2 requisite cytokines, namely M-CSF and receptor activator of NF- $\kappa\text{B}$  ligand (RANKL) (11). RANKL is a member of the TNF superfamily, physiologically expressed by osteoblasts and their precursors as a transmembrane protein that recognizes its receptor, RANK, on osteoclast lineage cells at various states of differentiation. GCs, which arrest osteoblast differentiation, also stimulate RANKL (12) and M-CSF (13) expression, which, in conjunction with the steroid's antiapoptotic effects on the mature cell (14), would seemingly increase bone-degradative activity. It is therefore curious that there is little evidence that accelerated bone resorption contributes to the osteoporosis complicating prolonged GC therapy (15). In fact, the histological appearance of bones of patients so treated indicates that bone resorption as well as formation is suppressed (1, 16, 17).

On the other hand, the conundrum of GC-inhibited bone resorption, in the face of stimulated RANKL and M-CSF expression and prolonged osteoclast lifespan, may reflect direct suppression by the steroid of the cell's resorptive machinery. We find, in fact, that while GCs do delay osteoclast apoptosis, they retard the cell's capacity to resorb bone, in vitro and in vivo, by disrupting its cyto-

**Nonstandard abbreviations used:** BMM, bone marrow macrophage; DEX, dexamethasone; GC, glucocorticoid; GR, GC receptor; *GR<sup>oc</sup>/-* mice, mice with GR deleted in osteoclast lineage cells; GST, glutathione-S-transferase; PTH, parathyroid hormone; RANKL, receptor activator of NF- $\kappa\text{B}$  ligand; TRACP5b, tartrate-resistant acid phosphatase 5b; TRAP, tartrate-resistant acid phosphatase.

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

**Citation for this article:** *J. Clin. Invest.* 116:2152–2160 (2006). doi:10.1172/JCI28084.

**Figure 1**

DEX impacts osteoclast lineage cells in a differentiation- and cytokine-dependent manner. WT and  $GR^{oc-/-}$  BMMs were cultured with M-CSF (50 ng/ml) in the absence (A) or presence (B) of RANKL (30 ng/ml) with various concentrations of DEX. After 3 days, proliferation was assessed by BrdU incorporation. (C) WT BMMs were incubated with or without DEX (100 nM) in the presence of M-CSF alone or with RANKL, TNF- $\alpha$  (10 ng/ml) or IL-1 $\alpha$  (10 ng/ml) for 3 days. Proliferation was evaluated by BrdU incorporation assay. (D) WT and  $GR^{oc-/-}$  BMMs were cultured with M-CSF and RANKL for 5 days to generate mature osteoclasts. DEX (100 nM) or vehicle was added for the entire 5-day culture period or the last 2 days (L2) or 1 day (L1). After 5 days, the relative apoptosis rate was determined by ELISA. \* $P < 0.01$ , \*\* $P < 0.001$  within each group.

skeleton. These effects are GC receptor (GR) mediated and specifically reflect blockade of M-CSF-stimulated cytoskeletal organization. Most importantly, the arrested resorption induced by GCs translates to dampened osteoblast activity, as mice in which the GR has been conditionally deleted in osteoclast lineage cells are protected from steroid-induced bone loss. Thus, suppression of the resorptive phase of remodeling contributes to the retarded bone formation central to GC-induced osteoporosis.

## Results

*Dexamethasone exerts variable effects during osteoclast differentiation.* To define the effects of GCs on osteoclast precursor proliferation and survival of the mature resorptive polykaryon, we assessed the impact of dexamethasone (DEX) on cells derived from WT mice and those with conditional disruption of the GR in a macrophage-specific manner ( $GR^{oc-/-}$  mice) (18). Validating this model, immunoblot analysis using GR-specific antibody showed that GR immunoreactivity is undetectable in lysates of  $GR^{oc-/-}$  bone marrow macrophages (BMMs) and mature osteoclasts (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28084DS1).

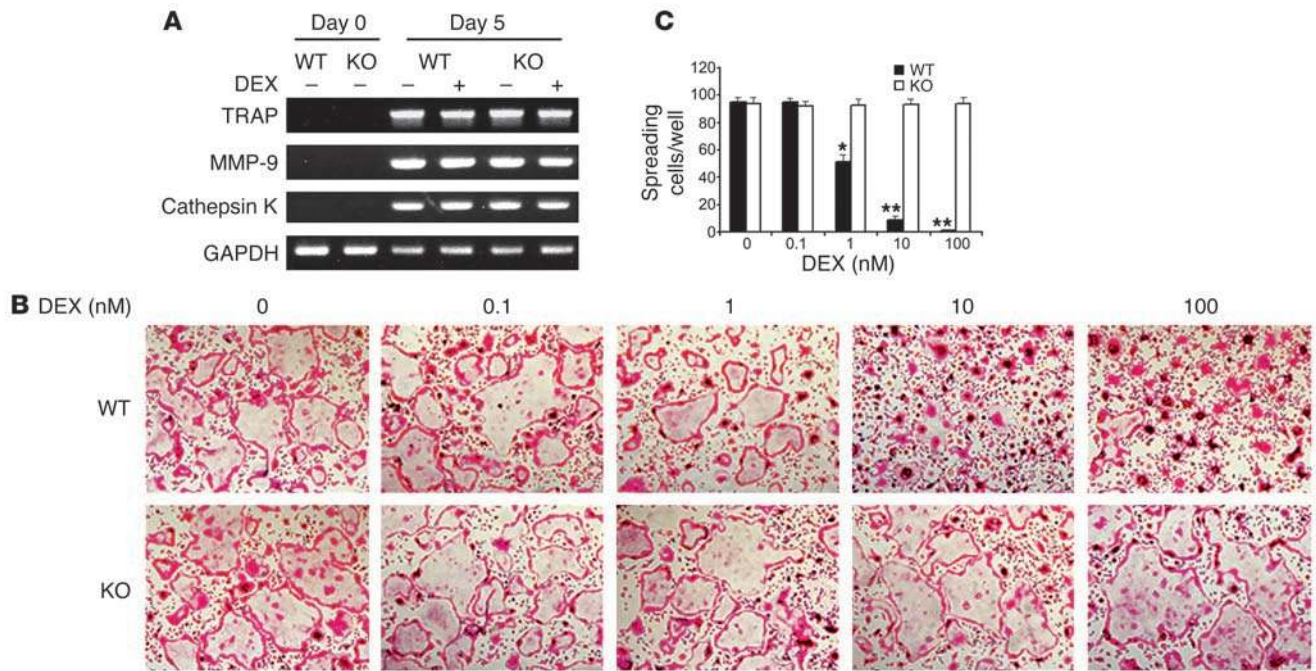
Turning to the effects of GCs during different phases of osteoclast differentiation, we found that 10 or 100 nM DEX dose-dependently inhibits WT BMM proliferation while having no effect on  $GR^{oc-/-}$  cells (Figure 1A). Interestingly, culture of WT BMMs in M-CSF and RANKL for 3 days, at which time the cells were committed to the osteoclast phenotype, completely abrogated the steroid's antiproliferative effects (Figure 1B). Next, WT BMMs were incubated with M-CSF alone or with the osteoclastogenic cytokines RANKL, TNF- $\alpha$ , or IL-1 $\alpha$ . Like RANKL, TNF- $\alpha$ , but not IL-1 $\alpha$ , blocks the inhibitory effect of DEX on M-CSF-induced BMM proliferation (Figure 1C).

Having explored the impact of GCs on osteoclast precursor proliferation, we turned to apoptosis of the mature, nonreplicating cell. Thus, at selected times, DEX was added to 5-day osteoclasto-

genic cultures consisting of BMMs in M-CSF and RANKL. Specifically, the steroid was present for the entire culture period (5 d) or added for the last 2 days or the final 24 hours. Assessment after 5 days, at which time naive cultures contained sheets of mature osteoclasts, established that DEX arrests apoptosis following exposure to the steroid as early as 1 day (Figure 1D). These results demonstrate that while DEX blunts BMM proliferation, it does not affect cells undergoing osteoclast differentiation for as few as 3 days and protects against apoptosis of mature osteoclasts.

*DEX alters osteoclast spreading but not differentiation.* We next asked whether GCs impact osteoclast differentiation. To this end, we assessed the steroid's effect on expression of 3 osteoclastic genes in WT and  $GR^{oc-/-}$  BMMs exposed to RANKL and M-CSF for 5 days. Neither *tartrate-resistant acid phosphatase (TRAP)*, *MMP-9*, nor *cathepsin K* mRNAs were present in naive BMMs, but each was induced indistinguishably in WT and  $GR^{oc-/-}$  cells as they underwent osteoclastogenesis in the presence or absence of DEX (Figure 2A). Thus, neither GCs nor the GR regulate osteoclast differentiation. On the other hand, the steroid had profound effects on osteoclast spreading. DEX, in concentrations as high as 100 nM, did not affect the appearance of osteoclasts lacking the GR. In contrast, inhibition of WT osteoclast spreading was profound at 1 nM of the steroid and universal at 100 nM (Figure 2, B and C). To determine the stage of osteoclast differentiation at which GCs inhibit spreading, we added DEX, for various periods, to osteoclastogenic cultures initiated with WT or  $GR^{oc-/-}$  BMMs (Figure 3A). Attesting to a direct effect on the mature osteoclast, exposure to the steroid during the last day of osteoclast differentiation was necessary and sufficient to arrest spreading (Figure 3B). Thus, DEX inhibits spreading of the mature osteoclast and its capacity to resorb mineralized matrix and does so independently of cell maturation.

*DEX specifically inhibits M-CSF-induced actin organization.* The morphological impact of DEX on WT osteoclasts, in the face of normal differentiation, suggests the steroid may alter actin organization. To determine whether such is the case, we assessed formation of



**Figure 2**

DEX-treated BMMs differentiate into osteoclasts but fail to spread. **(A)** WT and *GR<sup>oc-/-</sup>* BMMs were cultured with M-CSF and RANKL with or without DEX (100 nM). At day 0 and day 5, RNA was extracted, and the expression of osteoclastogenic markers was analyzed by RT-PCR. GAPDH served as loading control. **(B)** WT and *GR<sup>oc-/-</sup>* BMMs were cultured with M-CSF and RANKL with or without increasing concentrations of DEX. Five-day osteoclastogenic cultures were stained for TRAP activity. Magnification,  $\times 250$ . **(C)** Statistical analysis of the number of WT and *GR<sup>oc-/-</sup>* spread TRAP-positive multinucleated cells/well. \* $P < 0.001$ , \*\* $P < 0.0001$  versus DEX-untreated WT.

the actin ring, a cytoskeletal structure essential for optimal osteoclastic bone resorption (11). To this end, mature osteoclasts were generated on dentin. Control cells were maintained in M-CSF and RANKL, while others were exposed to cold PBS for 5 minutes. The cells were then stained with rhodamine-phalloidin to visualize the actin cytoskeleton. As seen in Figure 4A, all osteoclasts maintained consistently in M-CSF and RANKL exhibited characteristic actin rings, which disappeared following treatment with cold PBS. Furthermore, incubation of the PBS-exposed cells in cytokine-free medium for 5 hours failed to restore their actin rings.

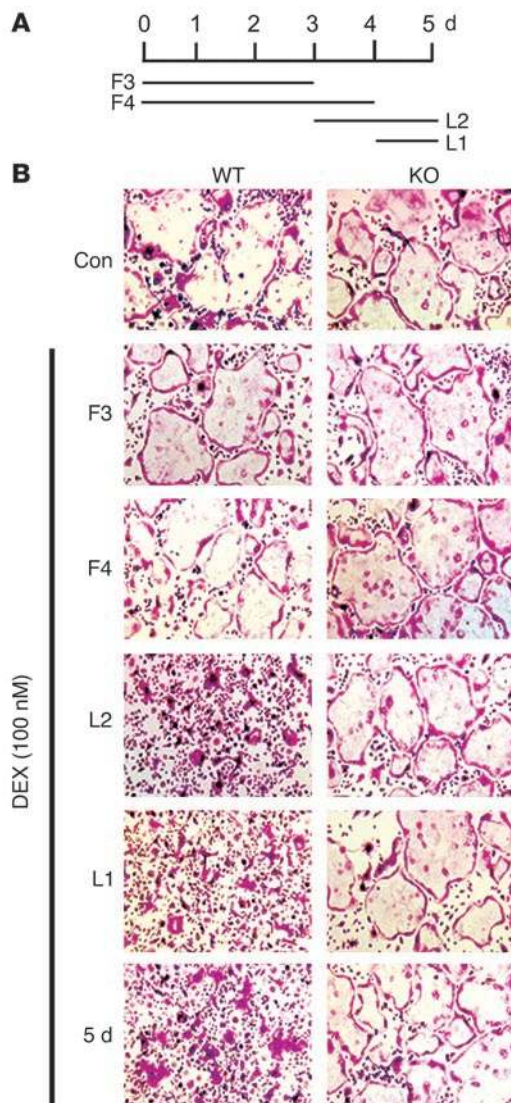
M-CSF, RANKL, TNF- $\alpha$ , and IL-1 $\alpha$  induce actin ring formation in mature osteoclasts (19, 20). Thus, we asked whether GCs regulate cytoskeletal organization in these cells in a cytokine-specific manner. WT and *GR<sup>oc-/-</sup>* osteoclasts were plated on dentin and cultured in osteoclast-generating medium for 5 days. After washing with cold PBS to disrupt actin rings, the resorptive polykaryons were incubated for 5 hours with various cytokines, with or without DEX. Each cytokine, in the absence of DEX, significantly restored actin ring formation, an event mirrored by *GR<sup>oc-/-</sup>* osteoclasts exposed to the steroid (Figure 4, B–F). While DEX did not alter RANKL-, TNF- $\alpha$ -, or IL-1 $\alpha$ -stimulated actin ring formation in WT osteoclasts (Figure 4, C–F), the steroid blocked M-CSF-induced actin ring formation by 73% (Figure 4, B and F).

*DEX arrests M-CSF-stimulated intracellular signaling.* Because GCs specifically blunt M-CSF-induced cytoskeletal organization in osteoclasts, we turned to the signal transduction pathway mediating this event. As seen in Supplemental Figure 2, M-CSF strongly activated ERK1/2 and modestly induced Akt in committed osteoclast precursors, but DEX failed to impact either signaling molecule.

Cytoskeletal organization in the osteoclast is regulated by the small GTPases RhoA (21) and Rac (22). We therefore assessed RhoA activation in M-CSF-treated WT committed preosteoclasts using a glutathione-S-transferase (GST) pull-down assay. While RhoA activity was enhanced approximately 5-fold within 5 minutes exposure to the cytokine, DEX completely blocked the induction (Figure 5A). Similarly, M-CSF rapidly activated Rac 2.5-fold above control, and this was, again, entirely abrogated by DEX (Figure 5B).

Vav3 is an osteoclast-specific guanine nucleotide exchange factor that targets Rac. Like RhoA and Rac, Vav3 is essential for osteoclast cytoskeletal organization (23). Consistent with its effect on Rac, DEX fully arrested the 5-fold increase in Vav3 tyrosine phosphorylation induced by M-CSF (Figure 5C). Hence, in keeping with its morphological and functional effects, DEX blunts a major cytoskeleton-organizing signaling pathway in osteoclasts. We also find that DEX-mediated suppression of M-CSF-activated Vav3 required 16 hours' exposure to the steroid, indicating that the event is likely to be genomic rather than nongenomic (Supplemental Figure 3).

*DEX inhibits osteoclastic bone resorption in vitro and in vivo.* To determine the functional implications of GC inhibition of M-CSF-induced cytoskeletal organization in the mature osteoclast, we assessed the capacity of steroid-treated cells to resorb mineralized matrix. First, BMMs were cultured on dentin slices in M-CSF and RANKL, with or without 100 nM DEX, for the entire 5 days required to maximize the number of mature osteoclasts. As shown in Figure 6A, DEX markedly decreased resorption lacuna formation by WT but not *GR<sup>oc-/-</sup>* cells. Because this result may reflect diminished osteoclast number due to suppressed BMM proliferation, we turned to cells committed to the osteoclast phenotype

**Figure 3**

DEX inhibits spreading in the later stage of osteoclast differentiation. WT and  $GR^{oc-/-}$  BMMs were cultured for 5 days in M-CSF and RANKL and stained for TRAP activity. The cells were treated with DEX (100 nM) for the entire 5 days, the first 3 days (F3), the first 4 days, the last 2 days, or the last day. Control cultures were maintained in the absence of DEX for 5 days. (A) Duration of exposure to Dex in days. (B) Osteoclastogenic cultures stained for TRAP activity. Magnification,  $\times 250$ .

inhibition occurs in the absence of the GR (Figure 7C). Thus, consistent with disruption of their cytoskeleton, DEX diminishes the activity of osteoclasts in vitro and in vivo.

*DEX-suppressed bone formation is mediated through osteoclasts.* To determine whether GC suppression of osteoclast function translates into arrested bone formation, we administered DEX (10 mg/kg) or vehicle to WT and  $GR^{oc-/-}$  mice daily for 14 days. Two courses of the fluorescent marker calcein were injected at 6-day intervals prior to sacrifice, at which time serum was collected.

As expected, DEX suppressed bone formation in WT mice. Specifically, the mineral apposition rate, a histomorphometric marker of the functional capacity of the average osteoblast, and total bone formation rate were reduced approximately two-thirds relative to control in DEX-treated WT mice (Figure 8, A–C). Moreover, serum levels of osteocalcin (Figure 8D) and alkaline phosphatase (Figure 8E), global biomarkers of bone formation, were similarly decreased in these animals. In contrast, the steroid did not impact either parameter of bone formation in mice whose osteoclasts lacked GRs. On the other hand, osteocyte apoptosis was indistinguishable in DEX-treated WT and  $GR^{oc-/-}$  mice (Figure 8F). Thus, DEX mediates its bone-suppressive effects, at least in part, indirectly via the osteoclast.

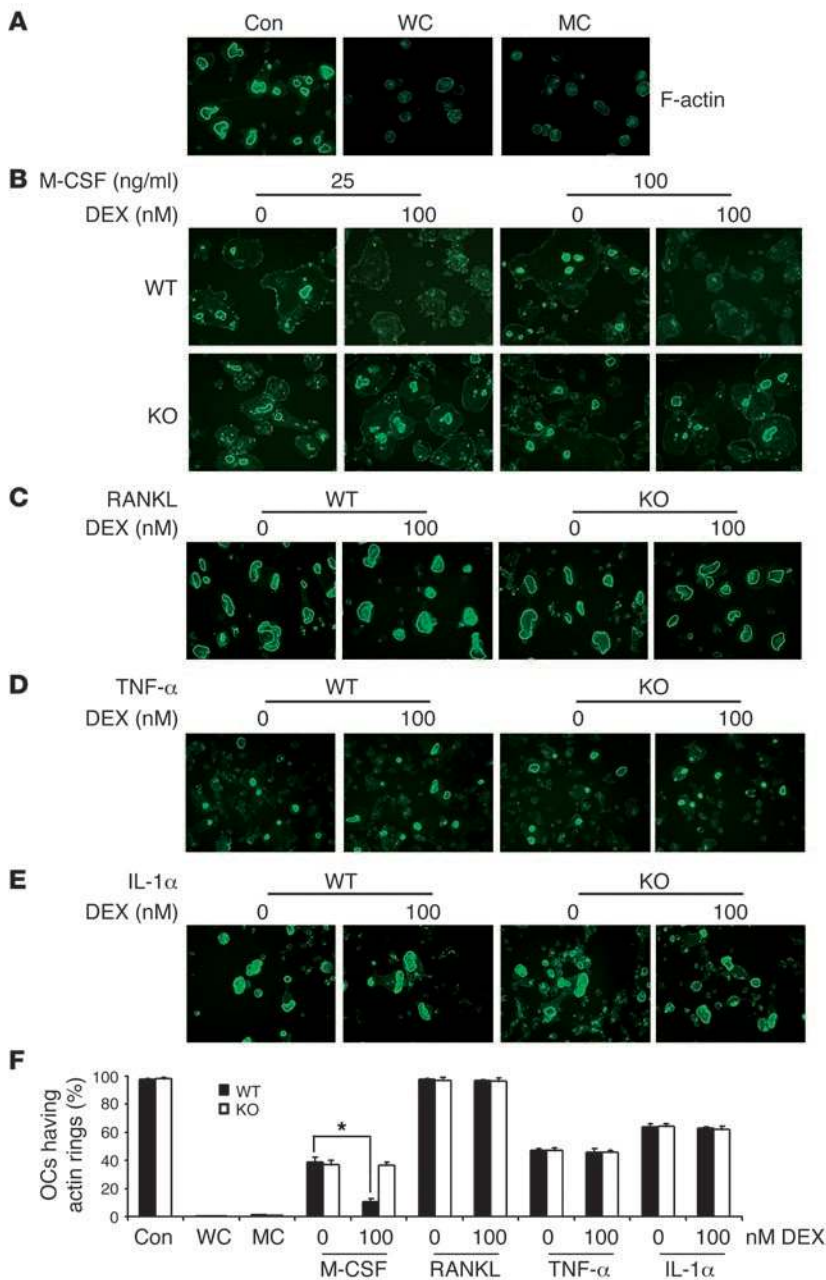
## Discussion

In 1990, Udagawa et al. first generated bone fide osteoclasts in culture by coculturing marrow macrophages and osteoblast precursors (24). The discovery that RANKL, produced by osteoblasts and their progenitors, is the key osteoclastogenic cytokine established that marrow stromal cells are essential for physiological osteoclastogenesis (25).

The alternative concept that osteoblast recruitment to sites of bone remodeling is mediated by the osteoclast is longer-standing yet suffers from a paucity of mechanistic insights (26). Two general theories of osteoclast regulation of the osteoblast present themselves. Specifically, the osteoclast itself may be the source of unidentified, osteoblast-trophic molecules. Buttressing this argument is the fact that certain forms of osteopetrosis, such as c-src deficiency, are characterized by abundant, albeit dysfunctional, osteoclasts, yet bone formation is accelerated (27). In other iterations of the disease, such as that due to lack of c-Fos, osteoclastogenesis is arrested and bone formation dampened (28, 29). An equally compelling argument holds that the osteoclast mobilizes matrix-residing growth factors, such as TGF- $\beta$  and IGFs, which, in turn, target and activate the osteoblast (30). Regardless of mechanism, the sequential tethering of osteoclastic and osteoblastic activity has important clinical implications. For example, antiresorptive agents, such as bisphosphonates, are attended by suppressed bone formation, a phenomenon that may eventuate in adynamic bone and its structural consequences (31). Similarly, the bone anabolic properties of PTH are diminished when it is administered with an antiresorptive drug (7, 8).

whose proliferative capacity was no longer impacted by the steroid (Figure 6B). To this end, BMMs were maintained on plastic for 2 days in M-CSF and RANKL. They were then lifted and equal numbers cultured on dentin in the presence or absence of DEX for 3 days. Once again, the steroid arrested resorptive pit formation by WT osteoclasts, while those lacking the GR receptor were spared.

Having established that GCs impair osteoclast function in vitro, we asked whether the same obtains in vivo. Hence, we treated WT and  $GR^{oc-/-}$  mice with parathyroid hormone<sub>1-34</sub> (PTH<sub>1-34</sub>), which, consistent with our in vitro observations, increased osteoclast number equally in both genotypes, independent of DEX (Figure 7A). On the other hand, the in vivo appearance of PTH<sub>1-34</sub>-induced osteoclasts exposed to the steroid was strikingly different from that of control. Whereas naive, PTH<sub>1-34</sub>-stimulated osteoclasts were largely juxtaposed to bone, with robust ruffled membrane formation, those in mice also receiving DEX typically failed to contact bone and did not develop this resorptive organelle (Figure 7B). Confirming that DEX suppresses stimulated bone resorption, the GC blunts the capacity of PTH<sub>1-34</sub> to enhance serum tartrate-resistant acid phosphatase 5b (TRACP5b) in WT mice while no such



**Figure 4**

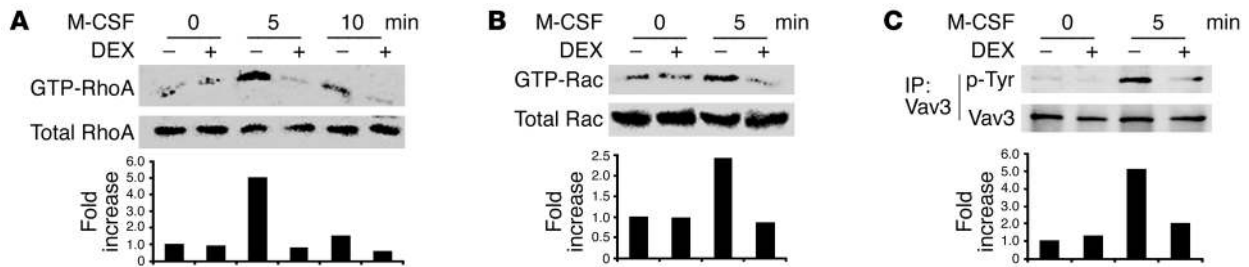
DEX inhibits M-CSF-induced actin ring formation. WT and *GR<sup>oc/-</sup>* BMMs were plated on dentin and cultured with M-CSF (10 ng/ml) and RANKL (100 ng/ml). (A) After 5 days, WT cells were fixed before (Con), after washing with cold PBS (washing control [WC]), or after washing and incubation in  $\alpha$ -MEM/10% FBS (media control [MC]) for 5 hours. The fixed cells were stained with FITC-phalloidin to visualize actin rings. (B–E) After 5 days WT and *GR<sup>oc/-</sup>* cells were washed with cold PBS and incubated with or without DEX (100 nM) in the presence of M-CSF (25 or 100 ng/ml) (B), RANKL (100 ng/ml) (C), TNF- $\alpha$  (10 ng/ml) (D), or IL-1 $\alpha$  (10 ng/ml) (E). After 5 hours incubation, the fixed cells were stained with FITC-phalloidin. (F) The percentage of osteoclasts (OCs) with recovered actin ring formation with or without DEX and various cytokines. \**P* < 0.001. Magnification,  $\times$ 250.

Prolonged GC therapy leads to yet another example of structurally inferior, adynamic bone, indicating that repressed osteoclast function may contribute to diminished formation. These observations are in keeping with the suggestion of Weinstein that steroid-induced osteoporosis reflects combined inhibition of both the osteoblast and osteoclast (1) and indicate that, as in other states of adynamic bone, GC-mediated inhibition of osteoclast function retards remodeling and, hence, dampens osteogenesis.

The issue as to whether GCs impact osteoclastic bone resorption has been controversial. The capacity of these steroids to suppress intestinal absorption and renal tubular reabsorption of calcium is consistent with a scenario of hormonally stimulated bone degradation (32). On the other hand, PTH levels are probably not increased in most GC-treated patients, and their skeletal response to PTH-suppressing agents, such as vitamin D, is

modest (32). Similarly, GCs attenuate production of sex steroids, low levels of which, in other circumstances, promote osteoclastic bone resorption (33). Hormone replacement therapy, however, does not retard GC-induced bone loss (34). Therefore, if GCs impact the osteoclast, they are likely to do so directly or within the context of locally produced osteoclast-regulating factors such as RANKL or M-CSF.

Our studies of the effects of GCs on various phases of osteoclast differentiation and function benefited from the availability of mice conditionally disrupting the GR in the entire BMM/osteoclast lineage, which permitted us to determine whether individual biological events are specific and GR mediated. These mutant mice appear indistinguishable from WT for at least 2 months, indicating that GC-responsive osteoclasts are not required for normal skeletal development. Probably reflecting the short dura-

**Figure 5**

DEX inhibits M-CSF-induced RhoA, Rac, and Vav3 activation. WT BMMs maintained in M-CSF and RANKL for 3 days were exposed to DEX (100 nM) or vehicle for 16 hours and then stimulated with M-CSF (100 ng/ml). GTP-RhoA and GTP-Rac were isolated by GST pull-down and immunoblotted with RhoA- (A) or Rac-specific (B) antibodies. (C) Vav3 was immunoprecipitated from the cell lysate and phosphotyrosine (p-Tyr) content of the immunoprecipitate determined by immunoblot with anti-phosphotyrosine antibody. In all circumstances, the data are expressed as fold difference in DEX-exposed (+) relative to vehicle-exposed (-) cells.

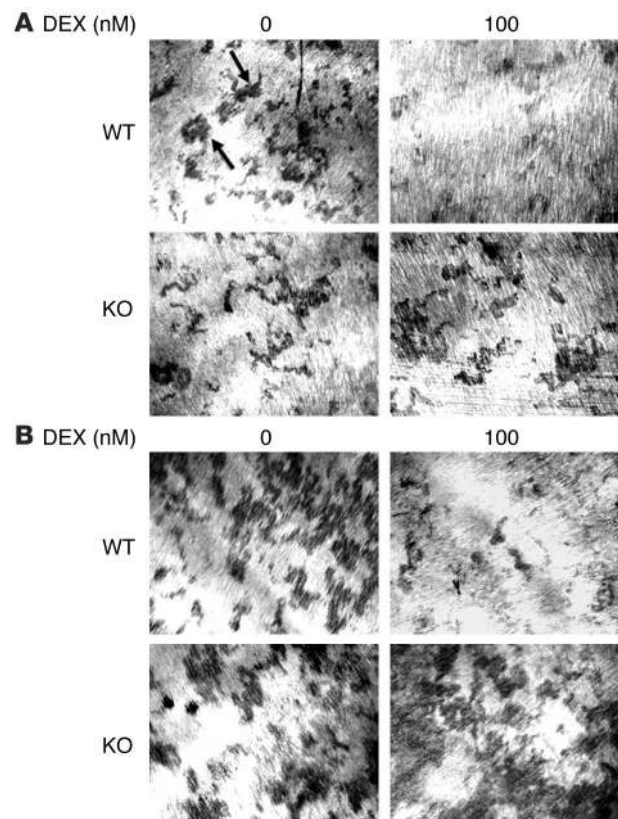
tion of treatment, histomorphometrically measured bone mass of WT and *GR<sup>oc-/-</sup>* mice, following 14 days of steroid exposure, was similar (data not shown).

We found that DEX regulates osteoclast precursor proliferation but does so in a differentiation-dependent manner. Thus, uncommitted BMMs or those stimulated only by M-CSF to develop into mature non-osteoclastogenic macrophages, underwent divisional arrest in the presence of DEX. IL-1 $\alpha$ , which alone is not osteoclastogenic (35), also failed to prevent DEX-suppressed BMM proliferation. In contrast, when the cells were maintained in osteoclastogenic medium containing M-CSF plus RANKL or TNF- $\alpha$ , the steroid no longer dampened proliferation. Thus, commitment to the bone-resorptive phenotype protects macrophages from the antiproliferative effects of GCs. Failure of DEX to suppress RANKL-treated osteoclast precursors was mirrored by unaltered activation of ERKs, which mediates replication of the cell.

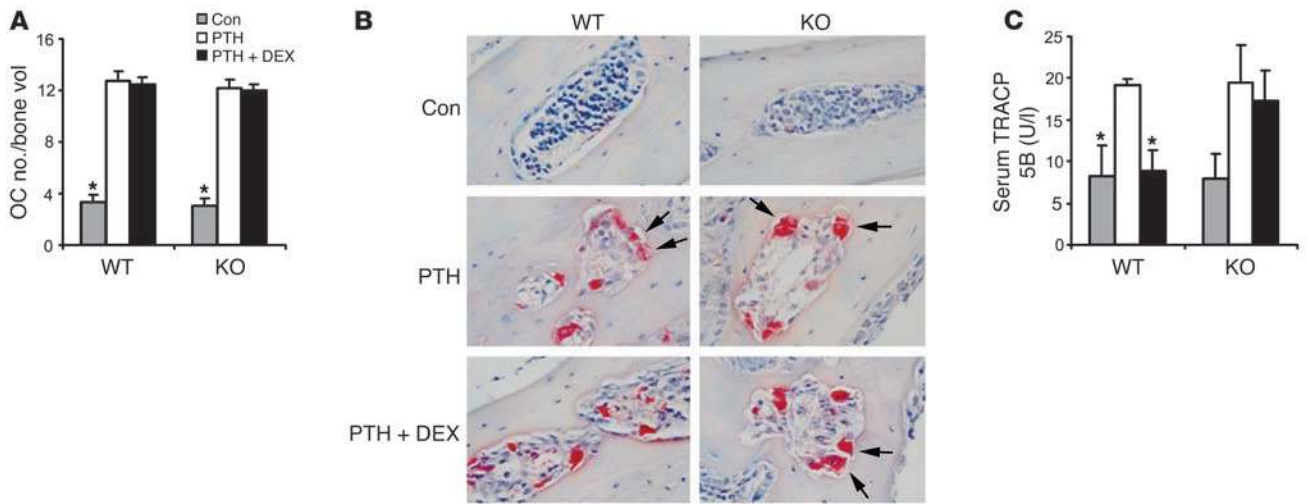
Weinstein et al. report that GCs prevent apoptosis of the mature osteoclast (14), which we confirm. The steroid's antiapoptotic properties are not attended by Akt activation, indicating that an alternative survival pathway is extant. We also note that the antiapoptotic effect requires only short exposure to the steroid in the latter phase of osteoclast differentiation. Despite their prolonged longevity, however, GC-treated osteoclasts have markedly suppressed bone-resorptive activity in vitro and in vivo. GCs induce RANKL and M-CSF expression and blunt synthesis of the osteoclast-inhibitory molecule osteoprotegerin (12, 13). These observations, taken with the normal proliferation of DEX-treated BMMs committed to the osteoclast phenotype, and the drug's antiapoptotic effect on the fully differentiated polykaryon, suggest the steroid would accelerate bone resorption in vivo. We found, however, that while the number of osteoclasts in PTH-stimulated WT mice treated with DEX mirrors the number in those that have not received the steroid, their

global bone-resorptive activity is suppressed in a GR-mediated fashion. This paradox of suppressed bone resorption in the face of unaltered osteoclast number indicates that the stimulatory effects of GCs on osteoclast survival are obviated by direct inhibition of the differentiated cell's capacity to degrade bone.

The osteoclast is characterized by a unique cytoskeleton, which undergoes continuous reorganization with different phases of the resorptive cycle (11). We find that DEX, in low nanomolar concentrations, disarranges the osteoclast cytoskeleton, yielding cells that fail to spread and ineffectively resorb mineralized matrix. Such features are characteristic of a variety of resorptive disorders, such as absence of the cytoskeleton-regulating proteins, *c-src* (13), or the  $\alpha\beta 3$  integrin (36). These in vitro GC-

**Figure 6**

DEX suppresses osteoclastic bone resorption in vitro. (A) WT and *GR<sup>oc-/-</sup>* BMMs were cultured on dentin with RANKL and M-CSF with or without DEX. After 5 days, resorption pits were stained with toluidine blue (arrows). (B) WT and *GR<sup>oc-/-</sup>* BMMs were cultured on plastic in the presence of M-CSF and RANKL. After 3 days the cells were lifted and replated on dentin in M-CSF and RANKL with or without DEX. Three days later, resorption pits were stained with toluidine blue. Magnification,  $\times 100$ .

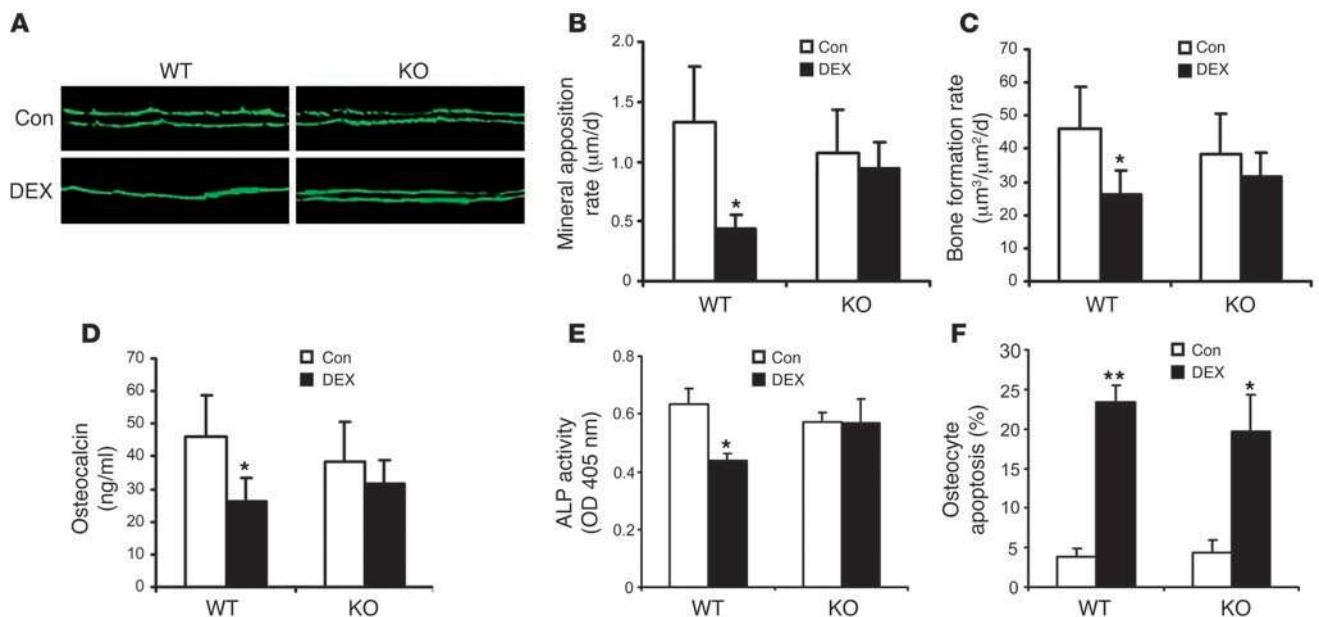


**Figure 7** DEX suppresses PTH-stimulated bone resorption in vivo. WT and *GRoc<sup>-/-</sup>* mice were injected daily for 4 days with vehicle (Con) or PTH or PTH plus DEX (*n* = 4). Histological sections of calvariae were stained for TRAP activity. (A) Osteoclast number/unit bone volume. (B) TRAP-stained histological sections of calvariae (arrows indicate osteoclasts with ruffled membranes in resorption lacunae). Magnification,  $\times 250$ . (C) Serum TRACP5b levels at sacrifice. \**P* < 0.01 versus PTH-treated WT.

induced events are reflected in vivo by small, irregular osteoclasts that fail to attach to or normally degrade bone and lack the cell's key resorptive organelle, its ruffled membrane.

Skeletal degradation occurs in an acidified microenvironment between the osteoclast and the bone surface that is isolated from the general extracellular space by an actin ring (11). Insufficient actin ring generation, in a number of circumstances, is accom-

panied by failure of the osteoclast to spread or adequately resorb bone. We describe what we believe to be a novel and rapid assay for actin ring formation in osteoclasts under the influence of individual cytokines. Interestingly, DEX did not alter the capacity of RANKL, TNF- $\alpha$ , or IL-1 $\alpha$  to establish actin rings in committed osteoclast precursors but substantially suppressed that stimulated by M-CSF. While M-CSF is classically viewed as a survival and pro-



**Figure 8** *GRoc<sup>-/-</sup>* mice are protected from DEX-suppressed bone formation. WT and *GRoc<sup>-/-</sup>* mice were injected with DEX (10 mg/kg) or vehicle daily for 14 days. Calcein was administered 8 and 2 days prior to sacrifice. (A) Fluorescent micrographs of representative calcein labels. Magnification,  $\times 100$ . (B) Measured mineral apposition rate. (C) Measured bone formation rate. (D) Serum osteocalcin levels at sacrifice. (E) Serum alkaline phosphatase (ALP) levels at sacrifice. (F) Percentage of osteocytes in histological sections of bone undergoing apoptosis. \**P* < 0.05, \*\**P* < 0.005 versus control.



liferative cytokine for osteoclast precursors, it also exerts cytoskeletal effects via a signaling pathway shared by the  $\alpha\beta3$  integrin (37). Thus, the spreading defect of  $\alpha\beta3$  integrin-deficient osteoclasts is rescued by high-dose M-CSF.

The osteoclast cytoskeleton is modulated by a series of small GTPases including RhoA (38) and Rac (23) that transit to their GTP bound state under the influence of guanine nucleotide exchange factors (GEFs). RhoA and Rac are activated in the osteoclast by  $\alpha\beta3$  integrin occupancy or M-CSF, both of which induce the cell-specific GEF isoform Vav3 (23). Inhibition of RhoA activity or genetic deletion of Rac (22) or Vav3 (23) arrests osteoclastic bone resorption, and we found that DEX prevents M-CSF-mediated induction of the 3 entities. Hence, GCs suppressed bone resorption by disrupting the cytoskeleton of the mature resorptive cell in an M-CSF/Vav3/RhoA/Rac-dependent manner.

GCs impact cells by genomic and nongenomic mechanisms. The steroid's nongenomic effects occur within seconds to minutes and are mediated by the GR or by other means such as G protein-coupled receptors (39). Because DEX-mediated suppression of Vav3 activation requires 16 hours, a genomic locus of action is likely. Whether this genomic effect involves direct DNA binding of the GR or represents GR associating with an intermediary DNA-interacting protein remains to be determined. In any event, our findings provide what we believe to be a novel paradigm for the pathogenesis of a common and often devastating form of osteoporosis that to date has remained largely refractory to therapeutic intervention.

## Methods

**Mice.** The loxP-floxed GR allele has been described previously (18). Mice used for these studies were progeny of lysM<sup>Cre+</sup>loxP<sup>phomo</sup> × loxP<sup>phomo</sup> matings. The lysM Cre<sup>+</sup> knockin was an F<sub>1</sub> cross of 129 × C57, as was the loxP allele (40). To avoid background drift effects of F<sub>2</sub> matings, several breeding pairs were analyzed and Cre<sup>-</sup> littermates were always used as controls for the Cre<sup>+</sup> mice from the same parents. All mice were 6–10 weeks old and maintained at the Animal Facility of Washington University School of Medicine. The Animal Ethics Committee of Washington University approved all experiments.

**Serum TRACP5b activity.** Serum TRACP5b activity was measured by ELISA (Immunodiagnostic Systems), after groups of 4 animals were subcutaneously injected with 10  $\mu$ g PTH (human fragment 1–34; Bachem) diluted in 25  $\mu$ l of vehicle (1 mM HCl plus 0.1% bovine serum albumin) or PTH plus DEX (10 mg/kg) once a day for 4 days. Vehicle alone served as control.

**Osteoclast cultures.** BMMs were prepared from whole bone marrow of 6- to 10-week-old mice as described previously (14) and cultured without or with DEX in the presence of GST-RANKL (30 ng/ml) and M-CSF (50 ng/ml) in  $\alpha$ -MEM containing 10% charcoal-stripped FBS. The medium was exchanged every 2 days. Cells were fixed and stained for TRAP activity after 5 days. To determine actin ring formation, BMMs were cultured on dentin with GST-RANKL (100 ng/ml) and M-CSF (10 ng/ml). After 5 days, some cells were fixed and stained with FITC-phalloidin.

**Proliferation.** Proliferation was determined using the Cell Proliferation ELISA system (Amersham Biosciences). After 3 days, BrdU was added to

each well, and cells were incubated for an additional 4 hours. BrdU incorporation was detected following the manufacturer's instructions.

**Osteoclast apoptosis.** Osteoclast apoptosis was assessed by ELISA (Cell Death Detection ELISA; Roche Diagnostics).

**Osteocyte apoptosis.** Osteocyte apoptosis was identified in bone sections by in situ nick-end labeling using the Klenow terminal deoxynucleotidyl transferase (EMD Biosciences) (2).

**RT-PCR.** Total RNA (1  $\mu$ g) extracted from cultured cells was used as a template for cDNA synthesis. Primers were synthesized on the basis of the reported mouse cDNA sequence. The following primers were used: for TRAP, 5'-ACAGCCCCCTCCACCCT-3' and 3'-TCAGGGTCTGGGTCTCCTTG-5'; for MMP-9, 5'-CCTGTGTGTTCCCGTTCATCT-3' and 3'-CGCTGGAATGATCTAAGCCCA-5'; for cathepsin K, 5'-GGAAGAAGACTACCAGAAGC-3' and 3'-GCTATATAGCCGCCTCACAG-5'; for GAPDH, 5'-ACTTTGTCAAGCTCATTTC-3' and 3'-TGCAGCGAAGCTTTATTGATG-5'. Amplification was conducted for 22–30 cycles, each of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. Ten microliters of each reaction mixture was analyzed by 1.5% agarose gel electrophoresis.

**Cell signaling.** mAbs obtained from Cell Signaling Technology were used to detect phosphorylated Akt and ERK by immunoblot assay. To assess Vav3 activation, cell lysates were immunoprecipitated with polyclonal antibody against Vav3 (Upstate USA Inc.), followed by Western blotting using anti-phosphotyrosine mAb (4G10; Upstate USA Inc.). Active Rho and Rac were measured using Rho and Rac1 Activation Kits (Pierce), following the manufacturer's protocol.

**Serum osteocalcin.** Serum osteocalcin levels were measured by ELISA (Biomedical Technologies Inc.).

**Serum alkaline phosphatase.** Serum alkaline phosphatase activity was assayed using QuantiChrom Alkaline Phosphatase Assay Kit (BioAssay Systems).

**Mineral apposition rate and bone formation rate.** Mice were administered fluorescent calcein dye (20 mg/kg) 8 and 2 days prior to sacrifice. Non-decalcified histological sections of calvariae were prepared and kinetic parameters determined by fluorescent microscopy using OsteoMeasure (Osteometrics).

**Statistics.** Data are presented as mean  $\pm$  SD. Statistical significance was determined by 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## Acknowledgments

This study was supported by grants from the NIH: AI50655 to L.J. Muglia; AR046852 and AR048812 to F.P. Ross; and AR032788, AR046523, AR048853, and DK056341 (from the Clinical Nutrition Research Unit) to S.L. Teitelbaum.

Received for publication January 31, 2006, and accepted in revised form June 6, 2006.

Address correspondence to: Steven L. Teitelbaum, Washington University School of Medicine, Department of Pathology and Immunology, Campus Box 8118, 660 South Euclid Avenue, St. Louis, Missouri 63110, USA. Phone: (314) 454-8463; Fax: (314) 454-5505; E-mail: teitelbs@wustl.edu.

- Weinstein, R.S. 2001. Glucocorticoid-induced osteoporosis. *Rev. Endocr. Metab. Disord.* **2**:65–73.
- Weinstein, R.S., Jilka, R.L., Parfitt, A.M., and Manolagas, S.C. 1998. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. *J. Clin. Invest.* **102**:274–282.
- Pereira, R.M., Delany, A.M., and Canalis, E. 2001. Cortisol inhibits the differentiation and apoptosis

- of osteoblasts in culture. *Bone.* **28**:484–490.
- Pereira, R.C., Delany, A.M., and Canalis, E. 2002. Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression. *Bone.* **30**:685–691.
- Aubin, J.E. 1999. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast dif-

- ferentiation. *J. Cell. Biochem.* **72**:396–410.
- Purpura, K.A., Aubin, J.E., and Zandstra, P.W. 2004. Sustained in vitro expansion of bone progenitors is cell density dependent. *Stem Cells.* **22**:39–50.
- Black, D.M., et al. 2003. The effects of parathyroid hormone and alendronate alone or in combination in postmenopausal osteoporosis. *N. Engl. J. Med.* **349**:1207–1215.
- Finkelstein, J.S., et al. 2003. The effects of parathy-





- roid hormone, alendronate, or both in men with osteoporosis. *N. Engl. J. Med.* **349**:1216–1226.
9. Manolagas, S.C., and Weinstein, R.S. 1999. New developments in the pathogenesis and treatment of steroid-induced osteoporosis. *J. Bone Miner. Res.* **14**:1061–1066.
10. Hirayama, T., Sabokbar, A., and Athanasou, N.A. 2002. Effect of corticosteroids on human osteoclast formation and activity. *J. Endocrinol.* **175**:155–163.
11. Teitelbaum, S.L. 2000. Bone resorption by osteoclasts. *Science.* **289**:1504–1508.
12. Hofbauer, L.C., et al. 1999. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. *Endocrinology.* **140**:4382–4389.
13. Rubin, J., et al. 1998. Dexamethasone promotes expression of membrane-bound macrophage colony-stimulating factor in murine osteoblast-like cells. *Endocrinology.* **139**:1006–1012.
14. Weinstein, R.S., et al. 2002. Promotion of osteoclast survival and antagonism of bisphosphonate-induced osteoclast apoptosis by glucocorticoids. *J. Clin. Invest.* **109**:1041–1048. doi:10.1172/JCI200214538.
15. Pearce, G., Tabensky, D.A., Delmas, P.D., Baker, H.W., and Seeman, E. 1998. Corticosteroid-induced bone loss in men. *J. Clin. Endocrinol. Metab.* **83**:801–806.
16. Prummel, M.F., Wiersinga, W.M., Lips, P., Sanders, G.T., and Sauerwein, H.P. 1991. The course of biochemical parameters of bone turnover during treatment with corticosteroids. *J. Clin. Endocrinol. Metab.* **72**:382–386.
17. Dempster, D.W. 1989. Bone histomorphometry in glucocorticoid-induced osteoporosis. *J. Bone Miner. Res.* **4**:137–141.
18. Brewer, J.A., et al. 2003. T-cell glucocorticoid receptor is required to suppress COX-2-mediated lethal immune activation. *Nat. Med.* **9**:1318–1322.
19. Faccio, R., Novack, D.V., Zallone, A., Ross, F.P., and Teitelbaum, S.L. 2003. Dynamic changes in the osteoclast cytoskeleton in response to growth factors and cell attachment are controlled by  $\beta 3$  integrin. *J. Cell Biol.* **162**:499–509.
20. Nakamura, I., et al. 2002. IL-1 regulates cytoskeletal organization in osteoclasts via TNF receptor-associated factor 6/c-Src complex. *J. Immunol.* **168**:5103–5109.
21. Chellaiiah, M.A., et al. 2000. Rho-A is critical for osteoclast podosome organization, motility, and bone resorption. *J. Biol. Chem.* **275**:11993–12002.
22. Korhonen, M., et al. 2005. Rac1 and Rac2 GTPases play distinct roles and are essential for full osteoclast differentiation. Paper presented at the 47th American Society of Hematology Annual Meeting. December 11. Atlanta, Georgia, USA.
23. Faccio, R., et al. 2005. Vav3 regulates osteoclast function and bone mass. *Nat. Med.* **11**:284–290.
24. Udagawa, N., et al. 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc. Natl. Acad. Sci. U. S. A.* **87**:7260–7264.
25. Lacey, D.L., et al. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* **93**:165–176.
26. Martin, T.J., and Sims, N.A. 2005. Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends Mol. Med.* **11**:76–81.
27. Marzia, M., et al. 2000. Decreased c-Src expression enhances osteoblast differentiation and bone formation. *J. Cell Biol.* **151**:311–320.
28. Grigoriadis, A.E., et al. 1994. c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science.* **266**:443–448.
29. Demiralp, B., Chen, H.L., Koh, A.J., Keller, E.T., and McCauley, L.K. 2002. Anabolic actions of parathyroid hormone during bone growth are dependent on c-fos. *Endocrinology.* **143**:4038–4047.
30. Rickard, D.J., Sullivan, T.A., Shenker, B.J., Leboy, P.S., and Kazhdan, I. 1994. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev. Biol.* **161**:218–228.
31. Odvina, C.V., et al. 2005. Severely suppressed bone turnover: a potential complication of alendronate therapy. *J. Clin. Endocrinol. Metab.* **90**:1294–1301.
32. Rubin, M.R., and Bilezikian, J.P. 2002. Clinical review 151: the role of parathyroid hormone in the pathogenesis of glucocorticoid-induced osteoporosis: a re-examination of the evidence. *J. Clin. Endocrinol. Metab.* **87**:4033–4041.
33. Lane, N.E., and Lukert, B. 1998. The science and therapy of glucocorticoid-induced bone loss. *Endocrinol. Metab. Clin. North Am.* **27**:465–483.
34. Weinstein, R.S., et al. 2004. The skeletal effects of glucocorticoid excess override those of orchidectomy in mice. *Endocrinology.* **145**:1980–1987.
35. Wei, S., Kitaura, H., Zhou, P., Ross, F.P., and Teitelbaum, S.L. 2005. IL-1 mediates TNF-induced osteoclastogenesis. *J. Clin. Invest.* **115**:282–290. doi:10.1172/JCI200523394.
36. McHugh, K.P., et al. 2000. Mice lacking  $\beta 3$  integrins are osteosclerotic because of dysfunctional osteoclasts. *J. Clin. Invest.* **105**:433–440.
37. Faccio, R., Zallone, A., Ross, F.P., and Teitelbaum, S.L. 2003. c-Fms and the  $\alpha v \beta 3$  integrin collaborate during osteoclast differentiation. *J. Clin. Invest.* **111**:749–758. doi:10.1172/JCI200316924.
38. Chellaiiah, M.A. 2005. Regulation of actin ring formation by rho GTPases in osteoclasts. *J. Biol. Chem.* **280**:32930–32943.
39. Dallman, M.F. 2005. Fast glucocorticoid actions on brain: back to the future. *Front. Neuroendocrinol.* **26**:103–108.
40. Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. 1999. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* **8**:265–277.