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Article

Glucocorticoids Suppress Bone Formation by Attenuating Osteoblast Differentiation via the Monomeric Glucocorticoid Receptor

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SUMMARY

Development of osteoporosis severely complicates long-term glucocorticoid (GC) therapy. Using a Cre-transgenic mouse line, we now demonstrate that GCs are unable to repress bone formation in the absence of glucocorticoid receptor (GR) expression in osteoblasts as they become refractory to hormone-induced apoptosis, inhibition of proliferation, and differentiation. In contrast, GC treatment still reduces bone formation in mice carrying a mutation that only disrupts GR dimerization, resulting in bone loss in vivo, enhanced apoptosis, and suppressed differentiation in vitro. The inhibitory GC effects on osteoblasts can be explained by a mechanism involving suppression of cytokines, such as interleukin 11, via interaction of the monomeric GR with AP-1, but not NF-κB. Thus, GCs inhibit cytokines independent of GR dimerization and thereby attenuate osteoblast differentiation, which accounts, in part, for bone loss during GC therapy.

INTRODUCTION

Although glucocorticoids (GCs) are widely used to treat allergic and autoimmune diseases, their application is accompanied by severe side effects, including detrimental effects on the skeleton (James et al., 2007). Reduction of bone mass by GCs is believed to involve systemic effects and/or direct effects on bone cells (Canalis et al., 2007), leading to induction of apoptosis in osteoblasts and osteocytes (Weinstein et al., 1998) and/or suppression of their differentiation. Whereas low doses of GCs stimulate (Shalhoub et al., 1992), high doses inhibit osteoblast differentiation, the latter being related to the inhibition of postconfluential proliferation (Smith et al., 2000). Suppression of osteoblast function could be a consequence of decreased expression of genes involved in bone formation, such as collagen type 1 (*Col1a1*) and *Runx2*, or could be due to antagonizing the BMP- and Wnt-signaling pathways (Canalis et al., 2007). Besides osteoblasts, modulation of osteoclast activity also appears to contribute to GC-induced osteoporosis (GIO). GCs induce the synthesis of receptor activator for nuclear factor (NF)-κB ligand (RANKL), an essential stimulator of osteoclastogenesis (Hofbauer et al., 1999). In addition, they prolong the longevity of osteoclasts in vivo but decrease their bone-degrading activity and seem to influence bone formation via the GR in osteoclasts (Jia et al., 2006; Kim et al., 2006).

The majority of GC effects are mediated via the glucocorticoid receptor (GR), a widely expressed member of the nuclear receptor superfamily. Following hormone binding, the GR alters gene expression via several modes of action, including its binding as dimers to GC-responsive elements (GRE) present in the promoter of hormone-responsive genes and interaction of the monomeric receptor with DNA-bound transcription factors such as NF-κB, AP-1, IRF-3, or STAT5 (Kassel and Herrlich,

2007). Currently, suppression of these transcription factors is believed to underlie, in part, the anti-inflammatory effects of GCs, and dimerization of the GR is hypothesized to contribute to many of the side effects. This view is supported by the finding that interaction with proinflammatory transcription factors is preserved in GR^{dim} mice carrying a dimerization-deficient GR, whereas induction of GRE-dependent transcription is abolished (Reichardt et al., 1998, 2001). However, there is a requirement for GR dimerization to achieve full suppression of inflammation in contact allergy, as GC treatment is therapeutically inefficient in GR^{dim} mice (Tuckermann et al., 2007).

The aim of this study was to attain a better understanding of the mode of GR action underlying GIO and the primary type of bone cells targeted by GCs. We demonstrate that the GR in osteoblasts is required to preserve full bone integrity under physiological conditions, that bone loss induced by therapeutic GC treatment is mediated by the GR in osteoblasts and independent of its dimerization, and that the monomeric GR is sufficient to impair osteoblast differentiation by suppressing osteoblast-derived cytokines such as interleukin 11 (IL-11).

RESULTS

Endogenous Glucocorticoid Action in Osteoblasts Increases Bone Mass, but Not Skeletal Development

We initially asked whether endogenous GCs influence the development of the skeleton by using GR knockout (GR^{null}) mice. Alizarin red/Alcian blue staining did not reveal any differences in cartilage and calcified tissues in GR^{null} compared to wild-type E18.5 fetuses, and X-ray analysis showed similar bone mineral densities (Figures S1A and S1B available online). The lack of gross alterations in growth plate dimensions and a similar extent of calcified bone revealed by von Kossa staining indicated that the development of skeletal tissues in GR^{null} fetuses is normal (Figures S1C and S1D).

To allow investigations of GC actions in adult bone, we disrupted the GR gene specifically in the osteoblast lineage by crossing GR^{flox} mice (Tronche et al., 1999) with an osteoblast-specific Cre-transgenic mouse line (*Runx2Cre*), thereby obtaining $GR^{Runx2Cre}$ mice. Of note, efficient recombination at all sites of endochondral and intramembranous bone formation, particularly in periosteal cells, osteoblasts, and osteocytes, but not in osteoclasts, was observed when *Runx2cre* mice were crossed to a Rosa26 reporter strain (Figure S2). In accordance with all known sites of *Runx2* expression, $GR^{Runx2Cre}$ displayed almost complete recombination of the GR-flox allele in long bones, to a large extent in calvaria, and to a minor part in cartilage. Recombination was undetectable in other mesenchymal tissues, i.e., muscle or fat (Figures S2G, S2I, and S2J). Thus, the GR is efficiently deleted in the osteoblastic lineage in $GR^{Runx2Cre}$ mice. Compared to control animals, females lacking the GR in osteoblasts exhibited no growth defects. Male mice showed a small increase in rump, but not in tibia length, indicating a potentially minor role of the GR in the growth of the spine in males (Table S1).

Microcomputer tomography (Micro-CT) analysis and bone histomorphometry of calcified vertebral sections of 3-month-old female and male $GR^{Runx2Cre}$ mice revealed a modest but highly significant decrease of bone density compared to GR^{flox} mice (Figures 1A, 1B, and data not shown). Whereas trabecular thickness was not altered, the decrease was, rather, due to diminished trabecular numbers and, consequently, an increase in trabecular spacing (Figure 1B). Numbers of osteoblasts, osteoblast surface, osteocytes, and osteoclast parameters were not significantly changed (Figure 1C). We therefore hypothesized that lack of the GR affects the differentiation toward functional osteoblasts, rather than their numbers per se. Indeed, as suggested by previous reports on mouse models deficient in GC action (Durbridge et al., 1990; Kalak et al., 2009; Sher et al., 2004, 2006), calvarial osteoblasts from GR-deficient mice displayed a diminished differentiation potential in terms of alkaline phosphatase (ALP) expression and bone nodule formation (Figure 1D). Accordingly, expression of marker genes for osteoblast differentiation and function, such as *Runx2*, *Col1a1*, and *Bglap2* (osteocalcin), was reduced (Figure 1E).

GR in Osteoblasts Mediates GC Suppression of Bone Formation

To address the role of the GR in osteoblasts in GC-induced bone loss, $GR^{Runx2Cre}$ and GR^{flox} mice were treated with the clinically relevant GR agonist prednisolone for 2 weeks. As expected, prednisolone led to a reduced bone mineral density in vertebral bones of wild-type mice due to decreased trabecular thickness. In contrast, these parameters were not affected in $GR^{Runx2Cre}$ mice (Figures 2A and 2B). Resorption, as assessed by deoxypyridinoline (DPD/Cr) levels, was not increased by prednisolone but, rather, was slightly decreased in mice of both genotypes (Figure 2C); however, osteoclast numbers were not affected (Figure 2D). In contrast, the numbers of osteocytes, osteoblasts (Figure 2D), and the osteoblast surface (data not shown) were decreased in GR^{flox} mice after prednisolone treatment, whereas the same parameters were unaltered in $GR^{Runx2Cre}$ mice.

Most importantly, whereas prednisolone caused an almost complete inhibition of bone formation as determined by dynamic histomorphometry following dual calcein labeling in vertebrae of wild-type mice, $GR^{Runx2cre}$ mice were fully protected (Figures 2E and 2F). Because resorption was only slightly altered after prednisolone treatment, this complete block of bone formation is presumably the major cause for prednisolone-induced bone loss in wild-type mice. Of note, prednisolone did not alter TUNEL and BrdU pulse labeling directly within bone tissue (Figure S3). But osteoblastogenesis, in terms of colony-forming units of osteoblasts (CFU OBs), was strongly impaired in GR^{flox} mice, but not in $GR^{Runx2Cre}$ mice, after 3 days of prednisolone treatment (Figure 2G). Most strikingly, the expression of the marker for functional osteoblasts, *Col1a1*, was strongly reduced after 3 days of prednisolone in GR^{flox} mice, whereas it remained unchanged in $GR^{Runx2Cre}$ mice (Figure 2H), indicating suppression of osteoblast differentiation and function via the GR in these cells.

The GR in Myeloid Cells Does Not Affect Bone Formation

To test whether the GR in osteoclasts influences bone formation during GC treatment, $GR^{LysMCre}$ mice lacking the GR in myeloid cells, such as monocytes, macrophages, neutrophils, and osteoclasts (Tuckermann et al., 2007; J.P.T., unpublished data), were treated with prednisolone for 2 weeks. The bone formation rate was similarly reduced in $GR^{LysMCre}$ and wild-type mice (Figures 2I and 2J), thus excluding a role for these cells in mediating GC-induced bone loss.

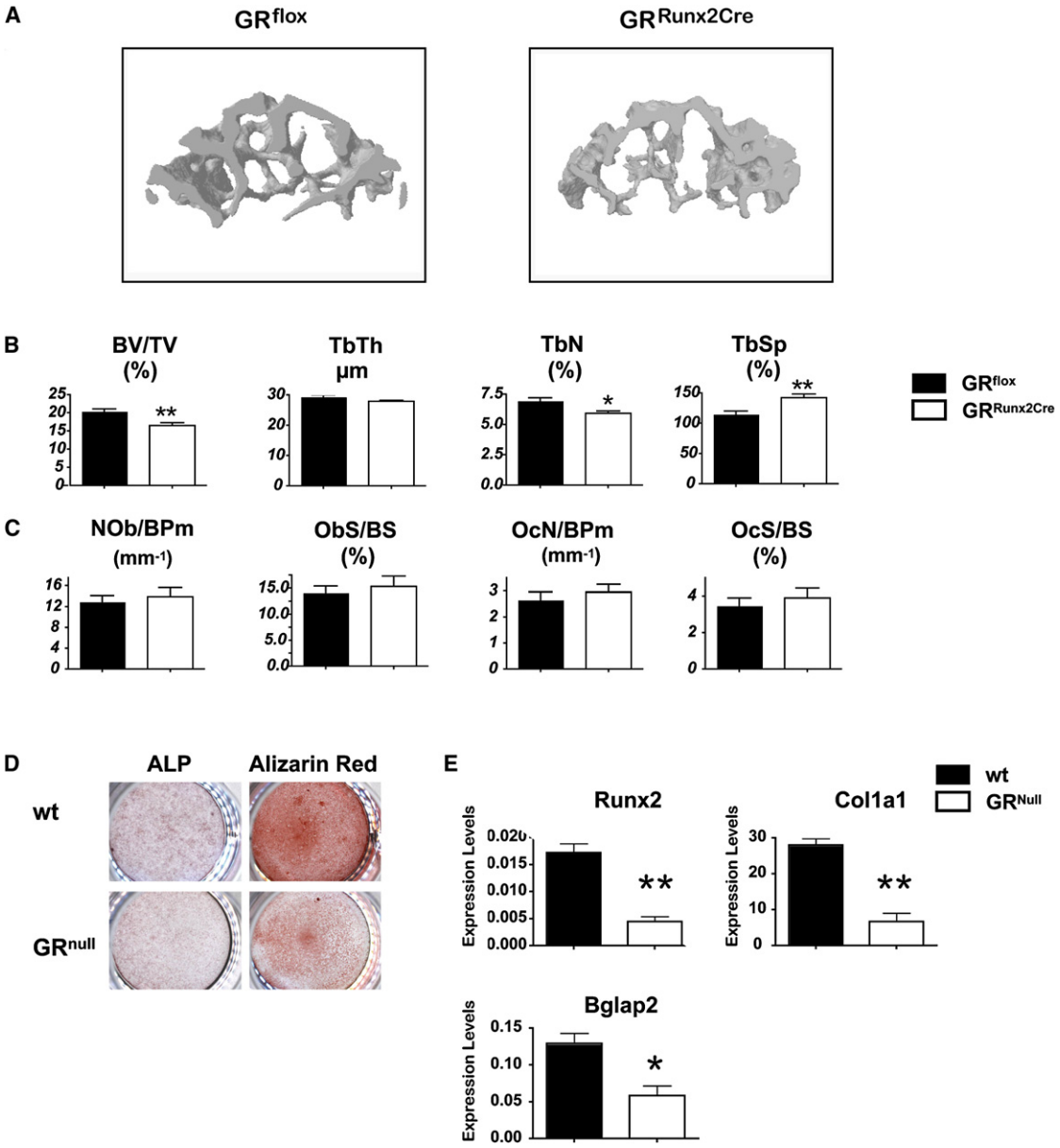


Figure 1. Reduced Bone Mass in GR^{Runx2Cre} Mice

(A) Micro-CT reconstruction of the trabecular part of vertebral L6 bodies of 10-week-old female GR^{Runx2cre} and GR^{flox} mice.

(B and C) Histomorphometry of (B) bone volume/tissue volume (BV/TV), trabecular thickness (TbTh), trabecular numbers (TbN), and trabecular spacing (TbSp) and (C) osteoblast number/bone perimeter (Ob.N/B.Pm), osteoblast surface/bone surface (Ob.S/BS), osteoclast number/bone perimeter (Oc.N/B.Pm), and osteoclast surface/bone surface (Oc.S/BS).

(D) Primary osteoblasts derived from wild-type (wt) and GR^{null} mice were induced with osteogenic differentiation medium, and differentiation was analyzed by ALP staining after 10 days and alizarin red after 20 days.

(E) Osteoblast mRNA expression levels (arbitrary units normalized to actin) for Runx2, Col1a1 (10 days of differentiation), and Bglap2 (20 days) determined by qRT-PCR.

Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 8 in (B) and (C). n = 3 in (E).

GC-Induced Bone Loss Occurs in the Absence of GR Dimerization

Although it had been hypothesized that GR dimerization was essential for many side effects of GC therapy, its role in bone loss has not been previously investigated. Therefore, GR^{dim} mice carrying a dimerization-defective GR were treated with prednisolone. In contrast to GR^{Runx2Cre} mice, bone formation was similarly repressed in GR^{dim} and wild-type mice, and bone mass and trabecular thickness of the vertebrae were reduced to a comparable degree (Figures 3A–3D). This was again accompanied by a strong reduction of osteoblast and osteocyte numbers (Figure 3E), as well as osteoblast surface (data not

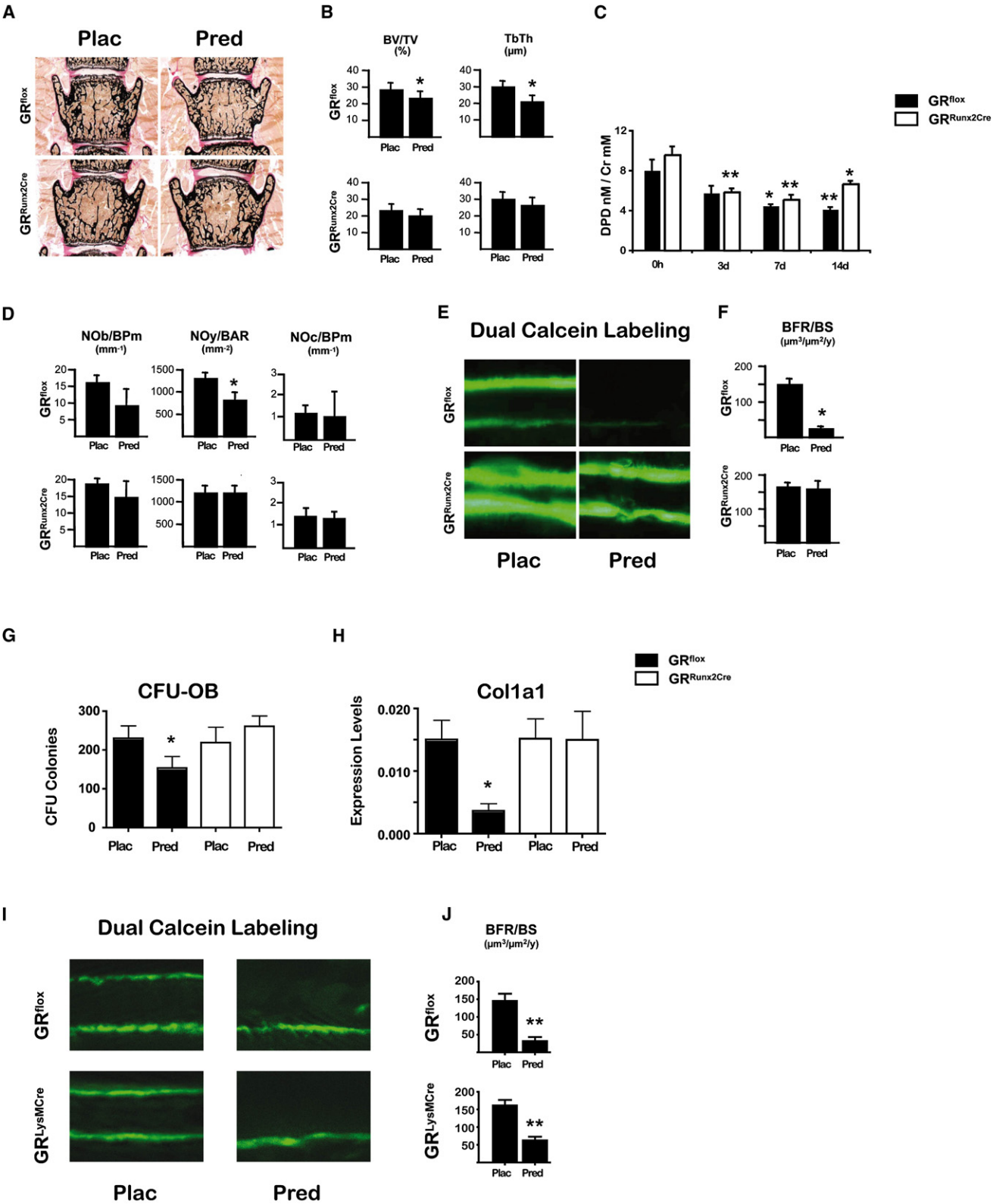


Figure 2. Prednisolone Reduces Bone Formation Dependent on the GR in Osteoblasts

(A and B) Vertebral bodies of 10-week-old female $GR^{Runx2cre}$ and GR^{flox} mice treated with placebo (Plac) or prednisolone (Pred; 12.5 mg/kg/day) for 2 weeks were analyzed by (A) von Kossa staining of undecalcified sections and (B) histomorphometry of BV/TV and TbTh.

(C) DPD/Cr levels in urine of mice after prednisolone treatment at indicated times.

shown) in both genotypes. Osteoblastogenesis in vitro was as efficiently suppressed in GR^{dim} as in wild-type mice, and *Col1a1* expression was similarly reduced in both genotypes (Figures 3F and 3G). Whereas osteoclast numbers were not changed, resorption was slightly decreased in both mouse strains (Figures 3E and 3H).

Dimerized GR Affects Osteoblast Proliferation, but Not Apoptosis and Differentiation

Primary osteoblasts from wild-type, GR^{null}, and GR^{dim} mice were cultured in vitro to determine the contribution of GR dimerization to GC effects on osteoblasts. Proliferation of preconfluent preosteoblasts isolated from neonatal calvaria was lower in cultures of both GR^{null} and GR^{dim} cells as compared to wild-type controls, suggesting that the GR promotes osteoblast expansion in the presence of basal GC concentrations (Figure 4A). In contrast, treatment with dexamethasone (Dex) completely prevented expansion of wild-type cells but did not affect GR^{null} and GR^{dim} cultures. Thus, GR dimerization is required for permissive and repressive GC activities on osteoblast expansion.

Postconfluential proliferation after exposure to osteogenic conditions has been reported to accompany osteoblastic differentiation in vitro (Smith et al., 2000). Ki-67-stained wild-type cells confirmed reduction of proliferation by Dex after 24 hr until 96 hr (Figure S4B). In contrast, postconfluential proliferation of GR^{dim} and GR^{null} cells was entirely unaffected by Dex (Figure 4B). In addition, Dex-induced apoptosis was observed in wild-type and GR^{dim} cells, but not in GR^{null} cells (Figures 4C and S4A). This is consistent with the slight reduction in postconfluential cell numbers by Dex in wild-type and GR^{dim}, but not in GR^{null} cells (Figures 4D and S4C). ALP activity and matrix mineralization were inhibited by Dex treatment of wild-type and GR^{dim}, but not of GR^{null} osteoblasts (Figures 4E and 4F). Efficient downregulation of the osteoblastic markers *Runx2*, *Akp1*, *Col1a1*, and *Bglap2* confirmed inhibition of osteoblast differentiation of wild-type and GR^{dim} cells. In contrast, no repressive effect of Dex was observed for GR^{null} osteoblasts (Figures 4G–4J).

The Monomeric GR in Osteoblasts and Osteoclasts Affects Osteoclastogenesis

Because osteoblasts can stimulate the formation of osteoclasts, cocultures of osteoblasts with wild-type, GR^{null}, and GR^{dim} fetal liver cells serving as osteoclast progenitors were established. Irrespective of whether the GR was present in osteoclasts, cocultures with GR^{null} osteoblasts under basal conditions led to the generation of fewer tartrate-resistant alkaline phosphatase-positive multinuclear cells (TRAP MNCs) as compared to wild-type osteoblasts (Figure 5A). This indicates that the GR in osteoblasts contributes to osteoclastogenesis under low-dose GC conditions. A pharmacological dose of Dex reduced the number of TRAP MNCs in cocultures independent of GR expression in the osteoclasts (Figure 5A), again highlighting a major contribution of the GR in osteoblasts. Of interest, Dex also repressed osteoclastogenesis when we cocultivated GR^{null} osteoblasts with wild-type fetal liver cells, but not when the GR was absent from both cell compartments (Figure 5A). Thus, the GR in both osteoblasts and osteoclasts mediates reduction of TRAP-positive cells by GCs.

Dimerization of the GR in osteoblasts was neither required for basal osteoclastogenesis nor for suppression of osteoclastogenesis by Dex (Figure 5B). It is noteworthy that GR^{dim} osteoclast differentiation from progenitor cells in the presence of M-CSF and RANKL was as efficiently prevented by Dex as in wild-type controls in terms of TRAP MNCs capable of resorbing bone surfaces (Figures 5D–5F). Therefore, repression of osteoclastogenesis by GCs occurs independently of GR dimerization in both osteoblasts and osteoclasts. Suppression of osteoblast differentiation, however, is cell autonomous and is not influenced by the GR in osteoclasts. In cocultures, ALP-positive osteoblasts were reduced only when the GR was present in osteoblasts but were independent of its presence in osteoclasts (Figure 5C).

GCs Inhibit Osteoblastogenesis in the Absence of NF-κB Activity

The dimerization-defective receptor in GR^{dim} mice is strongly impaired in direct DNA binding but is still able to suppress osteoblast differentiation. Thus, a GR dimer-independent tethering mechanism with other transcription factors, such as NF-κB, could be crucial in this process. To test this hypothesis, we employed primary osteoblasts expressing a nondegradable N-terminal truncated IκBα ($dN^{Runx2Cre}$). By preventing its phosphorylation by IκB kinases, this molecule acts as a dominant suppressor of p65/p50 activation (Schmidt-Ullrich et al., 2001), whereas GR activity is not affected. Indeed, NF-κB activity was strongly reduced, as indicated by the impaired nuclear translocation of NF-κB p65 after tumor necrosis factor (TNF)α treatment (Figure 6A). Independent of diminished NF-κB activity, osteoblast apoptosis was normal after Dex treatment (Figure 6B), and their differentiation was still efficiently inhibited by Dex based on reduced ALP activity and lower *Col1a1* and *Runx2* expression (Figures 6C–6F). Thus, it is unlikely that GCs inhibit bone formation via interference of the GR monomer with NF-κB activity.

Tethering of GR to AP-1 Sites Inhibits *Il11* Expression, Leading to Suppression of Osteoblast Differentiation

To determine the molecular mechanism used by the monomeric GR to suppress osteoblast differentiation, we performed quantitative PCR to identify genes potentially involved in this process. First, we confirmed lack of induction of known GRE-dependent genes by Dex in GR^{dim} osteoblasts, namely the GC-induced

(D) Histomorphometry of osteoblast number/bone perimeter (NOb/BPm), osteocyte number/bone area (NOy/BAR), and osteoclast number/bone perimeter (NOc/BPm).

(E and F) Fluorescent micrographs of (E) dual calcein labeling and (F) its quantitative analysis of bone formation rate/bone surface (BFR/BS).

(G and H) Number of (G) CFU OBs within the bone marrow/leg (femur and tibia) and (H) qRT-PCR-determined *Col1a1* mRNA expression in long bones of mice treated with placebo or prednisolone for 3 days.

(I and J) Fluorescent micrographs of (I) dual calcein labeling and (J) its quantitative analysis of BFR/BS in tibiae of $GR^{LysMcre}$ and corresponding GR^{flox} control mice.

Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 5–6.

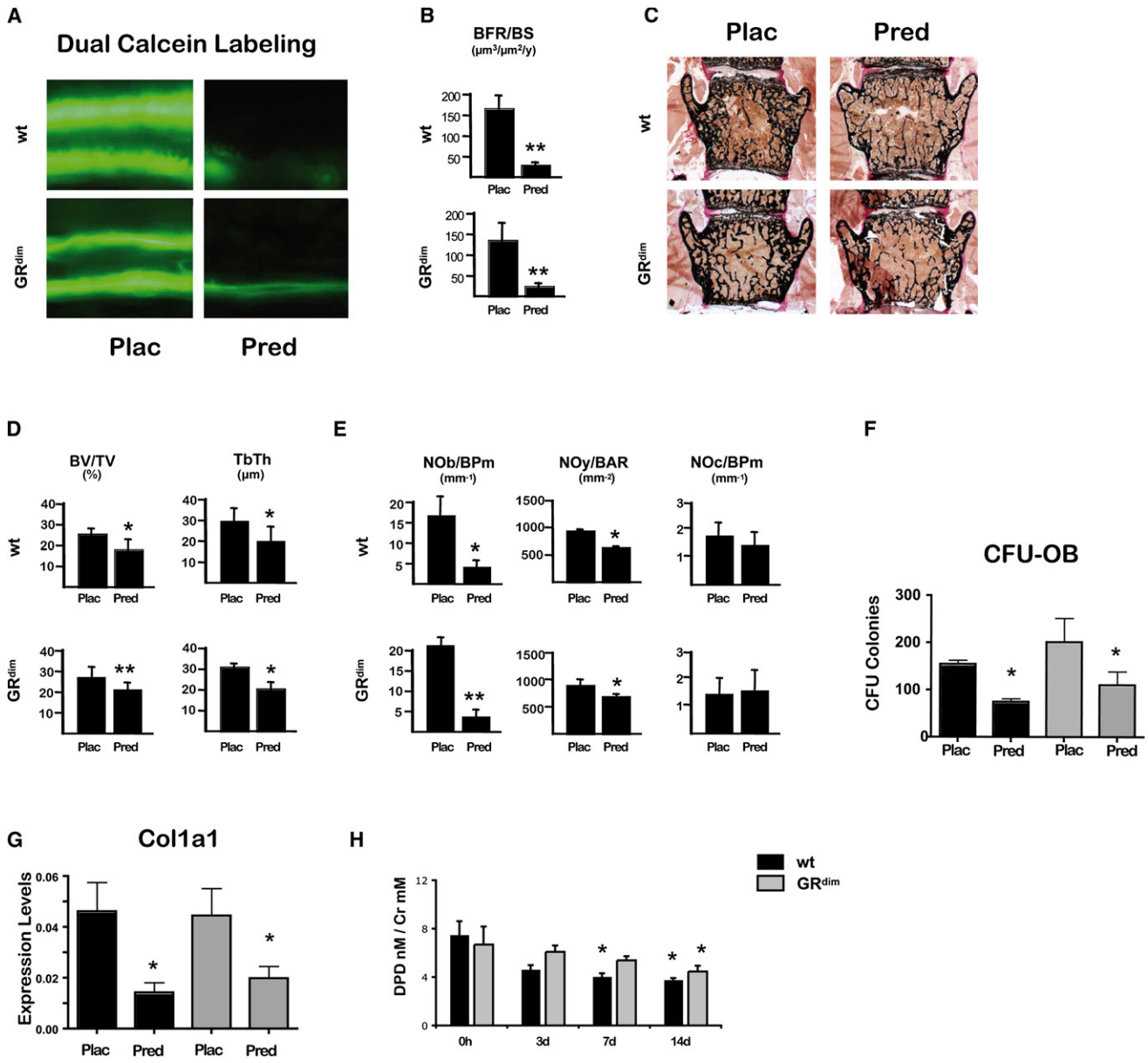


Figure 3. GR^{dim} Mice Respond to Prednisolone with Suppression of Bone Formation

(A and B) Fluorescent micrographs of (A) dual calcein labeling and (B) its quantitative analysis of BFR/BS in vertebrae of GR^{dim} and wild-type (wt) mice treated as described in Figure 2.

(C) Vertebral bodies were analyzed by von Kossa staining of undecalcified sections.

(D and E) Histomorphometry of (D) BV/TV, TbTh, (E) NOb/BPm, NOy/BAR, and NOc/BPm.

(F and G) Number of (F) CFU OBs within the bone marrow/leg and (G) qRT-PCR-determined *Col1a1* mRNA expression in long bones of mice treated with placebo or prednisolone for 3 days.

(H) DPD/Cr levels in urine of mice after prednisolone treatment at indicated times.

Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 5–6.

leucine zipper (*Tsc22d3*, *GILZ*), the circadian rhythm gene *Per1*, and the recently identified GR target gene *Klf15* (Yoshikawa et al., 2009) in GR^{dim} osteoblasts (Figures 7A–7C). In contrast, several growth factors and cytokines were similarly downregulated in both wild-type and GR^{dim} osteoblasts. Among them were the nerve growth factor 2 (*Ngf2*), the heparin binding-epidermal growth factor (*Hbegf*), interleukin-6 (*Il6*), the leukemia inhibitory factor (*Lif*), and interleukin-11 (*Il11*), which were downregulated by GCs in both wild-type and GR^{dim} osteoblasts (Figures S5A–S5C, 7D, and 7E).

Because defective IL-11 signaling causes reduced bone formation in mice (Sims et al., 2005), we analyzed GC regulation of *Il11* in embryonic bone organ cultures (detection of IL-11 in adult bones was difficult due to the small fraction of osteoblasts

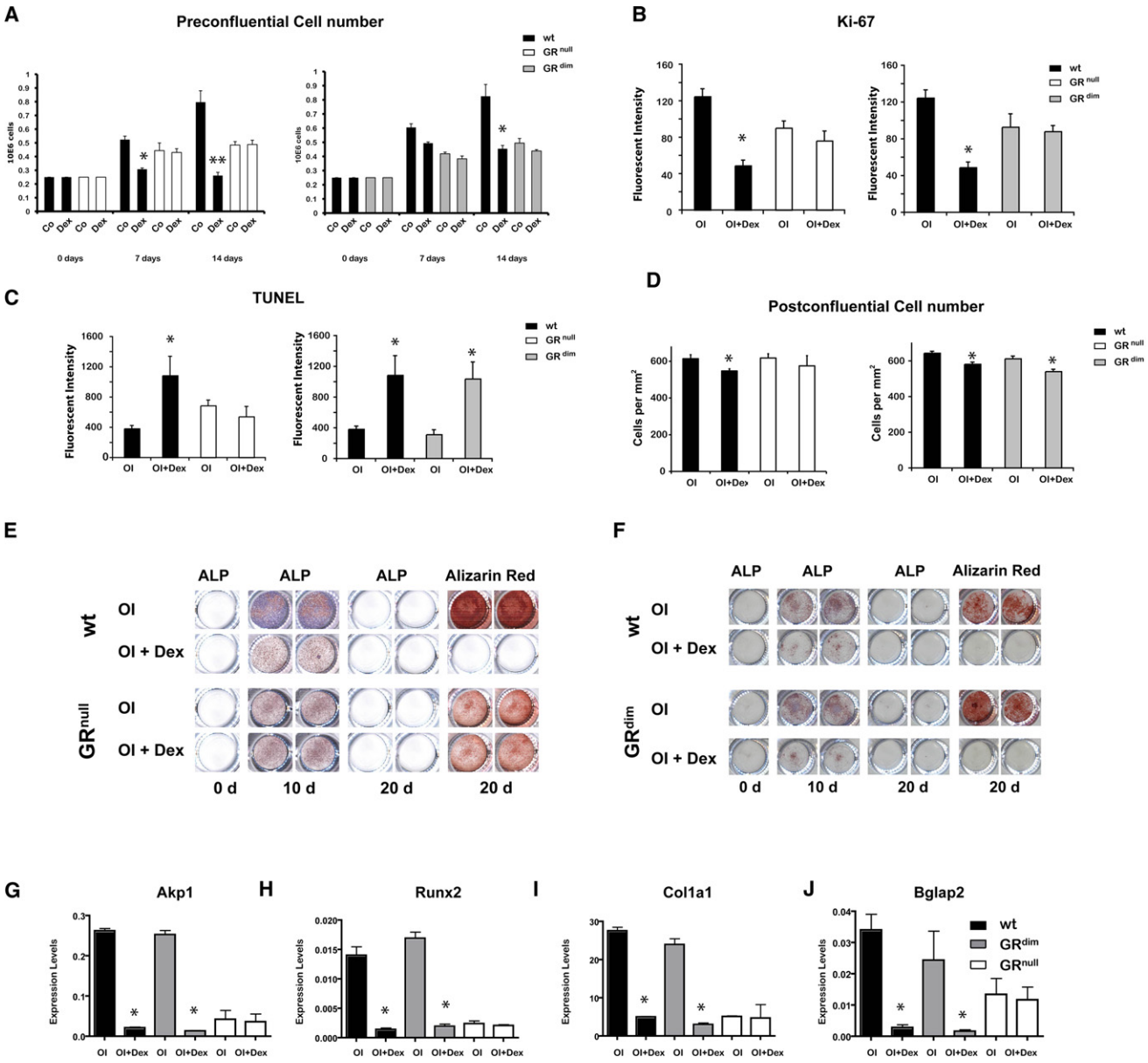


Figure 4. Dimerization of the GR Is Required for Inhibition of Osteoblast Proliferation, but Not for Induction of Apoptosis and Suppression of Differentiation

(A) Primary mouse osteoblasts were treated without (Co) or with 1 μM Dex (Dex), and cell numbers were assessed at indicated days.

(B–D) Primary osteoblasts were grown until confluence and were induced with osteogenic differentiation medium (OI) or in addition treated with 1 μM Dex (OI+Dex). At day 3, (B) Ki-67 content and (C) TUNEL labeling were analyzed, whereas (D) cell numbers were counted at day 10 using automated microscopy.

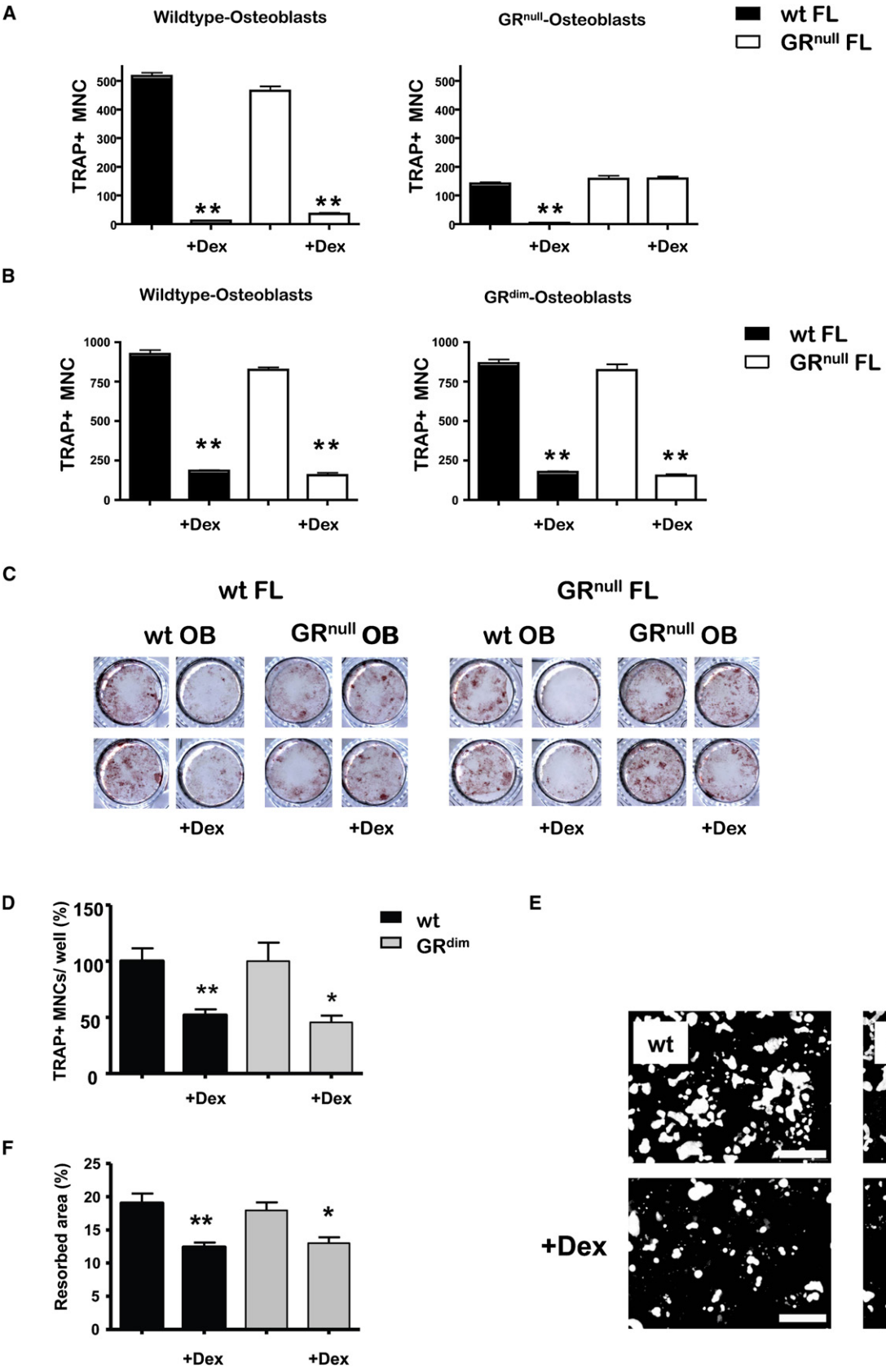
(E and F) Primary osteoblasts after osteogenic induction, with or without Dex, were stained for ALP activity and calcification with alizarin red at indicated days.

(G–J) qRT-PCR-determined osteoblast mRNA expression levels of *Akp1*(ALP), *Runx2*, *Col1a1* (10 days of differentiation), and *Bglap2* (20 days).

Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 3.

in the entire bone). Dex suppressed *Il11* expression in wild-type and GR^{dim} bones, but not in those from $GR^{Runx2Cre}$ mice (Figure 7F). Thus, *Il11* expression in the entire bone is similarly regulated by GCs as in isolated osteoblasts.

Suppression of *Il11* in cells and long bones from GR^{dim} mice points toward regulation by a tethering mechanism of the GR, e.g., binding to NF-κB or AP-1. Because Dex efficiently reduced *Il11* mRNA levels in osteoblasts that display strongly impaired NF-κB activity (Figure 7G), NF-κB is neither involved in *Il11* expression nor a target for suppression by the GR. In contrast, *Il11* expression under osteogenic conditions was strongly impaired in mouse embryonic fibroblasts (MEFs) lacking c-Jun/AP-1 in comparison to wild-type MEFs (Figure 7H). Furthermore, Dex diminished *Il11* mRNA levels in wild-type, but not in c-Jun knockout MEFs (Figure 7H), suggesting that c-Jun as a major component of the AP-1 complex drives *Il11* transcription


A
Wildtype-Osteoblasts
GRnull-Osteoblasts
wt FL
GRnull FL
TRAP+ MNC
+Dex
B
Wildtype-Osteoblasts
GRdim-Osteoblasts
wt FL
GRnull FL
TRAP+ MNC
+Dex
C
wt FL
GRnull FL
wt OB
GRnull OB
+Dex
D
wt
GRdim
TRAP+ MNCs/ well (%)
+Dex
E
wt
GRdim
+Dex
F
Resorbed area (%)
+Dex

under osteogenic conditions. To corroborate this finding, we performed chromatin immunoprecipitation of wild-type and GR^{dim} MEFs, in which *Il11* is repressed in a GR dimerization-independent manner (Figure S5D). A DNA element of the *Il11* promoter containing two adjacent functional AP-1-binding sites (Tohjima et al., 2003) was amplified from wild-type and GR^{dim} MEFs, confirming that the monomeric GR indeed interacts with the AP-1 site on the *Il11* promoter (Figure 7I). Thus, tethering of the GR monomer to AP-1 forms the basis of GC-mediated inhibition of *Il11*.

To investigate whether inhibition of cytokine and growth factor expression underlies the Dex-induced reduction of osteoblast number and differentiation, we ectopically added the previously identified factors to osteoblasts during osteogenic induction in the presence or absence of Dex. NGF2 and IL-6 rescued neither the reduced cell numbers nor the impaired differentiation (Figures S6A, S6B, S7A, and S7B). HB-EGF signaling evoked by epidermal growth factor in osteoblasts (Chien et al., 2000) counteracted the reduction in cell number caused by Dex (Figure S6C) but did not prevent the inhibition of differentiation (Figure S7C). In contrast, both LIF and IL-11, despite not rescuing the inhibitory effect of Dex on osteoblast cell counts (Figures S6D and S6E), potently antagonized the GC-mediated suppression of differentiation (Figures S7D, 7J, and 7K). In particular, Dex suppression of ALP activity, of *Runx2* expression, and of calcification was fully restored in the presence of IL-11 (Figures 7K and 7L). We conclude that LIF and, particularly, IL-11 are crucial factors in GIO.

DISCUSSION

GIO is a major side effect of GC therapy, but despite being aware of this problem for decades, the role of the GR in bone development, differentiation, and homeostasis has not been fully resolved. Using conditional and function-selective GR knockout mice, we establish an anabolic role of the GR in osteoblasts under physiological conditions and a catabolic function of the GR monomer at pharmacological exposure to GCs.

Anabolic Role of the GR in Bone Integrity

GR^{null} embryos did not reveal any major function of the GR in bone growth and architecture during development. Adult mice lacking the GR in osteoblasts also showed overall normal bone growth and geometry, with the exception of a slight increase in rump length in males. We cannot rule out that this is due to the minor recombination observed in the cartilaginous growth plates, which will be clarified with cartilage-specific GR mutant mice. In contrast, bone mass and trabecular number were lower in both female and male $GR^{Runx2Cre}$ mice. This is in line with the decreased bone mass observed in adrenalectomized rats (Durbridge et al., 1990) and in *Hsd11b2*-overexpressing mice in which GC activity is specifically disrupted in osteoblasts (Sher et al., 2004, 2006). In agreement with previously described stimulatory effects of GCs on osteoblastic cells (Ishida and Heersche, 1998; Shalhoub et al., 1992), we found reduced proliferation and a diminished differentiation capacity of cultured GR^{null} osteoblasts. GR^{dim} mice exhibited normal bone mass and trabecular numbers under physiological conditions. Given that GR^{dim} osteoblasts showed normal differentiation despite impaired proliferation, we conclude that effects on the former are more crucial for anabolic GC effects on bone.

GCs Inhibit Bone Formation via the GR in Osteoblasts

Our data support a model in which the GR in osteoblasts is essential for GIO because prednisolone failed to reduce the bone formation rate, bone mass, and trabecular thickness in $GR^{Runx2Cre}$ mice. In addition, GC-mediated suppression of *Col1a1* required the presence of the GR in osteoblasts. The requirement of the GR also clearly shows that the prednisolone dose used here, which was established to be effective in mice with the FVB/N background strain, displays no unspecific toxic effects. Osteoclast numbers were hardly affected by prednisolone, whereas osteoblast and osteocyte counts were diminished in wild-type, but not in $GR^{Runx2Cre}$, mice. In contrast to other studies that detected a marginal increase (Weinstein et al., 1998), we did not detect additional apoptosis of osteoblasts and osteocytes by prednisolone treatment in vivo. This discrepancy may be due to different mouse strains. Thus, apoptosis of mature osteoblasts would not explain the strong decrease in osteoblast numbers. This effect, rather, becomes effective at the osteoblast progenitor level because CFU OBs were already reduced in wild-type animals after 3 days of treatment. The observed reduction of osteocytes by ~20%–35%, in combination with no observable changes in empty lacunae (data not shown), also argues against an enhanced apoptosis and can only be explained by an absence of bone formation, allowing the removal of osteocyte-containing bone by still ongoing bone resorption.

In contrast to $GR^{Runx2Cre}$ mice, we found a similar reduction of bone mass, trabecular thickness, bone formation, osteoblast numbers, and CFU OBs by GCs in wild-type and GR^{dim} mice. These results clearly show that the GR in osteoblasts, but not its dimerization function, is required for inhibition of bone formation and, consequently, bone loss.

Figure 5. Osteoclastogenesis Is Suppressed by GCs by the GR in Osteoblasts and Osteoclasts, Independent of GR Dimerization, whereas Osteoblastogenesis Depends Solely on the Osteoblast GR

(A and B) (A) Wild-type and GR^{null} osteoblasts and (B) wild-type and GR^{dim} osteoblasts were cultured with fetal liver cells (FL) derived from wild-type or GR^{null} mice in the presence of vitamin D3, with or without Dex. After 7 days of culture, wells were stained with TRAP, and TRAP MNCs were counted.

(C) Wild-type and GR^{null} osteoblasts (OB) were cultured with fetal liver cells derived from wild-type or GR^{null} mice as in (A). ALP staining was performed after 10 days of coculture under osteogenic conditions with or without Dex.

(D) Wild-type- and GR^{dim}-derived bone marrow-derived macrophages (BMDMs) were cultured with M-CSF (50 ng/ml) and RANKL (30 ng/ml) in the absence or presence of Dex. TRAP MNCs were determined after 5 days.

(E and F) Wild-type and GR^{dim} BMDMs were cultured with M-CSF and RANKL in the presence or absence of Dex in osteological chambers coated with calcium phosphate to determine resorption area after 5 days of culture.

Data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. n = 4. Scale bar, 400 μm.

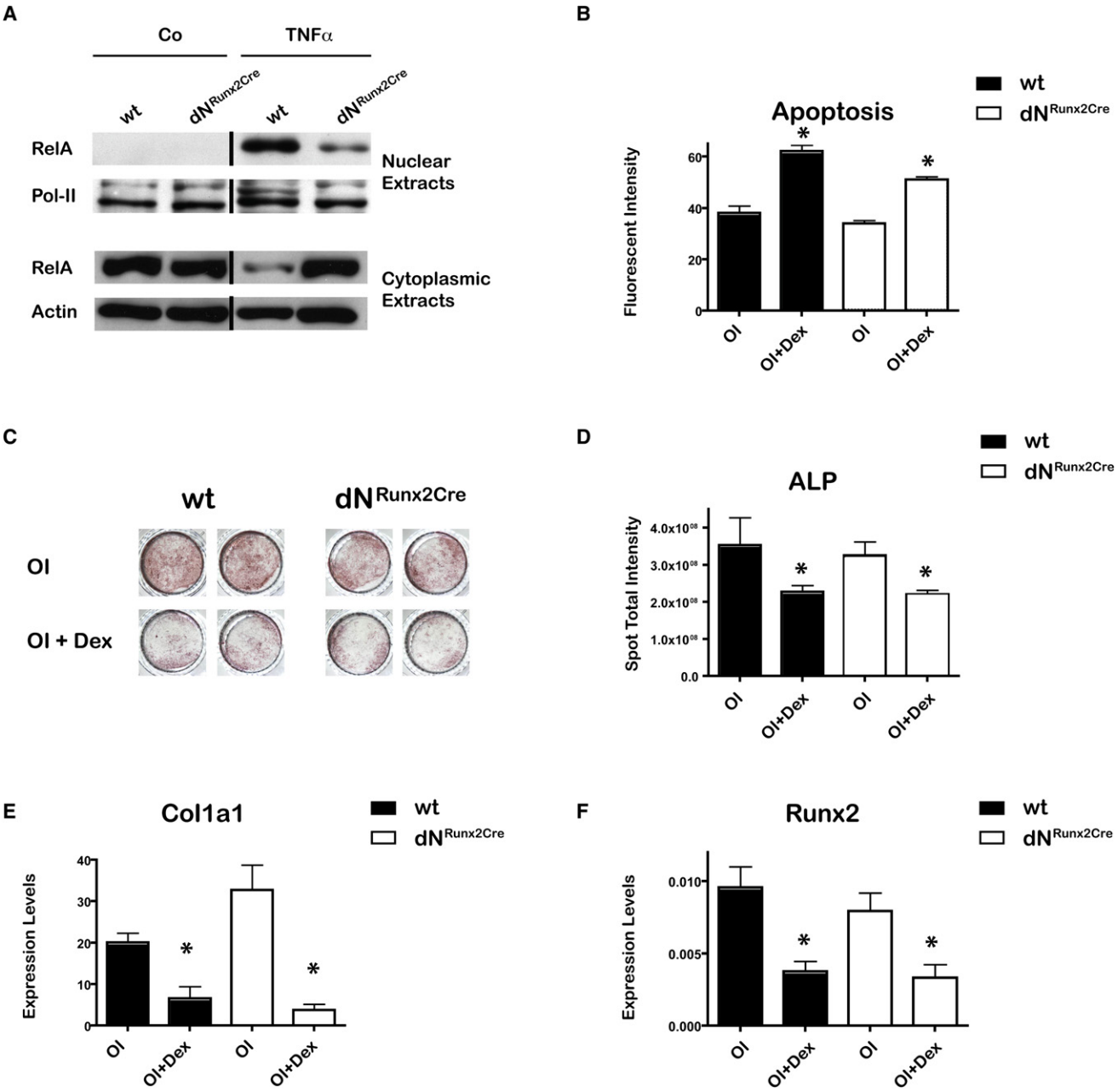


Figure 6. Lack of NF-κB Activity Does Not Change the Response of Osteoblasts Toward GCs

(A) Wild-type and dN^{Runx2Cre} osteoblasts were treated with TNFα for 30 min. Nuclear and cytoplasmic extracts were analyzed by immunoblotting for p65, actin, and polymerase II.

(B–D) Primary osteoblasts were grown until confluence and treated as described in Figure 4A. (B) TUNEL labeling was performed after 3 days. After 10 days, ALP staining (C) and quantitative ALP activity (D) were examined.

(E and F) qRT-PCR-determined osteoblast mRNA expression levels of *Col1a1* and *Runx2* (10 days of differentiation).

Data represent mean ± SEM. *p < 0.05. n = 3.

The Role of the GR in Osteoclasts

Cell-autonomous effects of the GR in osteoclasts can lead to GC-enhanced bone resorption (Jia et al., 2006; Weinstein et al., 2002; Yao et al., 2008). We found a slightly decreased resorptive activity during the entire 14 days of GC treatment. Nevertheless, in the absence of de novo bone formation, the residual resorptive activity after prednisolone administration was apparently sufficient to lead to GC-induced bone loss in wild-type and GR^{dim} mice. This explains why interfering with bone resorption using bisphosphonates (Weinstein et al., 2002) or inhibitory anti-RANKL antibodies (Hofbauer et al., 2009) ameliorates bone loss in GIO models.

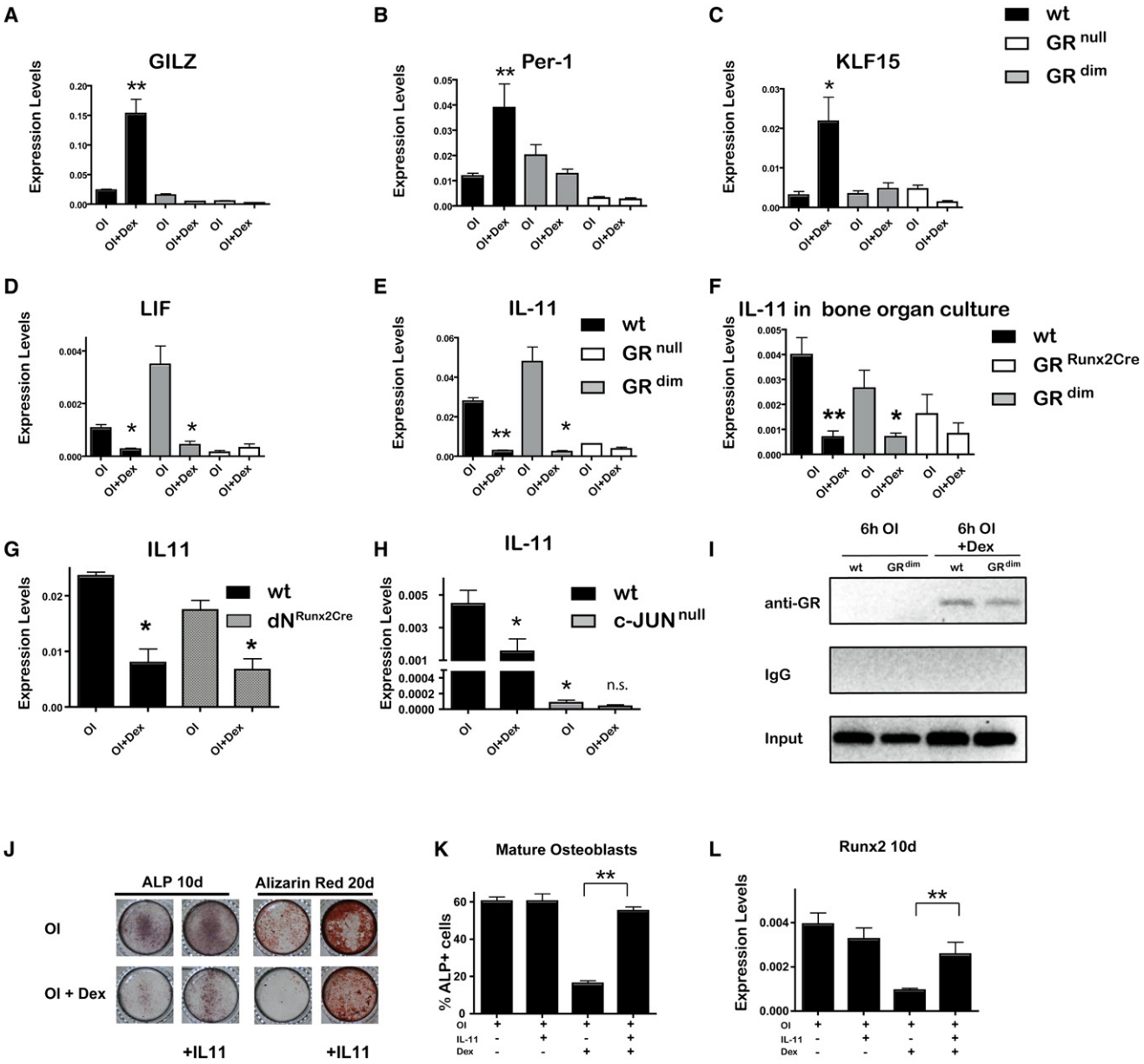


Figure 7. Differential Gene Regulation in GR^{dim} Osteoblasts and Bone Organs by GCs and Involvement of AP-1 in Suppression of IL-11 in Osteoblast Differentiation

(A–E) Primary osteoblasts were grown until confluence and treated as described in Figure 4A. qRT-PCR-determined mRNA expression levels of *Tsc22d3*, *Per1*, *Klf15*, *Lif*, and *Il11* after 6 hr.

(F–H) qRT-PCR-determined mRNA expression level of *Il11* in organ cultures of GR mutant fetal hind legs (F), in dN^{Runx2Cre} osteoblasts (G), and in c-*jun*^{null} MEFs (H) after 6 hr treatment.

(I) Chromatin immunoprecipitation of MEFs after 6 hr. Anti-GR precipitation revealed PCR-amplified fragments of the *Il11* promoter encompassing functional AP-1 sites. IgG precipitation served as negative control and amplification of the input as positive control.

(J) Primary wild-type osteoblasts were differentiated with or without Dex and in the presence or absence of recombinant IL-11 10 ng/ml. At day 10, ALP activity was determined, and at day 20, alizarin red staining was performed.

(K) Quantification of ALP-positive cells treated as in (J) after 10 days.

(L) qRT-PCR-determined *Runx2* mRNA expression level in cells treated as in (J) after 10 days.

Data represent mean ± SEM. *p < 0.05, **p < 0.01. n = 3.

In contrast to unaffected osteoclast numbers in vivo, pharmacological doses of GCs repressed osteoclastogenesis via the GR in osteoblasts and osteoclasts in vitro. The latter observation is in line with a report by Kim et al. (2006) showing that Dex inhibits osteoclast formation and spreading in vitro by a mechanism dependent on the GR in osteoclasts. The absence of changes

of osteoclast numbers in vivo might be explained by a slow turnover of bone-residing osteoclasts, thus concealing the inhibitory effect observed in the coculture experiments. Neither the direct inhibitory effects of GCs on isolated osteoclasts nor the indirect effects on osteoclastogenesis mediated by osteoblasts require GR dimerization.

Osteoclastogenesis proceeded normally in GR-deficient osteoclast progenitor cells. Likewise, we did not observe any differences in bone mass and trabecular thickness under physiological conditions in $GR^{LysMCre}$ mice lacking the GR in osteoclasts (data not shown). However, bone formation in $GR^{LysMCre}$ mice was similarly reduced upon GC treatment as in wild-type animals. This is in striking contrast to a previous report using the same animal model (Kim et al., 2006) but is in line with a study using *TRAP-Hsd11b2* transgenic mice in the sense that GCs reduce osteoblast numbers and bone formation even when GC actions in osteoclasts cannot occur (Jia et al., 2006). However, an important role of the GR in osteoclasts for the increased bone resorption observed in humans and some mouse strains, such as Swiss Webster mice, cannot be excluded.

Inhibition of Bone Formation and Osteoblastogenesis Is Independent of GR Dimerization

We have described complete suppression of osteoblast differentiation in vitro and GC-induced bone loss in vivo in the absence of GR-DNA binding. Osteoblast differentiation in GR^{dim} calvarial osteoblasts was as efficiently inhibited by GCs as in wild-type cells; however, only a low level of apoptosis was induced by GCs in wild-type and GR^{dim} osteoblasts in vitro. More strikingly, we failed to detect enhanced apoptosis in vivo at several time points, nor did we detect a change of empty lacunae, an indication of apoptotic loss of osteocytes (data not shown). These findings argue against an important role of cell death in GIO, as previously proposed (Weinstein et al., 1998).

Proliferation of GR^{dim} osteoblasts could not be inhibited by GCs either before or after confluence. In view of the previously reported role of proliferation for subsequent osteoblastic differentiation of MC-3T3 cells (Smith et al., 2000), the uncoupling of proliferation and differentiation in GR^{dim} osteoblasts is surprising. Because osteoblast numbers, osteoblastogenesis, *Col1a1* expression, and bone formation were reduced by GCs in GR^{dim} mice, we propose that inhibition of osteoblastogenesis, rather than inhibition of cell proliferation accounts for these effects.

Repression of AP-1-Dependent IL-11 Inhibits Osteoblastogenesis

Activation of bona fide GRE-dependent genes, such as *Tsc22d3*, *Per1*, and *Klf15* (Balsalobre et al., 2000; Rogatsky et al., 2003; Yoshikawa et al., 2009), was strongly impaired in GR^{dim} osteoblasts, whereas repression of several genes encoding growth factors and cytokines was not affected. Because inhibition of osteoblastogenesis by GCs was similar in wild-type and in GR^{dim} mice, we hypothesized that this was most likely achieved through repression of soluble factors via the monomeric GR. In line with this, we found that *Hbegf*, *Lif*, and *Il11* were inhibited by GCs independently of GR dimerization. In agreement to the observation that HB-EGF inhibits differentiation of mesenchymal stem cells (Krampera et al., 2005), it also exerted an inhibitory effect on osteoblast differentiation. Thus, HB-EGF is unlikely to play a role in GIO. In contrast, LIF and IL-11 counteracted suppression of osteoblast differentiation by Dex in culture. Although *Lif* is a target gene of AP-1 (Bozec et al., 2008) and induces aberrant bone growth in mice (Metcalf and Gearing, 1989), we focused on *Il11* because, in contrast to *Lif*, it was induced under osteogenic conditions in osteoblasts (data not shown). In entire embryonic bones, suppression of *Il11* by GCs requires the GR in osteoblasts, but not its dimerization. This points toward an interaction of the GR monomer with other transcription factors. Our analyses indicate that interaction with NF-κB is not essential for GIO because primary osteoblasts from $dN^{Runx2Cre}$ mice that are impaired in p65 nuclear translocation and thus NF-κB activity responded normally to GCs in terms of diminished cell numbers, induction of apoptosis, inhibition of differentiation, and suppression of *Il11*. Indeed, inhibition of NF-κB leads to enhanced osteoblast differentiation and bone mass instead of diminished osteoblast function (Chang et al., 2009). However, interaction of the monomeric GR with AP-1 appears to be crucial for inhibition of osteoblast differentiation by GCs. Unlike in wild-type cells, *Il11* was hardly induced in cells lacking c-Jun, and the residual expression was unaltered by Dex. Consistently, we demonstrated recruitment of the wild-type and the GR^{dim} receptor to two adjacent AP-1 sites in the *Il11* promoter (Tohjima et al., 2003). Finally, IL-11 was able to rescue Dex-mediated inhibition of osteoblast differentiation. In line with our results, IL-11 enhances bone formation when overexpressed in mice (Takeuchi et al., 2002), and targeted deletion of the IL-11 receptor alpha leads to decreased bone formation (Sims et al., 2005). Given that the AP-1 target genes *Il11* and *Lif* are able to rescue Dex-mediated inhibition of osteoblast differentiation, a tethering mechanism involving the monomeric GR and AP-1 presumably underlies repression of cytokines and growth factors during GIO.

Conclusions

We show in this study that the capacity of the monomeric GR to inhibit AP-1-controlled cytokines, such as IL-11 or LIF, is central to the suppression of osteoblast differentiation and that inhibition of bone formation by GCs is independent of GR dimerization. Consequently, tethering of the GR to AP-1 not only contributes to the anti-inflammatory effects of GCs, but is also essential for provoking GIO. In contrast, repression of NF-κB-dependent genes does not seem to be involved in osteoblast differentiation, whereas it plays a major role in the anti-inflammatory activities of GCs.

Therefore, our study proposes that selective GR agonists, which would fail to suppress AP-1-dependent gene expression but are still capable of inhibiting NF-κB-dependent gene regulation, could be beneficial in anti-inflammatory activities while not concomitantly inducing GIO.

EXPERIMENTAL PROCEDURES

Generation of Runx2Cre Mice

A 150 kb BAC covering the *Runx2* locus (Genome System BAC Library, RZPD, Germany) was modified by inserting a codon-improved Cre recombinase at the translational start site of the bone-specific distal promoter (P1) of the *Runx2* gene (Stock and Otto, 2005) by ET recombination, as previously described (Casanova et al., 2001). The modified BAC was microinjected into

the pronucleus of oocytes from FVB/N mice, and three different founder lines were established. One line was chosen for crossing with GR^{flox} mice based on its expression pattern.

Animal Experimentation

All animal experiments were performed in accordance with accepted standards of animal welfare and with permission of the responsible authorities of Baden-Württemberg and Thüringen. GR^{dim} (Reichardt et al., 1998) and GR^{flox} (Tronche et al., 1998) were backcrossed to the FVB/N background for at least four generations. dN^{flox} (Schmidt-Ullrich et al., 2001) and GR^{null} (Tronche et al., 1998) were on a miced background and 129/SvJ background, respectively. Cre activity was monitored using Rosa26 reporter mice by whole-mount β-galactosidase staining (Soriano, 1999). $GR^{LysMCre}$ mice (Tuckermann et al., 2007) were backcrossed to Balb/c for five generations. To induce osteoporosis, prednisolone (or a placebo control) was applied for 14 days by subcutaneous implantation of slow-release pellets, resulting in a calculated dose of 12.5 mg/kg/d (15 mg; 60 day release; Innovative Research of America, Inc.) in 10-week-old female mice.

Cell Culture and qRT-PCR Analysis

Primary osteoblasts were isolated from either embryonic (E18.5) or neonatal calvaria by sequential digestions and cultivated as previously described (David et al., 2005). c-Jun-deficient cells were obtained from previously described mice (Schreiber et al., 1999). Cells were passaged once before induction of osteogenic differentiation by 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate in the presence or absence of 1 μM Dex (Sigma). Differentiation of osteoblasts was determined by staining for ALP activity (ALP kit, Sigma or ELF Phosphatase Detection Kit, ATCC) or by alizarin red staining (Sigma) at indicated time points. Apoptosis was detected by In Situ Cell Death Detection Kit (Roche) and proliferation with fluorescent immunohistochemistry using mouse anti-Ki-67 (BD PharMingen) and Alexa Fluor 594-labeled donkey anti-mouse IgG (Molecular Probes). Quantification of the stainings and cell numbers according to DAPI labeling was performed by automated microscopy using Cellomics ArrayScan V^{TI} and software HCS Scan (Thermo-Fischer).

The factors EGF (Calbiochem), NGF2 (Sigma), IL-6 (ImmunoTools), IL-11 (Antigenix America, Inc.), and LIF (Chemicon) were added to the culture after differentiation at concentrations of 0.8, 4, and 20 ng/ml. qRT-PCR was performed as previously described (Tuckermann et al., 2007). Primer information can be provided upon request. Statistical differences between the groups of cultures (n = 3–6) were assessed by the Student's t test.

CFU OBs

CFU OBs per leg were obtained by counting alizarin red-positive colonies of 2×10^6 bone marrow cells (excluding erythrocytes) plated per 6-well after 20 days under osteogenic conditions.

Coculture Experiments

Cocultures were established as previously described (David et al., 2005). In brief, 0.5×10^6 fetal liver cells isolated from wild-type or GR^{null} embryos were placed on top of wild-type, GR^{dim}, or GR^{null} osteoblasts in the presence of 10 nM colecalciferol with or without 1 μM Dex per 24-well. Primary osteoclasts were derived from bone marrow of wild-type and GR^{dim} mice in the presence of 50 ng/ml recombinant murine M-CSF and 30 ng/ml recombinant murine RANKL (R&D Systems). TRAP-positive cells were stained after 7 days with the leukocyte acid phosphatase kit (Sigma) and counted per 24-well. Osteoclast apoptosis was measured with ELISA (Cell Death Detection ELISA, Roche). Resorption was assessed by seeding bone marrow cells on osteocyte chambers in the presence of RANKL and M-CSF, with or without Dex, and determination of resorption pits by von Kossa staining followed by microscopy.

Immunoblotting

Primary $dN^{Runx2Cre}$ osteoblasts were treated with TNFα (Sigma) for 30 min. Immunoblotting was performed with the nuclear (25 μg) and cytoplasmic (40 μg) protein fractions using rabbit anti-RelA, rabbit anti-PolII, and goat anti-β-actin (Santa Cruz).

Radiographic Analysis and Alizarin Red/Alcian Blue Staining

Whole skeletons were analyzed by contact radiography using a Faxitron X-ray cabinet (Faxitron X-ray Corp., Wheeling, IL). For differential staining of bone, E17.5 embryos were prepared according to the method of C. Arnott (Kaufmann, 2003).

Histomorphometry

Parameters of static and dynamic histomorphometry were quantified on toluidine blue-stained undecalcified proximal tibia and lumbar vertebral sections, as described previously (Amling et al., 1999), using the Osteo-histomorphometry system (Osteometrics, Atlanta, GA) (Parfitt et al., 1987). Osteocytes were assessed at the cortical bone by OsteoMeasure software. To assess dynamic histomorphometric indices, mice were given two injections of calcein, 9 and 2 days before dissection. Fluorochrome measurements for the determination of the bone formation rate were performed on two nonconsecutive 12 μm thick sections for each animal. Statistical differences between the groups (n = 5–6) were assessed by the Student's t test.

Osteoblast Proliferation and Apoptosis In Vivo

Mice were treated with prednisolone (12.5 mg/kg/d) for 1 day, and they got pulsed with 10 μg/g body weight BrdU 4 hr before dissection. Decalcified deparaffined femur sections were stained with the In Situ Cell Death Detection Kit (Roche) for apoptosis and rat anti-BrdU (Abcam) following peroxidase-coupled anti-rat IgG for proliferation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at doi:10.1016/j.cmet.2010.05.005.

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