Transcriptional regulation of connective tissue growth factor by cortisol in osteoblasts

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Pereira, Renata C., Deena Durant, and Ernesto Canalis. Transcriptional regulation of connective tissue growth factor by cortisol in osteoblasts. Am J Physiol Endocrinol Metab 279: E570–E576, 2000.—Glucocorticoids have important effects on osteoblastic function. Connective tissue growth factor (CTGF)/insulin-like growth factor binding protein-related protein 2 (IGFBP-rP2) plays a role in cell adhesion and function. We examined the regulation of CTGF/IGFBP-rP2 synthesis in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells). Cortisol caused a time- and dose-dependent increase in CTGF/IGFBP-rP2 mRNA levels in Ob cells. Cycloheximide did not preclude the effect, indicating that it was not protein synthesis dependent. Cortisol increased the rate of CTGF/IGFBP-rP2 transcription and, in transcriptionally arrested Ob cells, did not modify the decay of the transcript. Parathyroid hormone decreased, whereas transforming growth factor-β and, to a lesser extent, bone morphogenetic protein 2 increased CTGF/IGFBP-rP2 mRNA levels, but other hormones and growth factors had no effect. In conclusion, cortisol stimulates CTGF/IGFBP-rP2 transcription in Ob cells. Because CTGF/IGFBP-rP2 binds IGFs, its increased expression could be relevant to the actions of cortisol in bone.

skeletal cells; insulin-like growth factor; insulin-like growth factor binding proteins; glucocorticoids; bone formation

IN ADDITION TO THE SIX CHARACTERIZED insulin-like growth factor binding proteins (IGFBP), there are four proteins that share amino terminal structural homology with the IGFBPs and have been termed IGFBP-related proteins (IGFBP-rPs) (2, 34). IGFBP-rP1 is the product of the mac25 gene (28, 29, 42). The other three IGFBP-rPs are closely related and belong to a single CNN gene family. They are IGFBP-rP2, or connective tissue growth factor (CTGF), and IGFBP-rP3 and -rP4, which are encoded by the nephroblastoma overexpressed (nov 3) and the cysteine-rich 61 (cyr 61) genes, respectively (6). This family of genes also comprises expressed low in metastasis 1 (elm 1 or WISP-1), heparin-inducible CTGF/cyr 61/nov (CCN)-like protein or WISP-2, and WISP-3 (6). The products of these genes appear to play a role in cell differentiation and development. In addition, IGFBP-rP1 and CTGF/IGFBP-rP2 bind IGF-I and IGF-II, although their affinity for IGFs is lower than the affinity of IGFBPs (4, 29, 42).

Human CTGF is a protein of 348 amino acids with a molecular mass (Mr) of 36,000–38,000 Da, depending on its degree of glycosylation (6, 38). The murine homologue, first identified as fibroblast-inducible secreted protein 12 (Fisp-12), has 349 amino acids and is 91% homologous to the human protein (6, 35). CTGF/IGFBP-rP2 is expressed by a variety of cells and tissues, and its transcription is enhanced primarily by transforming growth factor-β (TGF-β) (5, 18, 43). During development, CTGF/IGFBP-rP2 is expressed in epithelial and secretory cells, liver parenchyma, vascular cells, and myoblasts and, to a lesser extent, in mesenchymal cells (39). CTGF/IGFBP-rP2 has been shown to have mitogenic properties, to play a role in cell adhesion mediated by the integrin receptors αvβ3 and αvβ5, and to induce angiogenesis (1, 20, 37). Some of its effects mimic those of TGF-β, suggesting that it might mediate selected TGF-β effects.

Glucocorticoids have complex effects on bone formation and resorption (7, 12). Some of these are probably due to direct actions of glucocorticoids on specific genes expressed by the osteoblast, whereas others may be indirect and mediated by locally produced growth factors or their binding proteins (7, 12). IGFs have important stimulatory effects on bone formation (19). Glucocorticoids inhibit the expression of IGF-I and IGFBP-5, a binding protein that may enhance IGF-I effects on bone cell function and increase the expression of IGFBP-6, a binding protein that selectively binds and blocks the effects of IGF-II on osteoblasts (11, 14, 15). In addition, glucocorticoids enhance the expression of mac25/IGFBP-rP1 in osteoblasts (32). These findings suggest that the IGF/IGFBP axis plays a central role mediating selected effects of glucocorticoids in bone.

Recently, a CTGF-like cDNA was cloned from human osteoblasts and was postulated to play a role in bone turnover (23). However, it is not known whether CTGF/IGFBP-rP2 is expressed and whether its expression is regulated in cells of the osteoblastic lineage. The present studies were undertaken to examine the effects

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of cortisol on CTGF/IGFBP-rP2 gene expression in cultures of osteoblast-enriched cells from 22-day fetal rat calvariae (Ob cells) and compare them to the action of other hormones and growth factors with known effects on bone cell function.

MATERIALS AND METHODS

Culture technique. The culture method was described in detail previously (27). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were killed by blunt trauma to the nuchal area or by CO₂ asphyxiation. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone by use of bacterial collagenase (CLS II, Worthington Biochemical, Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool and were previously shown to have osteoblastic characteristics (27). Ob cells were plated at a density of 8,000–12,000 cells/cm² and were cultured in a humidified 5% CO₂, 95% air atmosphere at 37°C until reaching confluence (~50,000 cells/cm²). Cells were cultured in DMEM supplemented with nonessential amino acids and 10% fetal bovine serum (both from Summit Biotechnology, Fort Collins, CO). Ob cells were grown to confluence, transferred to serum-free medium for 20–24 h, and exposed to test or control medium in the absence of serum for 0.5–24 h, as indicated in the text and legends. In one experiment, cells were cultured in the absence of an additional 24-h period to determine whether the effect of cortisol was sustained for 48 h. After this period of serum deprivation, >95% of the cells are viable, as determined by trypsin blue exclusion. For nuclear run-on assays, Ob cells were grown to subconfluence, trypsinized, replated, and grown to confluence when they were serum deprived and exposed to test or control solutions for 2–24 h. Cortisol, 17β-estradiol, testosterone (all from Sigma Chemical, St. Louis, MO), and 1,25 dihydroxy-vitamin D₃ (Biomol, St. Louis, MO), and 1,25 dihydroxy-vitamin D₃ (Biomol, St. Louis, MO) were dissolved in absolute ethanol and diluted 1:1,000 in DMEM. Recombinant human growth hormone (a gift from P. A. Kelly, Paris, France) was dissolved in distilled water and added to DMEM. Recombinant human TGF-β1 (a gift from Genentech, South San Francisco, CA), bone morphogenetic protein 2 (BMP-2; a gift from Genetics Institute, Cambridge, MA), fibroblast growth factor 2 (FGF-2), and pleat-derivated growth factor BB (PDGF-BB) (both from Austral, San Ramon, CA) were added directly to the medium. Recombinant human IGF-I (Austral) was dissolved in 20 mM sodium citrate and diluted 1:1,000 in DMEM. Cycloheximide and 5,6-dichlorobenzimidazole riboside (DRB) (both from Sigma) were dissolved in absolute ethanol and diluted 1:1,000 and 1:200, respectively, in DMEM. Control and experimental cultures were exposed to equal amounts of solvent. For RNA analysis, the cell layer was extracted with guanidine thiocyanate at the end of the incubation and stored at −70°C. For nuclear run-on assays, nuclei were isolated by Dounce homogenization.

Northern blot analysis. Total cellular RNA was isolated with an RNasea kit following manufacturer’s instructions (Qiagen, Chatsworth, CA). The RNA recovered was quantitated by spectrometry, and equal amounts of RNA from control or test samples were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize RNA standards and ribosomal RNA, documenting equal RNA loading of the various experimental samples. The RNA was then blotted onto Gene Screen Plus charged nylon (Du Pont, Wilmington, DE), and uniformity of transfer was documented by visualization of ribosomal RNA. A 1.56-kb EcoR I/Sph I I restriction fragment of the mouse CTGF/IGFBP-rP2 or Fisp-12 cDNA (kindly provided by Rolf-Peter Ryseck, Princeton, NJ) was purified by agarose gel electrophoresis (35). CTGF/IGFBP-rP2 cDNA was labeled with 50 μCi each [α-32P]deoxyctidine triphosphate and [α-32P]deoxyadenosine triphosphate at a specific activity of 3,000 Ci/nmol (Du Pont, Wilmington, DE) by means of the random hexanucleotide-primed second strand synthesis method (13). Hybridizations were carried out at 42°C for 16–72 h, and posthybridization washes were performed at 65°C in 0.2 × saline-sodium citrate (SSC) for 30 min. The blots were stripped and rehybridized with a 752 base pair (bp) BamH I/Sph I restriction fragment of the murine 18S cDNA (American Type Culture Collection, Rockville, MD) at 42°C for 72 h and washed in 1× SSC at 65°C in 0.1 × SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film, employing Cronex Lightning Plus intensifying screens (Du Pont). Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three or more cultures.

Nuclear run-on assay. To examine changes in the rate of transcription, nuclei were isolated by Dounce homogenization in a Tris buffer containing 0.5% IGEPAL CA-630 (Sigma). Nascent transcripts were labeled by incubation of nuclei in a reaction buffer containing 500 μM each of adenine, cytidine, and guanosine triphosphate, 150 U RNAse (Promega, Madison, WI), and 250 μCi [α-32P]uridine triphosphate (3,000 Ci/nM, Du Pont) (17). RNA was isolated by treatment with DNase I and protease K, followed by phenol-chloroform extraction and ethanol precipitation. Linearized plasmid pBluescript SK+ DNA containing ~1 μg of the CTGF/IGFBP-rP2 cDNA used for Northern blotting was immobilized onto GeneScreen Plus by slot blotting, according to manufacturer’s directions (Du Pont). The plasmid vectors pGL3-Basic (Promega) and pBluescript II KS (+) (Stratagene, La Jolla, CA) were used as a control for nonspecific hybridization, and 18S RNA cDNA or an 800 bp glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA (kindly provided by R. Wu, Ithaca, NY) was used to estimate loading of the radiolabeled RNA (41). Equal counts per minute of [32P]RNA from each sample were hybridized to cDNAs at 42°C for 72 h and washed in 1× SSC at 65°C for 20 min. Hybridized cDNAs were visualized by autoradiography. Nuclear run-on assay was done twice.

Western blot analysis. Medium aliquots from Ob cell cultures were precipitated with 10% trichloroacetic acid. The pellet was suspended in Laemml sample buffer to give a final concentration of 2% SDS and fractionated by PAGE on a 12% denaturing gel in the absence of reducing agents (24). Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA), blocked with 2% BSA, and exposed to a 1:2,000 dilution of an affinity affinity purified goat antibody raised against human CTGF (kindly provided by S. Williams, Miami, FL) in 1% BSA overnight. Blots were exposed to a rabbit anti-goat IgG antiserum conjugated to horseradish peroxidase and developed with a horseradish peroxidase chemiluminescent detection reagent. Western blots are representative of three or more cultures.

Statistical methods. Values are expressed as means ± SE. Slopes to determine mRNA decay were compared with a
GB-Stat software package (Dynamic Microsystems, Silver Spring, MD) (33).

RESULTS

Northern blot analysis of total RNA extracted from confluent cultures of Ob cells revealed a predominant CTGF/IGFBP-rP2 transcript of 2.4 kb (Fig. 1). Continuous treatment of Ob cells with cortisol caused a time-dependent increase in CTGF/IGFBP-rP2 steady-state mRNA levels. The effect was initially observed after 2 h of exposure to cortisol at 1 μM, was maximal after 6 h, and was sustained for 24 (Fig. 1) to 48 h (not shown). Treatment with cortisol at 1 μM for 2, 6, 24, and 48 h increased CTGF/IGFBP-rP2 mRNA levels by $2.5 \pm 0.6 \times 10^3$, $3.5 \pm 0.5 \times 10^3$, $3.1 \pm 0.3 \times 10^3$, and $2.9 \pm 0.5$-fold (means ± SE; $n = 7–14$), respectively, as determined by densitometry (Fig. 1). The effect of cortisol was dose dependent. Continuous treatment of Ob cells with cortisol for 6 h at 10 nM, 100 nM, and 1 μM increased CTGF/IGFBP-rP2 transcripts by $1.4 \pm 0.1$, $2.2 \pm 0.1$, and $3.5 \pm 0.5$-fold (means ± SE; $n = 3–9$), respectively (Fig. 2), and continuous treatment for 24 h at the same doses increased CTGF/IGFBP-rP2 mRNA by $1.5 \pm 0.1$, $3.4 \pm 0.4$, and $3.1 \pm 0.3$-fold, respectively (not shown).

Western blot analysis of conditioned medium from Ob cells revealed an immunoreactive protein with a Mr of 34–36 kDa, the known Mr of CTGF/IGFBP-rP2, which was increased by cortisol at 1 μM (Fig. 3) (6, 8). A recombinant human CTGF/IGFBP-rP2 standard migrated as a doublet with a Mr of 34 and 36 kDa due to various degrees of glycosylation. Additional high molecular weight “bands” were detected, but virtually all were also detected by the secondary antibody when tested in the absence of CTGF/IGFBP-rP2 antibody (not shown), indicating that they were nonspecific immunoreactive proteins. These were not modified by cortisol.

To determine whether or not the effects observed on CTGF/IGFBP-rP2 mRNA levels were dependent on protein synthesis, serum-deprived confluent cultures of Ob cells were treated with cortisol in the presence or absence of cycloheximide at 3.6 μM. In earlier experiments, cycloheximide at doses of 2 μM and higher was found to inhibit protein synthesis in Ob cell cultures by 80–85% (8). Northern blot analysis revealed that treatment with cycloheximide for 24 h did not prevent, and actually enhanced, the stimulatory effect of cortisol on CTGF/IGFBP-rP2 mRNA levels (Fig. 4).

To examine whether or not the effect of cortisol on CTGF/IGFBP-rP2 mRNA levels was due to changes in transcript stability, confluent cultures of Ob cells were exposed to DMEM or to DMEM plus cortisol for 1 h and then treated with the RNA polymerase II inhibitor DRB in the absence or presence of cortisol at 1 μM for 0.5–4 h (44). The half-life of CTGF/IGFBP-rP2 mRNA was 1–1.5 h in control cultures and in cortisol-treated samples, indicating that cortisol did not modify CTGF/IGFBP-rP2 transcript decay (Fig. 5). To determine whether or not cortisol modified the transcription of the CTGF/IGFBP-rP2 gene, nuclear run-on assays
CORTISOL STIMULATES CTGF/IGFBP-rP2

Fig. 4. Effect of cortisol at 1 μM in the presence (+) or absence (−) of cycloheximide (Cyclohex) at 3.6 μM on CTGF/IGFBP-rP2 mRNA expression in cultures of Ob cells treated for 24 h. Total RNA from control (−) or treated (+) cultures was subjected to Northern blot analysis and hybridized with α-32P-labeled CTGF/IGFBP-rP2 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. CTGF/IGFBP-rP2 mRNA was visualized by autoradiography and is shown in the top panel; 18S mRNA is shown below.

were performed on nuclei from Ob cells treated for 1, 2, or 24 h. This assay demonstrated that cortisol increased the rate of CTGF/IGFBP-rP2 transcription after 1 h, and the effect was sustained for 24 h (Fig. 6).

The induction of CTGF/IGFBP-rP2 was relatively selective to cortisol. 17β-Estradiol, testosterone, and 1,25 dihydroxy-vitamin D3, all tested at 10 nM for 2, 6, and 24 h, did not modify the expression of CTGF/IGFBP-rP2 mRNA in Ob cells (not shown). Although PTH at 10 nM for 2 and 6 h decreased CTGF/IGFBP-rP2 mRNA levels (Fig. 7A), we were unable to detect a decrease in protein levels (not shown), probably because CTGF/IGFBP-rP2 was barely detectable by Western blot analysis in control cultures. Other polypeptide growth factors, such as growth hormone at 0.5 μM and insulin at 100 nM, did not modify the expression of CTGF/IGFBP-rP2 in Ob cells treated for 2, 6, or 24 h (not shown). Confirming previous observations in non-skeletal cells, TGF-β1 caused a substantial increase in CTGF/IGFBP-rP2 mRNA levels (Fig. 7B) (5, 18, 43). The effect was initially observed after 2 h of TGF-β exposure, and after 6 and 24 h, TGF-β1 at 1.2 nM increased CTGF/IGFBP-rP2 mRNA levels by 20- to 40-fold. The related BMP-2 at 1 nM caused a smaller effect, and, after 2, 6, and 24 h, increased CTGF/IGFBP-rP2 transcripts by about threefold. TGF-β1 (Fig. 8), but not BMP-2 (not shown), increased CTGF/IGFBP-rP2 protein levels, as detected by Western blot analysis. Other polypeptide growth factors, such as IGF-I at 100 nM, PDGF-BB at 3.3 nM, and FGF-2 at 1.7 nM for 2, 6, or 24 h, did not modify CTGF/IGFBP-rP2 transcripts (not shown).

DISCUSSION

Recent studies have shown that cortisol has significant effects on the IGF/IGFBP axis in skeletal cells (7). Osteoblasts express IGF-I and IGF-II and various IGFBPs as well as IGFBP-rP1 (11, 16, 26, 30, 32). The present investigation was undertaken to determine whether osteoblasts express CTGF/IGFBP-rP2 and to examine its regulation by cortisol and other hormones and factors known to act on skeletal cells. We demonstrated that cortisol increases CTGF/IGFBP-rP2 mRNA levels in Ob cells in a time- and dose-dependent manner, concomitant with an increase in polypeptide levels, indicating that cortisol induces CTGF/IGFBP-rP2 synthesis. The effect of cortisol on CTGF/IGFBP-rP2 mRNA does not require de novo protein synthesis. In fact, the effect of cortisol was enhanced modestly by cycloheximide, possibly due to an inhibitory effect on the synthesis of RNA degrading enzymes. Experiments in transcriptionally arrested Ob cells using the RNA polymerase II inhibitor DRB revealed that cortisol did not modify the stability of CTGF/IGFBP-rP2 transcripts (44). This, in conjunction with an increase in the rate of transcription, indicates that cortisol stimulates CTGF/IGFBP-rP2 expression at the transcriptional level.

In our study, the effects of cortisol on CTGF/IGFBP-rP2 synthesis were observed at doses that modify other parameters of metabolic function in Ob cells, suggesting that the stimulation of CTGF/IGFBP-rP2 synthesis by cortisol is physiologically relevant. IGF-I and IGF-II are abundant in skeletal tissue and have important effects on bone formation (19). Because CTGF/IGFBP-rP2 has the potential to bind IGFs, its induction by cortisol could be a mechanism to modulate their actions in bone (34). Glucocorticoids have complex effects on bone remodeling and have a major impact on the IGF/IGFBP axis. The effects of these steroids on bone formation are opposite to those of IGF-I and IGF-II, suggesting that some of their effects could be mediated...
by changes in the synthesis of IGF-I, IGF-II, or IGFBPs (7). Glucocorticoids decrease the synthesis of IGF-I and IGFBP-5, a binding protein that can stimulate bone cell growth, and increase expression of IGFBP-6, a binding protein that selectively binds IGF-II (11, 14, 15). Glucocorticoids increase CTGF/IGFBP-rP1 and IGFBP-rP2 expression to a similar extent, and these proteins may mediate actions of these steroids in bone. There is evidence to indicate that CTGF/IGFBP-rP2 enhances cell attachment, and this could play a role in cell differentiation (1, 19). Similarly, IGFBP-rP1 has been implicated in cell differentiation, and the two proteins may act in conjunction in this process (40). Although glucocorticoids decrease bone formation, they can induce the differentiation of cells of the osteoblastic lineage, an effect that is, to an extent, mediated by BMPs (3, 4, 25, 36). Therefore, the stimulatory effects of cortisol on IGFBP-rP1 and the effects of the steroid and BMP-2 on CTGF/IGFBP-rP2 could facilitate cell attachment and differentiation. However, it is important to note that the exact function of the two IGFBP-rPs in bone has not been established, and there is no direct evidence that they play a role in the osteoblast response to glucocorticoids.

Our studies confirm reports on nonskeletal cells demonstrating that TGF-β1 is the major inducer of CTGF/IGFBP-rP2 and caused a more pronounced absolute stimulation than cortisol (5, 18, 43). TGF-β1 stimulates CTGF/IGFBP-rP2 degradation in endothelial cells, a phenomenon not examined in the current studies (5). However, if it does occur, it does not preclude an increase in CTGF/IGFBP-rP2 in osteoblasts. Glucocorticoids have complex effects on the TGF-β axis in osteoblasts, and it is not probable that TGF-β mediates the effects of cortisol on CTGF/IGFBP-rP2 synthesis in rat Ob cells. In human osteoblasts, glucocorticoids activate TGF-β, but in rat Ob cells, cortisol has no

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Fig. 6. Effect of cortisol at 1 μM on the rate of CTGF/IGFBP-rP2 transcription in cultures of Ob cells treated for 1, 2, or 24 h. Nascent transcripts from control (–) or cortisol-treated (+) cultures were labeled in vitro with [α-32P]UTP, and the labeled RNA was hybridized to immobilized cDNA for CTGF/IGFBP-rP2. 18S RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA were used to demonstrate loading, and pGL3-Basic or pBluescript II KS (+) vector DNA was used as a control for nonspecific hybridization.

Fig. 7. Effect of parathyroid hormone (P) at 10 nM (A), bone morphogenetic protein-2 (B) at 1 nM and transforming growth factor-β (T) at 1.2 nM (B) on CTGF/IGFBP-rP2 mRNA expression in cultures of Ob cells treated for 2, 6, or 24 h. Total RNA from control (C) or treated cultures was subjected to Northern blot analysis and hybridized with α-32P-labeled CTGF/IGFBP-rP2 cDNA. The blot was stripped and rehybridized with labeled murine 18S cDNA. CTGF/IGFBP-rP2 mRNA was visualized by autoradiography and is shown in the top panels; 18S mRNA is shown below.

Fig. 8. Effect of transforming growth factor-β (TGF-β) at 1.2 nM on CTGF polypeptide levels in cultures of Ob cells treated for 24 h. A 1.2-ml aliquot of conditioned medium from control (–) and TGF-β-treated cultures (+) was precipitated and subjected to Western immunoblot analysis, and CTGF was detected with an anti-CTGF antibody and a chemiluminescence detection system. Migration of molecular mass markers in kDa is indicated on the left, and CTGF is identified by the arrow on the right.
effect on TGF-β1 mRNA levels and decreases TGF-β1 activity by decreasing the binding of TGF-β1 to signaling receptors (8, 9, 31). The stimulation caused by cortisol on CTGF/IGFBP-rP2 synthesis seems to be relatively specific to mesenchymal cells, because it occurs in osteoblasts and fibroblasts (10). PTH was the only other hormone found to regulate CTGF/IGFBP-rP2 expression. The effect was opposite to that of cortisol, inhibiting CTGF/IGFBP-rP2 mRNA levels, and it is probably related to an induction of cAMP by PTH in osteoblasts. cAMP was shown to inhibit the TGF-β1 induction of CTGF/IGFBP-rP2 mRNA in fibroblasts, although cAMP had no effect on CTGF/IGFBP-rP2 transcripts in vascular cells (5, 22). The opposite effects of PTH and cortisol on CTGF/IGFBP-rP2 expression are not surprising, because PTH and other cAMP inducers and cortisol have opposite effects on other aspects of the IGF/IGFBP axis in osteoblasts (7). For instance, PTH induces, whereas glucocorticoids inhibit, IGF-I expression in Ob cells (7).

In conclusion, the present studies demonstrate that cortisol stimulates CTGF/IGFBP-rP2 transcripts in skeletal cells through mechanisms that involve increased transcription. An increased level of CTGF/IGFBP-rP2 in the bone microenvironment may be relevant to the actions of cortisol on bone formation.

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