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Opposing Effects by Glucocorticoid and Bone Morphogenetic Protein-2 in Fetal Rat Bone Cell Cultures

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Abstract Glucocorticoid in excess produces bone loss in vivo. Consistent with this, it reduces the stimulatory effect of transforming growth factor β (TGF-β) on collagen synthesis in osteoblast-enriched cultures in vitro, where it also suppresses TGF-β binding to its type I receptors. Analogous studies with bone morphogenetic protein-2 (BMP-2) show directly opposite results. These findings prompted us to assess the effect of glucocorticoid on BMP-2 activity in cultured bone cells, and whether either agent had a dominant influence on TGF-β binding or function. BMP-2 activity was retained in part in osteoblast-enriched cultures pre-treated or co-treated with cortisol, and was fully evident when glucocorticoid exposure followed BMP-2 treatment. In addition, BMP-2 suppressed the effects of cortisol on TGF-β activity, on TGF-β binding, and on gene promoter activity directed by a glucocorticoid sensitive transfection construct. While BMP-2 also alters the function of less-differentiated bone cells, it only minimally prevented cortisol activity in these cultures. Our studies indicate that BMP-2 can oppose certain effects by cortisol on differentiated osteoblasts, and may reveal useful ways to diminish glucocorticoid-dependent bone wasting. J. Cell. Biochem. 67:528–540, 1997.

Key words: transforming growth factor β (TGF-β); collagen; receptors; gene promoters; osteogenesis; metabolic bone diseases

Bone loss can result from an imbalance in the normally symmetrical bone remodeling sequence of osteoclastic resorption and osteoblastic bone formation [Martin et al., 1989; Baron, 1996]. Because bone mass invariably decreases with pathologic or pharmacologic glucocorticoid excess, many studies have focused on ways by which this steroid affects bone metabolism. Glucocorticoid alters the activity of peripheral tissues that transport, produce, or modify molecules that affect bone formation, but it also has direct effects on bone cells. Physiological levels or transient exposure to cortisol enhance some aspects of osteoblast differentiation in bone-derived cell and organ cultures [Dietrich et al., 1978; Bellows et al., 1987; Lian and Stein, 1993; Kream et al., 1997; among others]. However, other variations in dose, duration, and route of glucocorticoid administration determine whether its effects are stimulatory or inhibitory in vivo and in vitro. In this regard, chronic exposure to high levels of glucocorticoid produces clear decreases in osteoblast activity and increases in osteoclast function. The largest share of bone loss that accompanies glucocorticoid excess occurs in a short period of time, and in situations and at tissue sites with high bone turnover rates [Bockman and Weinerman, 1990; Lukert and Raisz, 1990; Adler and Rosen, 1994; Kerstjens et al., 1994; Goans et al., 1995; Briner et al., 1995; Lukert, 1996].

Bone growth and remodeling are also controlled by local (bone-derived) factors, which have only been identified and well characterized in the last dozen years [Centrella et al., 1995a; Canalis, 1995]. Initial work with local growth factors described their individual effects on bone cell activity, but more recent efforts reveal interactions between local and systemic agents [Pfeilschifter and Mundy, 1987;

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Petkovitch et al., 1987; Centrella et al., 1988, 1989, 1991; Komm et al., 1988; McCarthy et al., 1989, 1990; Marusic et al., 1991; Chen et al., 1991; Lian and Stein, 1993]. Regarding glucocorticoid, changes in osteoblast function may in part derive from its ability to alter local growth factor synthesis, activation, binding, or signal transduction [McCarthy et al., 1990; Centrella et al., 1991; Chen et al., 1991]. Among these are effects on transforming growth factor-βs (TGF-βs), one of the more active and concentrated families of local bone regulators. Effects by TGF-βs on osteoblast activity vary with the state of bone cell differentiation, and many in vivo and in vitro studies verify their complex role in osteogenesis [Noda and Camilliere, 1989; Marcelli et al., 1990; Beck et al., 1991; Centrella, 1993; Centrella et al., 1995b; Sumner et al., 1995; and reviewed in Centrella et al., 1995a]. We found that glucocorticoid potently regulates TGF-β activity in bone cell cultures and induces a striking re-distribution in binding by TGF-β to its cell surface receptors. Specifically, exposure to high levels (100 nM or greater) of cortisol suppresses the stimulatory effect of TGF-β on osteoblast proliferation and collagen synthesis. In parallel, TGF-β binding to signal transducing TGF-β receptor I (TRI) rapidly decreases, and its association with the cell surface proteoglycan termed betaglycan is enhanced. Notably, these changes in TGF-β binding and function on osteoblasts approximate the TGF-β receptor and activity profiles that occur in less-differentiated bone cell cultures [Centrella et al., 1988, 1991, 1995b; Centrella, 1993] (also unpublished results).

In addition to TGF-βs, several members of the bone morphogenetic protein (BMP) family are secreted by bone cells and accumulate in the bone matrix. First defined functionally, BMPs promote osteogenesis at ectopic sites in vivo. Although the BMPs share extensive sequence homology with TGF-βs [Celeste et al., 1990; Rosen and Thies, 1992; Centrella et al., 1995a], they use similar, but independent cell surface receptor systems [Kingsley, 1994; Massague et al., 1994; Miyazono et al., 1994; Centrella et al., 1996a]. Most evidence suggests that BMPs can act early in the process of osteogenesis, and in many instances precede the effects of TGF-βs [Rosen and Thies, 1992; Rosen et al., 1994; Centrella et al., 1995a,b]. Our more recent studies reveal that BMPs also potently alter the TGF-β receptor profile on osteoblasts. In marked contrast to the effects of glucocorticoid, previous exposure to BMP-2 rapidly decreases the proportion of TGF-β binding to betaglycan and TGF-β receptor II (TRII), while maintaining high levels of binding to TRI. In so doing, BMP-2 alters the effects of TGF-β on proliferation, collagen synthesis, and alkaline phosphatase in patterns that are consistent with a progressive increase in osteoblast differentiation [Centrella et al., 1995b] (summarized in Table I).

Several studies are therefore consistent with the hypothesis that the TGF-β receptor profile may change with osteoblast differentiation, and may be re-designed by systemic and local agents in ways that are consistent with variations in osteoblast function. Because of the similarities between TGF-βs and BMPs and between their cell surface receptor systems, we first considered that glucocorticoids might also regulate BMP function in bone. Having previously noted the contrasting effects of glucocorticoid and BMP-2 on osteoblast activity, we also wished to determine if certain effects by these agents may converge on TGF-β in bone cells. Our current studies reveal a dominating role for BMP-2. Significantly, several glucocorticoid-induced effects are directly opposed by BMP-2. In particular, interacting effects between these agents on TGF-β activity appear to be initiated, at least in part, at the level of TGF-β receptors.

METHODS

Cell Cultures

Using procedures approved by the Yale Animal Care and Use Committee, parietal bones from 22-day-old rat fetuses (Sprague Dawley®

TABLE I. Dissimilar Effects by Glucocorticoid and BMP-2 on Bone Cell Activity*

Parameter	Glucocorticoid	BMP-2
Cell replication	Decrease	Increase
Matrix synthesis	Decrease	Increase
Alkaline phosphatase	No effect	Increase
TGF-β induced collagen	Decrease	Increase
TGF-β receptor I	Decrease	Increase
Betaglycan	Increase	Decrease

*This table summarizes several conspicuous differences between the effects of high dose or chronic exposure to glucocorticoid and BMP-2 in some in vivo or in vitro models used to examine bone cell metabolism. Changes in TGF-β activity, TGF-β receptor I, and betaglycan are derived from studies in the osteoblast-enriched fetal rat cell cultures as in the present study.

rats, Charles River Breeding Laboratories, Wilmington, MA) were dissected free of sutures and digested for five 20-min intervals with collagenase. Cells from the first digestion (population 1) are heterogeneous and appear less differentiated by several biochemical criteria, while cells from the last three digestions (osteoblast-enriched populations 3–5) exhibit characteristics associated with differentiated osteoblasts. By comparison to population 1, populations 3–5 express higher levels of type I collagen, alkaline phosphatase, PTH receptors, and sensitivity, and an increase in osteocalcin in response to $1,25(OH)_2D_3$. Histochemical staining shows that ≈80% of population 3–5 cells express alkaline phosphatase, although this itself is not entirely osteoblast-specific. However, by these criteria, by differential sensitivity to TGF-β, BMP-2, various prostaglandins, and the ability to form mineralized nodules in vitro [McCarthy et al., 1988; Centrella et al., 1988, 1994, 1995b, 1996a,b; Hughes et al., 1995] populations 3–5 are well distinguished from population 1. Moreover, these cells are only examined in primary culture to reduce the problems of phenotypic drift and aneuploidy that occur in many continuous or osteosarcoma-derived cell culture models. Primary cultures were plated at $6–9 \times 10^3$ cells/cm^2 in Dulbecco's modified Eagle's medium containing 20 mM Hepes buffer, pH 7.2, 100 µg/ml ascorbic acid, penicillin and streptomycin, and 10% fetal bovine serum. At confluence (approximately $5–6 \times 10^4$ cells/cm^2) cultures were refed identical medium lacking serum, and supplemented with various agents for the times indicated in each experiment.

Reagents

Cell culture reagents were obtained from Life Technologies (Bethesda, MD). TGF-β1 was a recombinant simian preparation identical in amino acid sequence to human TGF-β1, examined in collaboration with Bristol-Myers Squibb, Inc., Seattle, WA and exhibited binding and biological characteristics indistinguishable from our earlier studies with other native or recombinant preparations [Centrella et al., 1996b]. TGF-β1 produces dose-related stimulatory effects on collagen and noncollagen protein synthesis that are maximal in at 120 pM (3 ng/ml) in fetal rat bone-derived cell cultures [Centrella et al., 1987, 1988, 1991, 1995b]. Cortisol (hydroxycortisone) was obtained from Sigma Chemical Co. (St. Louis, MO). Earlier studies demonstrated dose-dependent inhibitory effects on TGF-β binding and activity that plateau at 100 nM cortisol [Centrella et al., 1991]. Recombinant human BMP-2 was expressed in CHO cells and purified to homogeneity in our laboratories at Genetics Institute, Cambridge, MA [Rosen et al., 1994]. Our previous studies determined its optimum effective dose at 1–3 nM in this and other in vitro cell culture models [Rosen et al., 1994; Centrella et al., 1995b, 1996a].

Protein Synthesis

Cultures (2 cm^2) were pulsed with 5 µCi/ml [^{3}H-2,3]proline (2.5 Ci/mmol; DuPont NEN, Boston, MA) for the last 2 h of culture. Cell layers were lysed by freeze-thawing, and extracted in 0.5% Triton X-100 (Sigma). Samples were precipitated with 10% trichloroacetic acid, chilled, and insoluble material was collected by centrifugation. Precipitates were acetone extracted, dried, redissolved in 0.5 N acetic acid, and neutralized with sodium hydroxide. [^{3}H]proline incorporation into collagen (collagenase-digestible protein) and noncollagen protein (all other proteins) was measured using bacterial collagenase free of nonspecific protease activity. Percent collagen synthesis was calculated from the ratio of [^{3}H]proline labeled collagen and noncollagen protein, after correcting for the 5.4 enrichment of proline in collagen [Peterkofsky and Diegelmann, 1971].

DNA Synthesis

Cultures (0.32 cm^2) were pulsed with 5 µCi/ml [^{3}H-methyl]thymidine (80 Ci/mmol; DuPont NEN) for the last 2 h of culture, lysing the cells in 0.1 M sodium dodecylsulfate/0.1 N sodium hydroxide, collecting the insoluble material formed by precipitation with 10% trichloroacetic acid, and scintillation counting [Centrella et al., 1987, 1988, 1991, 1995b].

Alkaline Phosphatase Activity

Enzyme activity was assessed in extracts from 2 cm^2 cultures, prepared by lysis in 0.5% Triton X-100 (<1% of total alkaline phosphatase is released to the medium; unpublished result). Hydrolysis of *p*-nitrophenylphosphate was measured at 410 nm after a 30-min incubation at 37°C [Lowry, 1957]. Data are expressed as pmol of *p*-nitrophenol released per minute per µg of

cell protein, determined by the method of Bradford [1976].

TGF-β Binding

TGF-β1 was radioiodinated with chloramine T to a specific activity of 4,000–4,500 Ci/mmol. Radioligand was separated from unincorporated ^{125}I through Sephadex G-50 in a solution of 0.1 M acetic acid and 4 mg/ml bovine serum albumin. Binding was examined by incubation with serum-free medium containing 4 mg/ml bovine serum albumin and 100 pM ^{125}I-TGF-β1 for 3 h at 4°C. To visualize TGF-β binding complexes, cultures were rinsed with chilled binding medium, cross-linked with 0.2 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, IL), extracted, fractionated by polyacrylamide gel electrophoresis, and examined by autoradiography [Centrella et al., 1988, 1991, 1995b]. Densitometry was assessed using a ScanMan densitometer and SigmaGel® (Jandel, San Rafael, CA).

Transfections

To examine a biochemical effect dependent on the presence of a functional TGF-β type I receptor [Carcamo et al., 1995; Wieser et al., 1995], subconfluent cultures (4.8 cm^2) were transfected with 0.5 μg p3TPLux, a plasmid encoding 3 repeats of a phorbol ester response element cloned upstream of the TGF-β responsive sequence derived from the human plasminogen activator inhibitor I promoter, and fused to the reporter gene luciferase (kindly provided by Dr. J. Massague, Memorial Sloan-Kettering Cancer Center, New York). To examine a biochemical effect dependent on the presence of a functional glucocorticoid receptor [Truss and Beato, 1993; Yen et al., 1997], cultures were transfected with 0.5 μg of pMMTV-Luc, which contains promoter DNA derived from the mouse mammary tumor virus (MMTV) also fused upstream of luciferase (kindly provided by Dr. Ronald Evans, The Salk Institute, La Jolla, CA). After 1 day of p3TPLux or pMMTV-Luc expression, cultures were treated with the reagents indicated in each experiment. Cell extracts were collected, and aliquots were used to measure luciferase activity with commercial reagents (Promega, Madison, WI), and corrected for protein content [McCarthy et al., 1996; Ji et al., 1996].

Statistical Analysis

Data were analyzed in multiple samples after multiple determinations and, where appropriate, are expressed as means ± SEM. In experiments with more than one variable or group, differences were assessed by analysis of variance with limits set by the Student-Newman-Keuls test, using commercial software (SigmaStat®). In experiments where one group was compared to its control, analysis defaulted to Student's *t*-test. Differences were considered significant when P values were <0.05.

RESULTS

Effects on Collagen Synthesis

Pre-exposure to cortisol. BMP-2 alters bone cell activity in specific ways that appear to depend on their differentiation status. In primary cultures of population 1 and osteoblast-enriched populations 3–5, 0.3–3 nM BMP-2 dose-dependently enhances collagen synthesis [Centrella et al., 1995b, 1996a]. Therefore, the effect of cortisol in response to BMP-2 was first examined on collagen synthesis in both cell populations. Cells were initially treated with 100 nM cortisol for 24 h (which maximally suppresses TGF-β1 activity) [Centrella et al., 1991] and then supplemented with a maximally stimulatory dose of 3 nM BMP-2 for the next 24 h. Treatment for the total 48-h period with cortisol alone produced a significant decrease in basal collagen synthesis rates, and suppressed the stimulatory effect of BMP-2 in both cell cultures (Fig. 1A). In populations 3–5, cortisol reduced the percent of collagen vs. noncollagen synthesis by 35–40% without (control: 7.7 ± 0.3, vs. cortisol: 5.0 ± 0.5; $P < 0.05$) or with BMP-2 (BMP-2: 8.6 ± 0.5, vs. cortisol plus BMP-2: 5.2 ± 0.5; $P < 0.05$). Nonetheless, the rate of collagen synthesis was still significantly enhanced by BMP-2 after cortisol treatment in populations 3–5, but not in population 1.

Simultaneous exposure to cortisol and BMP-2. Pretreatment with cortisol could irreversibly damage bone cells and reduce their general metabolic potential. Therefore, its ability to modify BMP-2 activity was next examined when cultures were treated simultaneously with both factors. Cortisol alone only moderately suppressed basal collagen synthesis in populations 3–5 after 24 h of exposure. However, it still significantly reduced the effect of BMP-2 on new collagen synthesis when the cultures were exposed to both agents simulta-

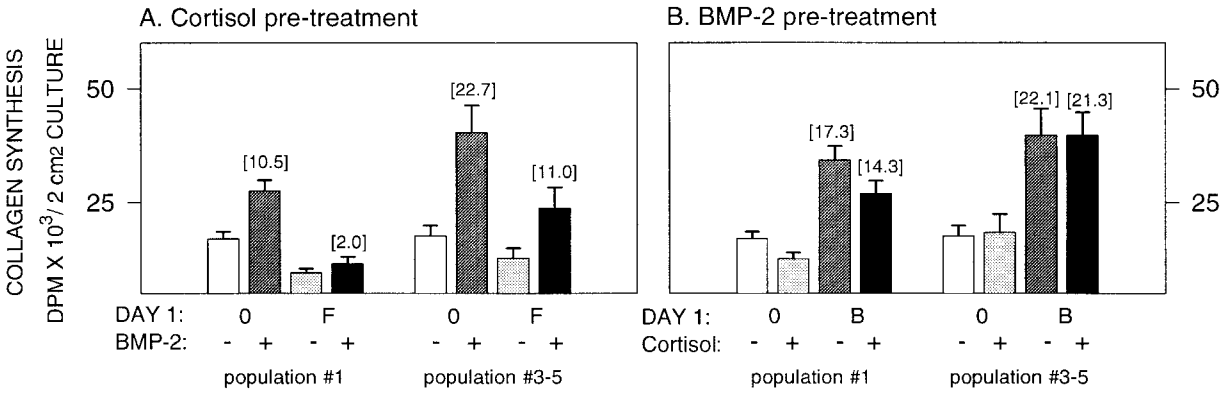

Fig. 1. Effect of cortisol on collagen synthesis in BMP-2 treated fetal rat bone cells. Confluent, serum deprived population 1 and population 3–5 cultures were pre-treated for 24 h with vehicle (0) or 100 nM cortisol (F) in **A,** or with vehicle or 3 nM BMP-2 (B) in **B.** Cultures were then supplemented with vehicle, BMP-2, or cortisol during the next 24 h, as indicated. Collagen synthesis was measured in extracts from cells labeled with [^{3}H]proline during the last 2 h of incubation. Numbers in brackets indicate the amount of new collagen synthesis induced by BMP-2. By analysis of variance, pre-treatment with cortisol significantly reduced basal and BMP-2 induced collagen synthesis, BMP-2 significantly enhanced collagen synthesis in population 3–5 cultures pre-exposed to cortisol, and pretreatment with BMP-2 opposed cortisol activity. Data are from 4 studies and a minimum of 24 replicate culture wells per condition.

TABLE II. Co-Treatment With Cortisol and BMP-2 on DNA and Protein Synthesis in Osteoblast-Enriched Cultures*

Treatment	DNA synthesis (dpm × 10^{-3}/culture)	Collagen synthesis (dpm × 10^{-3}/culture)	Noncollagen synthesis (dpm × 10^{-3}/culture)	Percent collagen
None	15.2 ± 1.5	8.8 ± 0.7	21.4 ± 1.6	7.8 ± 0.8
BMP-2	39.7 ± 6.7[a]	22.9 ± 1.9[a]	48.4 ± 2.6[a]	7.9 ± 0.4
Cortisol	13.0 ± 1.7	7.4 ± 0.6	24.2 ± 3.2	5.9 ± 0.5[b]
Both	43.7 ± 8.2[c]	12.9 ± 1.0[c]	34.2 ± 1.7[c]	6.5 ± 0.2[b]

*Confluent, serum deprived cultures were treated for 24 h with vehicle, 3 nM BMP-2, 100 nM cortisol, or both factors simultaneously. DNA synthesis was measured in 0.32 cm^2 cultures by pulsing with 5 μCi/ml [^{3}H]thymidine, and collagen and noncollagen protein synthesis were measured in 2 cm^2 cultures by pulsing with 5 μCi/ml [^{3}H]proline during the last 2 h of incubation. By analysis of variance, [a]significant stimulatory effect by BMP-2; [b]significant inhibitory effect by cortisol; [c]simultaneous treatment with cortisol and BMP-2 was significantly greater than cortisol alone. There were no differences in total protein content among the groups. Data are results from 3 studies and a minimum of 14 replicate cultures per condition.

neously. In contrast, it did not reduce the mitogenic effect of BMP-2 (Table II). Therefore, while cortisol could reduce collagen synthesis alone or in response to subsequent treatment or co-treatment with BMP-2, it did not have non-specific or generally toxic effects on bone cell activity.

Pre-exposure to BMP-2. To determine if the stimulatory effects induced by prior exposure to BMP-2 were also sensitive to cortisol, cultures were treated for 24 h with BMP-2 and then supplemented with cortisol during a second 24-h period. The stimulatory effect of BMP-2 by itself on collagen synthesis persisted in both cultures during the total incubation period. Notably, subsequent exposure to cortisol had no effect on BMP-2-induced collagen synthesis in populations 3–5, although it still in part suppressed the stimulatory effect of BMP-2 in population 1 (Fig. 1B).

Effects on Alkaline Phosphatase

Alkaline phosphatase is only minimally expressed and is not appreciably increased by BMP-2 in population 1. In contrast, while this marker of osteoblast activity is significantly higher in more differentiated population 3–5 cultures, it is also rapidly enhanced by BMP-2 [Centrella et al., 1995b]. Therefore, studies to examine interacting effects of cortisol and BMP-2 on alkaline phosphatase activity were focused on populations 3–5. As shown in Table III, treatment with cortisol during the first or second 24-h period did not affect basal alkaline phosphatase activity, confirming that at 100 nM cortisol does not have a general inhibitory

TABLE III. Effects of Cortisol and BMP-2 on Alkaline Phosphatase Activity in Osteoblast-Enriched Cultures*

	Treatment intervals		Alkaline phosphatase (pmol/min/μg protein)
	First 24 h	Second 24 h	
Control	None	None	468 ± 56
Cortisol pre-treatment	Cortisol	None	517 ± 68
	None	BMP-2	884 ± 96[a]
	Cortisol	BMP-2	644 ± 73[b]
Co-treatment	None	BMP-2/ Cortisol	607 ± 45[b]
BMP-2 pre-treatment	BMP-2	None	1,262 ± 176[a]
	None	Cortisol	465 ± 69
	BMP-2	Cortisol	1,144 ± 171[a]

*Confluent, serum deprived 2 cm^2 cultures were treated for 24 h with vehicle, 3 nM BMP-2, or 100 nM cortisol during the intervals indicated. Alkaline phosphatase activity was measured in extracts and corrected for protein content. By analysis of variance, [a]significant stimulatory effect by BMP-2; [b]pre-treatment or co-treatment with cortisol significantly reduced the stimulatory effect of BMP-2. Data are results from 4 studies and a minimum of 18 replicate cultures per condition.

effect on bone cell metabolism. Alkaline phosphatase activity rose during the 24- to 48-h treatment period with BMP-2, and, like collagen synthesis, this effect was partly suppressed by pre-treatment or co-treatment with cortisol. Again, in cultures pre-treated with BMP-2, its full stimulatory effect was evident when they were subsequently exposed to cortisol. Together with the collagen synthesis studies, these results indicate that some biochemical effects in populations 3–5, having first been induced by BMP-2, are refractory to subsequent inhibition by cortisol.

Regulation of TGF-β Activity

TGF-β is one of the most potent enhancers of matrix collagen synthesis in skeletal tissue. Initial studies revealed that pre-treatment with glucocorticoid reduces the stimulatory effect of TGF-β on new collagen synthesis in osteoblast-enriched populations 3–5 cultures [Centrella et al., 1991]. This contrasts directly with the effect of BMP-2, which synergistically enhances this aspect of TGF-β activity [Centrella et al., 1995b]. Based on the protective effects of BMP-2 seen in Figure 1B and Table III, we examined if BMP-2 could prevent the inhibitory effect of cortisol on TGF-β-induced collagen synthesis. Cultures were first treated with BMP-2 or cortisol before exposure to TGF-β1. Analogous to earlier studies, TGF-β alone potently enhanced new collagen synthesis. Even though TGF-β was approximately 2.5–3-fold more effective in population 3–5 than in population 1 cultures, pre-exposure to cortisol suppressed TGF-β activity by 30–40% in both populations (Fig. 2A). However, when population 3–5 cultures were exposed to BMP-2 before the addition of cortisol, the full stimulatory effect of TGF-β on new collagen synthesis was restored. Re-establishment of TGF-β activity was less evident in population 1, suggesting that this effect also was at least partly restricted to more differentiated bone cells (Fig. 2B).

Variations in TGF-β Receptors

Effects on individual TGF-β receptors. Cortisol and BMP-2 also alter TGF-β binding to bone cells in very different ways [Centrella et al., 1991, 1995b]. As shown in Figure 3, BMP-2 rapidly decreases TGF-β binding to betaglycan and TRII in population 3–5 cultures, and in so doing enhances the proportion of TGF-β binding to TRI. In effect, BMP-2 tends to establish the TGF-β receptor profile found on highly differentiated rat osteoblasts, coincident with an increase in osteoblast-like activity [Centrella et al., 1995b]. Within the same time frame, cortisol enhances TGF-β binding to betaglycan and reduces its binding to TRI. Cortisol also causes a slight decrease at TRII [Centrella et al., 1991]. Therefore, cortisol induces a re-distribution of TGF-β receptors in osteoblast-enriched cultures that contrasts sharply with the effect of BMP-2. Both agents are less effective regulators of these events in population 1 cultures.

Combined effects of cortisol and BMP-2. When population 3–5 cultures were co-treated with BMP-2 and cortisol, BMP-2 still inhibited TGF-β binding to betaglycan and TRII, in effect maintaining a higher ratio of binding to TRI (Fig. 4). After 2 days of treatment with cortisol alone, its effect on the TGF-β receptor profile persisted. Even so, BMP-2 was able to modify in part the TGF-β binding profile when population 3–5 cultures were first pre-treated with cortisol and then treated with BMP-2, or with both agents simultaneously (Fig. 5), verifying its ability to oppose the action of cortisol. In contrast, no protective effects were observed

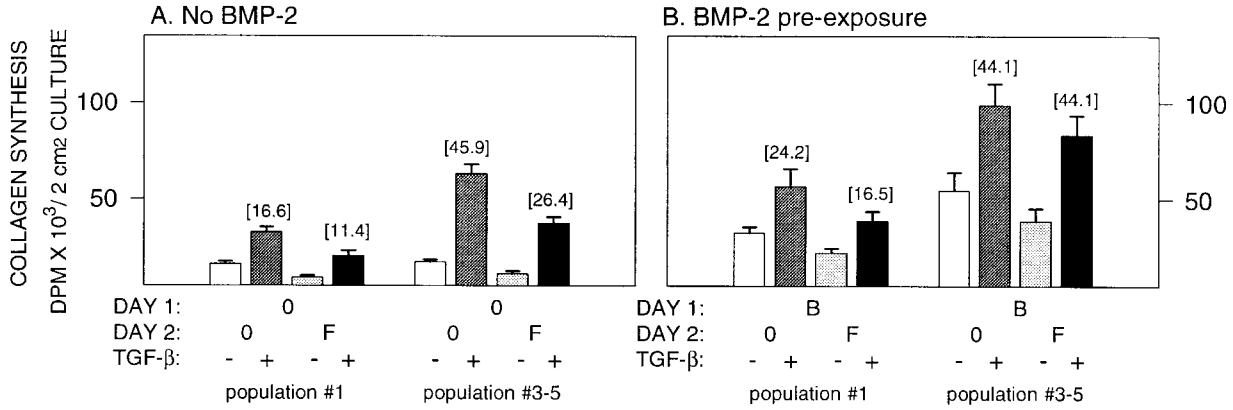

Fig. 2. Effect of BMP-2 and cortisol on collagen synthesis in response to TGF-β in fetal rat bone cells. Confluent, serum-deprived population 1 and population 3–5 cultures were treated for 24 h with vehicle (0) in **A,** or 3 nM BMP-2 (B) in **B,** and with vehicle or 100 nM cortisol (F) during the next 24 h. Cultures were then supplemented with vehicle or with 120 pM TGF-β1 as indicated, during a final 24-h period. Collagen synthesis was measured as in Figure 1. Numbers in brackets indicate the amount of new collagen synthesis induced by TGF-β. By analysis of variance, TGF-β significantly enhanced collagen synthesis in all conditions, pre-treatment with cortisol significantly reduced collagen synthesis in untreated and TGF-β1 treated cultures, and pretreatment with BMP-2 opposed cortisol activity. Data are from 3 studies and a minimum of 18 replicate culture wells per condition.

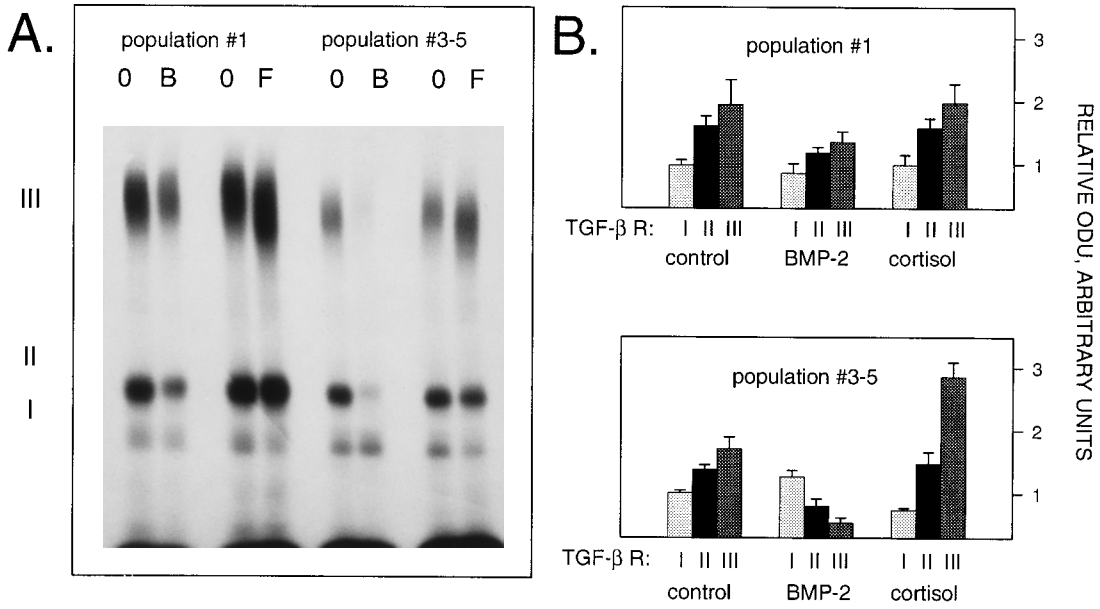

Fig. 3. Effect of BMP-2 and cortisol on TGF-β binding in fetal rat bone cells. **A:** Confluent, serum-deprived population 1 and population 3–5 cultures were treated with vehicle (0), 3 nM BMP-2 (B), or 100 nM cortisol (F) for 24 h. Cultures were incubated with ^{125}I-TGF-β1, cross-linked, and extracts were fractionated on a polyacrylamide gel and visualized by autoradiography. Complexes were designated by analogy to other cells, where betaglycan (III) migrates at >200 kDa, TRII (II) at 85 kDa, and TRI (I) at 65 kDa (14), relative to protein standards. Each lane shows the result of 2 pooled replicate cultures. **B:** Autoradiograms were scanned and analyzed by densitometry. Values are shown by comparison to TRI in untreated cultures, designated as 1.0 relative OD unit. Data are from 4 studies using ^{125}I-TGF-β1 labeled on 3 separate occasions.

with any treatment schedule in population 1 (Fig. 4B and other data not shown).

Effects on 3TPLux Expression

TGF-β enhances expression of the promoter/reporter construct 3TPLux, and in chemically mutated mink lung epithelial cells this is dependent on TGF-β binding to and activation of TRI [Carcamo et al., 1995; Wieser et al., 1995]. In bone cells, TGF-β also induces dose-dependent increases in 3TPLux expression that are maximal at 0.12–0.4 nM (Fig. 6, left). Analogous to

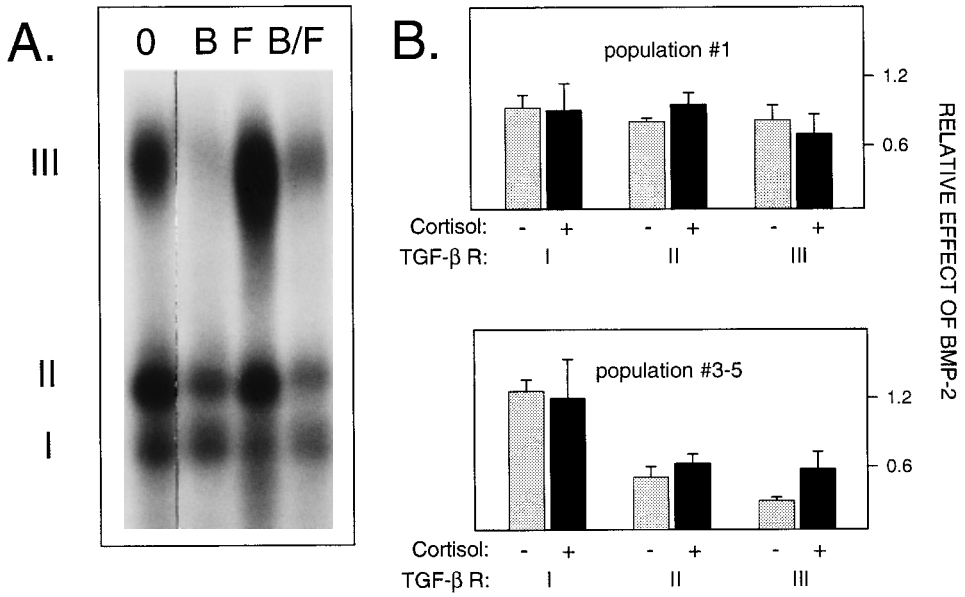

Fig. 4. Opposing effect by BMP-2 on cortisol-dependent changes in TGF-β binding in osteoblast-enriched fetal rat bone cell cultures. **A:** Confluent, serum-deprived population 3–5 cultures were treated with vehicle (0), 3 nM BMP-2 (B), 100 nM cortisol (F), or both agents (B/F) for 24 h. Receptor complexes containing ^{125}I-TGF-β1 were measured as in Figure 3. Each lane shows the result of 2 pooled replicate cultures. **B:** Parallel studies were performed in population 1 and population 3–5 cultures that were treated, extracted, and examined in parallel, as in A. Autoradiograms were scanned and analyzed by densitometry. Values are shown as the relative effect of BMP-2 in cultures without (−) or with (+) cortisol. Each lane shows the result of 2 pooled replicate cultures. Data are results from 3 studies using ^{125}I-TGF-β1 labeled on 2 separate occasions.

other biochemical effects [Centrella et al., 1987, 1991, 1995a,b, 1996a,b], a more potent increase in reporter enzyme expression occurs in populations 3–5 than in population 1. Although BMP-2 causes a striking decrease in TGF-β binding to TRII on osteoblasts, it failed to suppress the stimulatory effect of TGF-β on relative 3TPLux expression. In other studies not shown, BMP-2 at up to 3 nM never itself induced 3TPLux expression, while it synergistically enhanced the response to lower amounts (4–40 pM) of TGF-β. In contrast, 24-h pre-treatment with cortisol, which decreases TGF-β binding to TRI and to a lesser extent TRII, reduced the effect of TGF-β on 3TPLux expression by 40 ± 9% (not shown), and 48 h pre-treatment caused a 70 ± 4% reduction (Fig. 6, right). When cortisol-treated cultures were supplemented with BMP-2 during the 24-h period before exposure to TGF-β, BMP-2 partly opposed cortisol activity. However, treatment for the entire 72-h interval with BMP-2 by itself suppressed basal as well as TGF-β-induced 3TPLux expression, and in this context it failed to modify the inhibitory effect of cortisol (data not shown).

Effects on GRE-Dependent Promoter Activity

Because BMP-2 tended to oppose the inhibitory effect of cortisol on several aspects of osteoblast activity, it may intercept glucocorticoid-dependent effects directly. Therefore, we examined the effect of BMP-2 on reporter gene expression under control of a glucocorticoid sensitive promoter. Treatment for 24–48 h with cortisol induced a progressive increase in promoter activity in populations 3–5, and an attenuated response after 24 h in population 1 cultures. Similar to other changes noted throughout these studies, BMP-2 suppressed the effect of cortisol by 60 ± 5% in populations 3–5 (Fig. 7), but had no significant effect in population 1 (data not shown).

DISCUSSION

Periodic glucocorticoid synthesis and release are essential components of normal tissue metabolism and a physiological response to stress. Indeed, in an in vitro model of osteogenesis, formation of mineralized nodules in osteoblast-enriched cultures is enhanced by early treat-

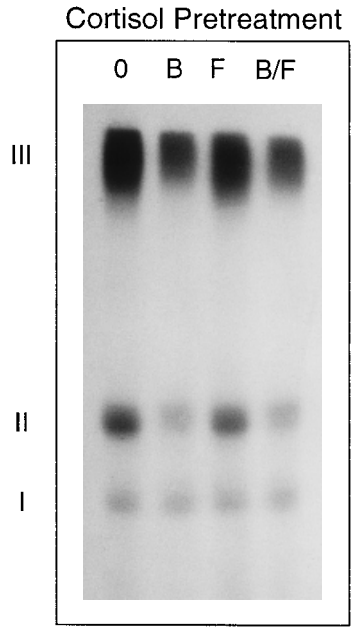

Fig. 5. Effect of pre-treatment with cortisol on BMP-2-dependent changes in TGF-β binding in osteoblast-enriched fetal rat bone cell cultures. Confluent, serum-deprived population 3–5 cultures were treated with 100 nM cortisol (F) for 24 h and then supplemented with vehicle (0), with 3 nM BMP-2 (B), with cortisol, or with both agents simultaneously (B/F) for an additional 24 h. Receptor complexes containing ^{125}I-TGF-β1 were measured as in Figure 3. Each lane shows the result of 2 pooled replicate cultures. Data are results from 2 studies using ^{125}I-TGF-β1 labeled on 2 separate occasions.

ment with a physiological concentration of glucocorticoid [Bellows et al., 1987; Lian and Stein, 1993]. However, uncontrolled or chronic exposure to high levels of glucocorticoid is detrimental to connective tissue integrity, and skeletal morbidity associated with glucocorticoid-induced osteopenia is a significant clinical problem for as many as 50% of individuals requiring long-term hormone therapy. Certain bone anomalies associated with glucocorticoid excess are associated with a decrease in the amount and the quality of bone matrix. This may derive at least in part from a decrease in collagen synthesis by osteoblasts [Dietrich et al., 1978; Kream et al., 1997], which therefore fail to replace actively remodeling bone [Bockman and Weinerman, 1990; Lukert and Raisz, 1990; Adler and Rosen, 1994; Lukert, 1996].

Because TGF-β, an abundant local bone growth factor, is one of the most potent stimulators of collagen synthesis by osteoblasts, we considered that some glucocorticoid-dependent effects are also focused within skeletal tissue. Our earlier studies demonstrated that collagen synthesis in response to TGF-β was inhibited by glucocorticoid in parallel with a re-distribution in the TGF-β receptor profile [Centrella et al., 1991]. Further studies then revealed that BMP-2 induced certain converse effects, suggesting that it could mitigate glucocorticoid function. Because glucocorticoid and BMP-2 produced very different effects on TGF-β binding to specific cell surface receptors, we considered that this might account for how some of these effects occur. Our present results reveal that, in addition to its direct effects on TGF-β receptors and activity, BMP-2 can suppress the ability of glucocorticoid to inhibit TGF-β function. These changes are more pronounced in osteoblast-enriched cultures. Importantly, BMP-2 also suppressed reporter gene expression controlled by a glucocorticoid sensitive promoter, suggesting that BMP-2 has a direct and dominant influence over glucocorticoid, rather than simply an independent effect that acts downstream of TGF-β receptors or TGF-β activity. These are the first studies that we know of demonstrating skeletal tissue-restricted effects by BMP-2 on glucocorticoid activity.

Other aspects of bone cell function or TGF-β activity may also be altered by glucocorticoid, but not all of them may be moderated by BMP-2. For example, glucocorticoid and BMP-2 each reduce the mitogenic effect of TGF-β in isolated bone cells [Centrella et al., 1991, 1995b]. However, decreases in TGF-β induced proliferation may occur for different reasons with each agent. The effect by glucocorticoid may relate to a simultaneous decrease in TRI, and to a lesser extent TRII, reducing an intracellular signaling system that requires both receptors in bone cells. With regard to BMP-2, its ability to suppress TGF-β binding to betaglycan and TRII specifically, in combination with its potent stimulatory effect on osteoblast-related activities such as alkaline phosphatase, PTH receptor expression, and mineralization [Beck et al., 1991; Rosen and Thies, 1992; Thies et al., 1992; Rosen et al., 1994; Centrella et al., 1995, 1996a; and others], suggest that the decreased mitogenic effect of TGF-β in this situation may accompany a re-direction in bone cell function towards a more differentiated status.

BMP-2 activity occurs through type I BMP receptors that share extensive sequence homology to TRI. Each receptor contains an intracellular serine/threonine kinase domain that must be activated to induce downstream events

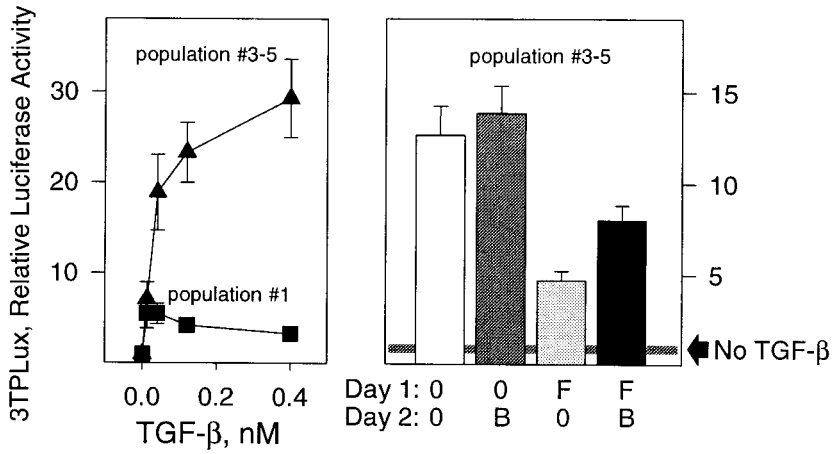

Fig. 6. Effects of cortisol and BMP-2 on 3TPLux expression in fetal rat bone cell cultures. Subconfluent cultures were transfected with the TGF-β-sensitive promoter/reporter construct 3TPLux, cultured to confluence, and serum deprived. **Left:** Population 1 and population 3–5 cultures were treated for 24 h with the amounts of TGF-β1 indicated. **Right:** Population 3–5 cultures were treated for 24 h with vehicle (0) or 100 nM cortisol (F), with vehicle or 1 nM BMP-2 (B) for a second 24-h period, and then with vehicle or 120 pM TGF-β1 for the final 24-h interval. Cell extracts were used to measure reporter (luciferase) expression and corrected for protein content. Values indicate the relative effect of each agent or combination by comparison to control (no TGF-β). By analysis of variance, TGF-β significantly enhanced reporter gene expression in all conditions, and pre-treatment with cortisol significantly reduced reporter gene expression in untreated and BMP-2-treated cultures. By Student's *t*-test, BMP-2 significantly opposed the inhibitory effect of cortisol. Data are results from at least 3 studies and a minimum of 9 replicate culture per condition.

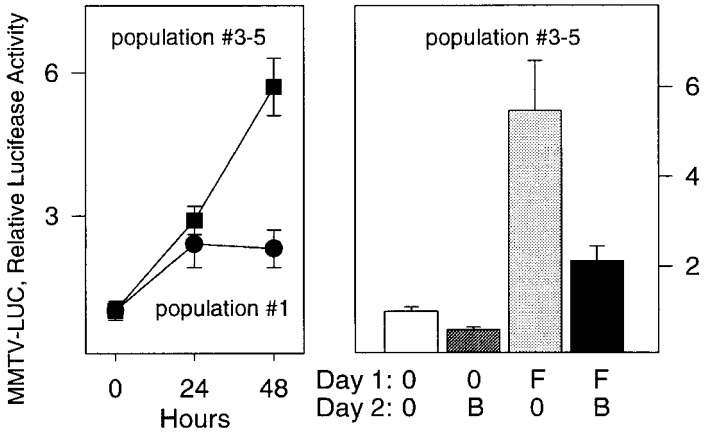

Fig. 7. Effects of cortisol and BMP-2 on MMTV-LUC expression in fetal rat bone cell cultures. Subconfluent cultures were transfected with the glucocorticoid-sensitive promoter/reporter construct MMTV-LUC, cultured to confluence, and serum deprived. **Left:** Population 1 and population 3–5 cultures were treated for 0, 24, or 48 h with 100 nM cortisol as indicated. **Right:** Population 3–5 cultures were treated for 24 h with vehicle (0) or 100 nM cortisol (F), and with vehicle or 1 nM BMP-2 (B) for a second 24-h period. Cell extracts were used to measure reporter (luciferase) expression and corrected for protein content. Values indicate the relative effect of each agent or combination by comparison to control (no addition). By analysis of variance, cortisol significantly enhanced reporter gene expression in control and BMP-2-treated cells, and BMP-2 significantly opposed cortisol activity. Data are results from 6 studies and 18 replicate cultures per condition.

[Kingsley, 1994; Massague et al., 1994; Miyazono et al., 1994; Centrella et al., 1996a; Mayer et al., 1996]. Alterations in certain amino acids within or near the kinase domain produce mutated receptors that are nonfunctional or constitutively active [Wieser et al., 1995; Zou and Niswander, 1996], suggesting similar mechanisms of activation. Regardless of the high level of similarity between the ligands and their receptor systems, each factor causes certain alterations in bone cell activity that are in part phenotype or differentiation dependent. Our results further distinguish TGF-β and BMP-2 function with regard to the TGF-β sensitive

promoter reporter plasmid 3TPLux in cells endogenously sensitive to both growth factors. Moreover, our earlier studies reveal that exposure to BMP-2 alters the TGF-β binding pattern on osteoblasts [Centrella et al., 1995b], although as in other systems, it does not compete directly for TGF-β receptor occupancy [Kingsley, 1994; Massague et al., 1994; Miyazono et al., 1994; Centrella et al., 1995b, 1996a]. In so doing, BMP-2 decreases the mitogenic effect of TGF-β and increases its ability to enhance new collagen synthesis and alkaline phosphatase in fetal rat bone cells [Centrella et al., 1995b, 1996a]. These results indicate that BMP-2 may in part re-define TGF-β activity in a pool of osteoblasts that express an intermediate phenotype, where certain downstream effectors utilized in response to TGF-β may be depleted after previous exposure to BMP-2. Thus BMP-2 and perhaps other BMPs have this and other effects that precede TGF-β function during bone formation.

Several targets have been identified for the kinase domain contained within type I receptors for TGF-β or BMPs [Yamaguchi et al., 1995; Chen et al., 1995; Derynck and Zhang, 1996]. In this context different downstream substrates appear to be affected in different ways by each factor. New evidence that intracellular proteins termed Smads may be specific receptor kinase substrates and that some Smads may have individual type I receptor specificities adds a new level of complexity to this system. Our present studies show that pre-exposure to cortisol blocks in whole or in part certain effects by either BMP-2 or TGF-β in cultured bone cells. Nevertheless, while cortisol reduces TGF-β binding to its own type I receptor, we have found no decreases in ligand binding to a type I receptor that binds BMP-2 and BMP-4 (unpublished results). Changes in individual Smads, of which some may be specific for individual type I receptors and some shared, might therefore explain situations where variations in the effects of these agents might occur even in the presence of apparently normal receptor complexes. Therefore, glucocorticoid may alter type I receptors for each factor and at least some of their downstream effects in different ways.

BMP may oppose cortisol activity through a glucocorticoid receptor mediated mechanism. While we found that BMP-2 directly suppresses glucocorticoid-dependent gene expression, we have not yet examined if this occurs through changes in glucocorticoid receptor number or affinity, or directly at the level of DNA binding and gene transcription. By themselves, BMP-2 and glucocorticoid each alter the steady-state levels of mRNA encoding betaglycan core protein in opposite ways, preceding and consistent with their dissimilar effects on TGF-β binding at that site. Furthermore, glucocorticoid alters or fails to alter the levels of TRI and TRII gene promoter activity, mRNA, and immunoreactive protein in ways that are consistent with its effects on ligand binding at each site (unpublished results), but additional studies are required to examine all of these parameters in the presence of both factors. Nonetheless, BMP-2 might activate other factors that themselves intercept events induced by glucocorticoid, which, left unchecked, would otherwise dampen TGF-β activity.

Agents or methods that prevent or reverse the damaging effects of glucocorticoid on bone cells could have important implications. Systemic administration of BMPs may not be practical for the management of metabolic bone loss. However, its ability to suppress specific aspects of glucocorticoid activity verifies its potential in select aspects of bone replacement therapy. Because little is still known about the molecular control of osteogenesis by growth factors and hormones, additional studies in models such as ours could help to explore these issues further. Regardless of how they occur, our present results demonstrate for the first time clear, nonsymmetrical, and opposing effects between BMP-2 and glucocorticoid on bone cells. Surprisingly, some actions by both of these agents converge on TGF-β activity in bone. A better understanding of these events could predict novel procedures or mimetics that prevent the inhibitory effects of glucocorticoid, perhaps specifically within the skeletal system.

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