

Differential Effects of Transforming Growth Factor- β on the Synthesis of Extracellular Matrix Proteins by Normal Fetal Rat Calvarial Bone Cell Populations

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Abstract. To determine the effects of transforming growth factor- β (TGF- β) on the different cell types that exist in bone, cell populations (I-IV), progressively enriched in osteoblastic cells relative to fibroblastic cells, were prepared from fetal rat calvaria using timed collagenase digestions. TGF- β did not induce anchorage-independent growth of these cells, nor was anchorage-dependent growth stimulated in most populations studied, despite a two- to threefold increase in the synthesis of cellular proteins. In all populations the synthesis of secreted proteins increased 2-3.5-fold. In particular, collagen, fibronectin, and plasminogen activator inhibitor synthesis was stimulated. However, different degrees of stimulation of individual proteins were observed both within and between cell populations. A marked preferential stimulation of plasminogen activator inhibitor was ob-

served in each population, together with a slight preferential stimulation of collagen; the effect on collagen expression being directed primarily at type I collagen. In contrast, the synthesis of SPARC (secreted protein acidic rich in cysteine/osteonectin) was stimulated approximately two-fold by TGF- β , but only in fibroblastic populations. Collectively, these results demonstrate that TGF- β stimulates matrix production by bone cells and, through differential effects on individual matrix components, may also influence the nature of the matrix formed by different bone cell populations. In the presence of TGF- β , osteoblastic cells lost their polygonal morphology and alkaline phosphatase activity was decreased, reflecting a suppression of osteoblastic features. The differential effects of TGF- β on bone cell populations are likely to be important in bone remodeling and fracture repair.

TRANSFORMING growth factor- β (TGF- β)¹ is a 25-kD dimeric polypeptide that can affect the growth, differentiation, and activity of a variety of different cell types (17, 32). Although present in most normal and transformed cells studied, TGF- β is stored in relatively high amounts in platelets, suggesting a role for this growth factor in wound healing (1). TGF- β is also concentrated in bone, where it was originally identified as a cartilage-inducing factor (29) that exists in two forms, CIF-A and CIF-B. Recently, two major forms and a minor form of porcine TGF- β have been isolated (5). The major forms are distinct homodimers, whereas the minor form is a heterodimer. Amino acid sequence data indicate that the major forms of TGF- β correspond to CIF-A and CIF-B (30).

TGF- β promotes a wound healing response in vivo that is characterized by increased cellular proliferation and protein synthesis (25, 31). Studies of connective tissue cells in vitro have demonstrated that TGF- β stimulates the synthesis of matrix protein including collagen and fibronectin (7, 9, 25,

39). In studies of bone, TGF- β has been shown to stimulate bone resorption (33), to be mitogenic for cells in fetal rat calvariae, and, on transitory exposure, to stimulate collagen and protein synthesis by calvarial cells in organ culture (3). More recently, the mitogenic effect of TGF- β on osteoblast-enriched populations of fetal calvarial cells in monolayer cultures was shown to be independent of the stimulation of collagen synthesis (4). Several factors known to regulate bone metabolism, such as parathormone and 1,25(OH)₂ vitamin D₃, can stimulate TGF- β activity in cultures of rodent calvariae (22), and in particular, 1,25(OH)₂ vitamin D₃ has been shown to stimulate TGF- β -like activity in a rat osteoblastic cell line (21). The concentration of TGF- β in bone matrix and its stimulation in osteoblastic cells indicate that TGF- β may function in the modulation of resorption and formation that occurs during bone remodeling and in the process of bone repair.

In this study we have analyzed the effects of TGF- β on matrix protein synthesis by bone cells derived from fetal rat calvaria. Because bone cells are highly heterogeneous (2), we have isolated distinct populations of bone cells that range in

I. Abbreviations used in this paper: SPARC, secreted protein acidic rich in cysteine; TGF- β , transforming growth factor- β .

phenotype from predominantly fibroblastic to osteoblastic. The effects of low levels (0.1 and 1.0 ng/ml) of TGF- β on the osteoblastic cells could then be differentiated from responses of fibroblastic cells. These studies have concentrated on the effect of TGF- β on the synthesis of the secreted proteins, collagen and fibronectin, that are involved in the formation of connective tissue matrix. In addition, we have studied the effect of TGF- β on osteonectin, a prominent calcium-binding glycoprotein synthesized by osteoblastic and fibroblastic cells (20, 37, 40) that is relatively abundant in bone and rapidly remodeling connective tissues (35). Osteonectin appears to be highly homologous to SPARC protein (6a, 8), a 40-kD glycoprotein originally recognized as the major protein induced by retinoids in the differentiation of parietal endoderm cells, and to a 43-kD "culture shock" protein synthesized by bovine endothelial cells (15, 16, 26, 27), and the basement membrane protein BM-40 (14). The expression of SPARC/osteonectin may, therefore, be related to the wound healing response and could also be an indicator of cellular differentiation.

Materials and Methods

The isolation of fetal rat calvarial cell populations, the conditions of cell culture (23, 38), and methods used for protein synthesis were carried out essentially as described in detail elsewhere (30) and are reported here in an abbreviated form.

Isolation and Culture of Bone Cell Populations

Frontal and parietal bones from 21-d-old rat fetuses were cleaned of periosteum and loose soft connective tissue and washed in ice-cold PBS containing antibiotics as described previously (23). Approximately 30 calvariae were minced with scissors into small pieces and digested at 37°C with 5 ml of a sterile bacterial collagenase solution. After 10 min, the released cells were collected and immediately mixed with an equal volume of FBS. The procedure was repeated to release cells at 20, 30, 50, and 70 min after the initial digestion. Since few cells were obtained at 70 min they were combined with the 50-min digestion to produce four populations (I-IV). The individual cell populations were filtered through a 200-mesh sieve to remove debris and the cells collected by centrifugation at 400 *g* for 5 min. The cells were then resuspended in α minimal essential medium (α -MEM) containing 15% (vol/vol) FBS and antibiotics and plated in T-75 tissue culture flasks and grown to confluence.

Fetal rat calvarial cell populations prepared as described above exhibited a range of morphologies from spindle shaped (fibroblastic) to polygonal (osteoblastic). Further, the later-eluting populations were enriched with osteoblastic cells, as indicated by greater cAMP response to parathormone, higher alkaline phosphatase levels, and synthesis of lower amounts of type III collagen.

After trypsinization, 1-3 $\times 10^4$ cells from each population were seeded into triplicate 35-mm culture dishes and grown to confluence. Cells were synchronized by incubation for 24 h in 1% (vol/vol) FBS before being incubated with TGF- β (purified from human platelets as described previously [1] and generously supplied by Dr. M. B. Sporn, National Cancer Institute, National Institutes of Health, Bethesda, MD) at 0.1 and 1.0 ng/ml or with vehicle (1 mg/ml BSA in 4 mM HCl) alone in α -MEM containing 1% (vol/vol) FBS for 24 h in a humidified incubator at 37°C. The choice of a 24-h incubation time was based on a time course study of TGF- β on human gingival fibroblasts in which the 24-h time point appears representative of long-term effects of TGF- β on protein synthesis (Wrana, J. L., and J. Sodek, manuscript in preparation).

Cell Growth Analysis

Anchorage-dependent growth of bone cells was determined after a single 24-h exposure to TGF- β . Cells were removed from the tissue culture dishes by incubating at 37°C for 10-15 min with 1.0 ml 0.15% (wt/vol) trypsin in citrate-saline buffer and quantitatively transferred into 19 ml of Isoton (Coulter Electronics, Inc., Hialeah, FL), and the cell number determined on a Coulter Counter (model ZF; Coulter Electronics Inc., Hialeah, FL).

For anchorage independent growth, colony formation in 0.3% (wt/vol) soft agar was measured using the method of DeLarco and Todaro (6) except that a 7 ml basal layer of 0.5% (wt/vol) agar was used with fully supplemented α -MEM as the incubation medium. Mixed populations with cell densities of 10^5 cells/2 ml 0.3% (wt/vol) agar in triplicate 60-mm petri dishes were used for these studies.

Analysis of Protein Synthesis

To analyze the synthesis of cell-associated and secreted proteins, confluent cells were pulse-chased with [³⁵S]methionine. After a 24-h incubation period with or without TGF- β , triplicate 35-mm dishes were washed in methionine-free DME over a 15-min period, and each dish incubated at 37°C in 1.0 ml of the same medium containing 50 μ g/ml sodium ascorbate and 75 μ Ci [³⁵S]methionine (NEG-009H; sp act > 400 Ci/mmol; New England Nuclear, Boston, MA) for 30 min. After the pulse period the cells were washed twice in α -MEM containing 0.2% (vol/vol) FBS and incubated for a further 4 h at 37°C in 2.0 ml of the same medium. The chase media were dialyzed in the presence of proteolytic enzyme inhibitors against three changes of distilled water. Cell layers were washed three times with ice-cold TBS, pH 7.4, then scraped into 1.0 ml of 50 mM Tris/HCl, pH 7.4, containing protease inhibitors, and sonicated. Aliquots of the radiolabeled media and cell layer were analyzed by scintillation counting and by fluorography after PAGE in the presence of SDS on 5-20% cross-linked gradient gels and 7.5% cross-linked linear gels as described previously (30).

Analysis of Collagen Synthesis

Equal aliquots of the culture media containing 1-5 $\times 10^4$ dpm were freeze dried and reconstituted in 1.0 ml assay buffer (50 mM Tris/HCl, 5 mM CaCl₂, pH 7.5). Samples of 0.5 ml were incubated with and without 25 μ g highly purified bacterial collagenase in the presence of 2.5 mM *N*-ethylmaleimide at 37°C for 2 h. The undigested noncollagenous protein was then precipitated in the presence of 50 μ g BSA by ice-cold TCA, 7% (wt/vol) final concentration. The supernatant was quantitatively removed and extracted with diethyl ether to remove the TCA and then the radioactivity determined by scintillation counting. The radioactivity incorporated into collagenous proteins was determined by subtracting the radioactivity in the control from the collagenase-digested sample. The radioactivity in the non-collagenous protein was determined after extracting any residual collagen (<3% total radioactivity) from the pellet with hot TCA and dissolving the remaining pellet in 100 μ l of 70% (vol/vol) formic acid before scintillation counting.

Analysis of Collagen Types

Aliquots of culture media containing 0.5-2 $\times 10^4$ dpm were freeze-dried and redissolved in 100 μ l 0.5 M acetic acid adjusted to pH 2.2 with HCl. 50 μ g pepsin in 100 μ l of the same solution was added and the digestion carried out for 4 h at 15°C. The solution was immediately freeze-dried before analyzing by SDS-PAGE on 7.5% cross-linked gels using delayed reduction. The collagen α chains corresponding to collagen types I, III, and V were visualized by fluorography and quantitated from several exposures (12) using a laser densitometer (LKB Ultrascan XL; Fisher Scientific, Toronto, Ontario, Canada), interfaced with a Macintosh Plus. Integration of densitometric scans was performed using the Curves program developed by Dr. P. N. Lewis (Department of Biochemistry, University of Toronto, Ontario, Canada).

Analysis of Fibronectin Synthesis

Fibronectin was selectively and quantitatively bound to gelatin-Sepharose (Pharmacia, Inc., Piscataway, NJ) pre-equilibrated in TBS and contained in individual minicolumns. Aliquots of media containing 1-4 $\times 10^4$ dpm radioactivity were brought to 0.5% (vol/vol) Triton X-100, using a 20% (vol/vol) Triton stock solution in water, and allowed to percolate slowly (1 h at 22°C) through 100 μ l gelatin-Sepharose resin held in a 1-ml plastic micro-pipette tip with a small plug of glass wool. The affinity column was washed with 1.0 ml of 0.5 M NaCl in TBS followed by a 1-ml wash in TBS alone. The fibronectin was then eluted with 100 μ l of double strength SDS-PAGE sample buffer and aliquots taken for scintillation counting and SDS-PAGE analysis. Cell-associated fibronectin could not be purified sufficiently by this procedure for scintillation analysis and was, therefore, quantitated by densitometric analysis of fluorographs of the radiolabeled protein separated on SDS-PAGE using 5-20% cross-linked gradient gels. In each case the exposure of fluorographs were adjusted to permit densitometric analysis in the linear range.

Analysis of SPARC/Osteonectin Synthesis

Equal aliquots (0.5 ml) of radiolabeled culture medium were diluted with an equal volume of immunoprecipitation buffer (0.3% [vol/vol] NP-40, 0.3% [vol/vol] sodium deoxycholate, 0.15% [wt/vol] BSA in TBS, 0.02% [wt/vol] sodium azide) at a twofold concentration, and incubated with 5 μ l preimmune serum in a 1.5 ml plastic Microfuge tube (Bio-Rad Laboratories, Richmond, CA) for 2 h at 4°C. Immune complexes formed were removed from solution by adding 100 μ l protein A-Sepharose (Pharmacia, Inc.) and incubation for 1 h at 4°C. After centrifuging for 5 min at 10,000 g, the supernatant was incubated overnight at 4°C with 3 μ l rabbit anti-SPARC/osteonectin antiserum raised against porcine bone osteonectin (6a). A second 100 μ l aliquot of protein A-Sepharose was then added and incubated for 1 h. The specific immunoprecipitate obtained was pelleted by centrifugation for 5 min and washed four times with immunoprecipitation buffer. The radiolabeled protein in the final pellet was dissolved in 100 μ l electrophoresis sample buffer containing 75 μ g dithiothreitol by heating to 95°C for 10 min. Aliquots were taken for scintillation counting and analysis by SDS-PAGE on 12% cross-linked gels. Because of a slight contamination of high relative molecular mass material in the specific immunoprecipitates, densitometric analysis of fluorographs was used for quantitation.

Alkaline Phosphatase Activity

To assay for alkaline phosphatase activity, cells in 35-mm dishes were washed three times with ice-cold TBS and scraped into 0.5 ml 50 mM Tris/HCl buffer, pH 7.4, and the dishes rinsed with 0.5 ml Tris buffer. The cell layer suspension was sonicated on ice with two 10-s pulses on a sonifier at setting No. 5 (Branson Sonic Power Co., Danbury, CT). After centrifugation for 5 min at 10,000 g, 25 μ l of the supernatant was mixed with 100 μ l of substrate (7.5 mM *p*-nitrophenyl-phosphate [Sigma Chemical Co., St. Louis, MO] in 2.6 mM MgCl₂ in 0.33 M 2-amino-2-methyl-1-propanol, pH 10.3 [buffer No. 221, Sigma Chemical Co.]) in individual wells of a 96-well tissue culture plate. The plate was then incubated for 30–60 min at 30°C, the reaction being stopped by the addition of 100 μ l 0.5 N NaOH. The color generated was read at 405 nm on a Multiskan (Titertek, Elfab Oy, England) and compared against a standard curve obtained with standard alkaline phosphatase (Sigma Chemical Co.).

Results

In these studies, cell populations prepared from several separate calvarial isolates were used. The results presented below were obtained from one particular preparation of cells and were typical of the results obtained. The various bone-cell populations isolated showed different morphologies, the early released populations being enriched with fibroblastic cells and the later released cells being enriched in osteoblastic cells (23). After a 24-h exposure to TGF- β , slight morphological changes were apparent in the osteoblast-enriched populations that were more clearly observed after 4 d (Fig. 1). Initially the osteoblastic cells were predominantly polygonal in shape but in the presence of TGF- β changed to a variety of morphologies that ranged from polygonal to spindle shaped. In contrast, the fibroblastic populations retained a predominantly spindle-shaped morphology in the presence of TGF- β .

Over the same time period little difference was observed in cell numbers compared with controls, except in population III where a slight increase in cell number was apparent in the presence of 1.0 ng/ml TGF- β (Table I). TGF- β alone, or in combination with 2.5 ng/ml epidermal growth factor, did not stimulate colony formation when mixed populations of the bone cells were grown on soft agar, whereas NRK-49F indicator cells showed good anchorage-independent growth in the combined presence of TGF- β and epidermal growth factor (results not shown).

Protein synthesis, measured by incorporation of [³⁵S]methionine, in cell-layer proteins of control cultures was similar for each of the four bone cell populations, ranging from 5

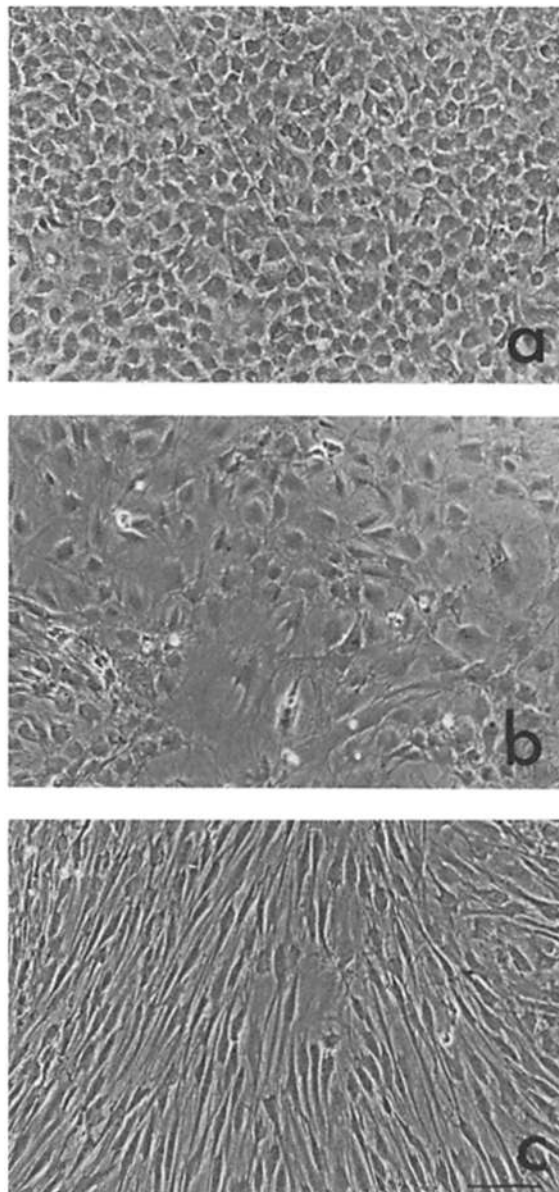


Figure 1. Phase-contrast micrographs of bone cell population IV. Cells from population IV were plated at 20,000 cells/dish and grown to confluence before incubation with TGF- β . Control cultures of population IV cells were enriched in polygonal cells, typical of osteoblastic cells in culture (a), and retained their original morphology after 96 h whereas cells incubated in the presence of 1.0 ng/ml TGF- β exhibited a variety of morphologies (b and c).

to 6 dpm/cell (Fig. 2 a). TGF- β at 0.1 ng/ml stimulated protein synthesis 1.3–1.6-fold, and at 1.0 ng/ml by 2.3–2.9-fold. Similar responses were observed for each population and, on examination of the radiolabeled proteins separated by SDS-PAGE on 5–20% gradient gels (Fig. 3 A), essentially identical patterns were apparent for all control populations and their TGF- β -stimulated counterparts. In contrast, considerable differences were seen in the radiolabeled proteins secreted by these cells. As shown in Fig. 2 b, synthesis of secreted proteins was least in population I (0.5 dpm/cell) being almost one-half the level in populations II–IV. Significant stimulation of secreted protein synthesis was evident at both

Table I. Cell Growth after 24-h Incubation with TGF- β

Treatment	Bone cell population			
	I	II	III	IV
	(cell number $\times 10^6$)	(cell number $\times 10^6$)	(cell number $\times 10^6$)	(cell number $\times 10^6$)
Control	0.62 \pm 0.08	0.62 \pm 0.06	0.59 \pm 0.08	0.50 \pm 0.08
TGF- β (1.0 ng/ml)	0.65 \pm 0.01	0.63 \pm 0.14	0.69 \pm 0.0*	0.48 \pm 0.01

* Significantly different from control ($P < 0.01$).

levels of TGF- β , ranging from 1.6–2.5-fold at 0.1 ng/ml TGF- β to 2.2–3.5-fold at 1.0 ng/ml with maximal stimulation of protein synthesis (2.75 dpm/cell) observed in population II. Analysis of the secreted proteins by SDS-PAGE on 5–20% gradient (not shown) and 7.5% linear (Fig. 3 B) gels, revealed similar profiles for all the populations with prominent radioactive bands in regions of fibronectin (220 kD), procollagens (110–160 kD), and SPARC/osteonectin (40 kD). In TGF- β -treated cultures, quantitative differences in various protein bands were apparent. The most prominent change was the appearance of a 48-kD protein which could be quantitatively immunoprecipitated with an antiserum specific for the endothelial type plasminogen activator inhibitor (results not shown). From densitometric scans, this protein was increased at least 3–12-fold, with greater stimulation observed at the higher concentration of TGF- β in populations I and IV (Fig. 2 c).

To analyze more specifically the effect of TGF- β on the synthesis of secreted proteins, individual proteins were separated and quantitated. The amount of collagen synthesized was determined by selective digestion of collagenous proteins with highly purified bacterial collagenase. Because the collagen in the cell layer represented only a small proportion of the total radiolabeled protein (<5%) the results were quite variable. Consequently, only the data for the collagen in the culture media are reported. In control cultures, the proportion of radioactivity in collagen relative to the total radiolabeled protein secreted varied from 20% in population I to 24% in populations III and IV (Table II). Upon stimulation with TGF- β , the proportion of radiolabeled collagen increased slightly, but significantly, in populations I and II with a maximal value of 27.6% observed in population II. Since the proportion of collagen was comparable between populations, the relative amounts of radiolabeled collagen synthe-

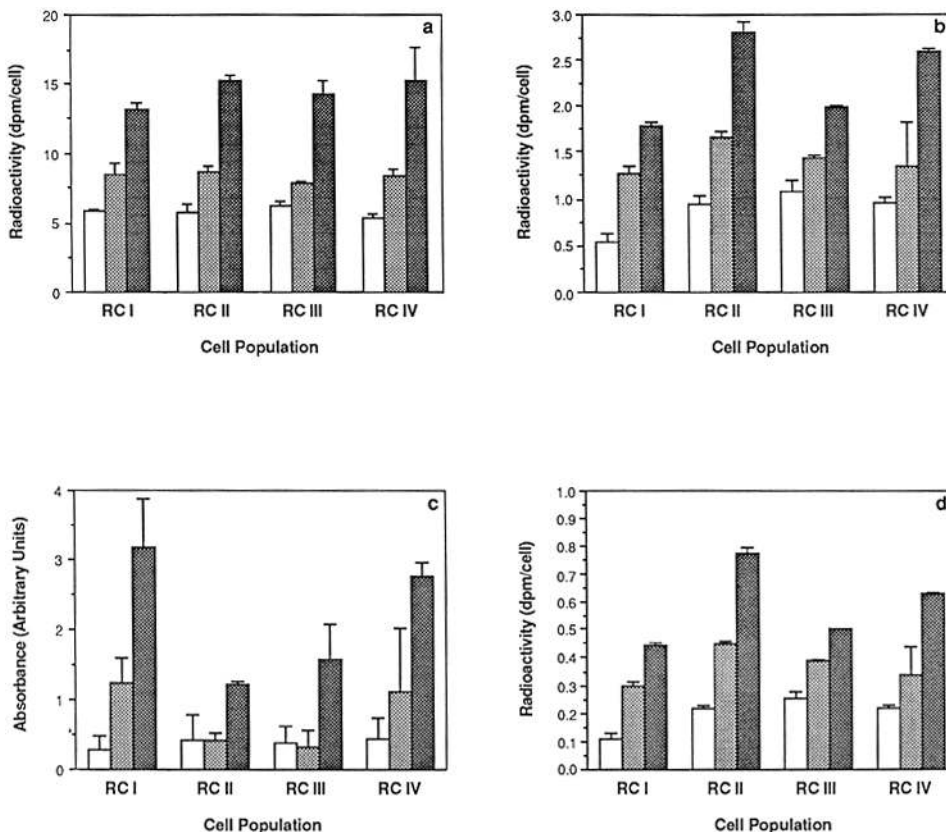


Figure 2. Quantitation of [35 S]-methionine-labeled proteins. The stimulation of protein synthesis after a 24-h incubation with 0.1 (lightly stippled bars) and 1.0 ng/ml (heavily stippled bars) TGF- β was determined from pulse-chase experiments using [35 S]methionine and compared with controls (open bars) for each of the bone cell populations. (a) Cell-associated proteins; (b) secreted proteins; (c) 48-kD protein measured by densitometry; (d) collagen measured by selective digestion with bacterial collagenase. Differences with respect to controls were analyzed for significance using Student's *t*-test. All increases were significant ($P > 0.01$ – 0.001) with 1.0 ng/ml and also for 0.1 ng/ml TGF- β except for RC IV population in b, RC II, RC III, and RC IV populations in c, and RC IV population in d.

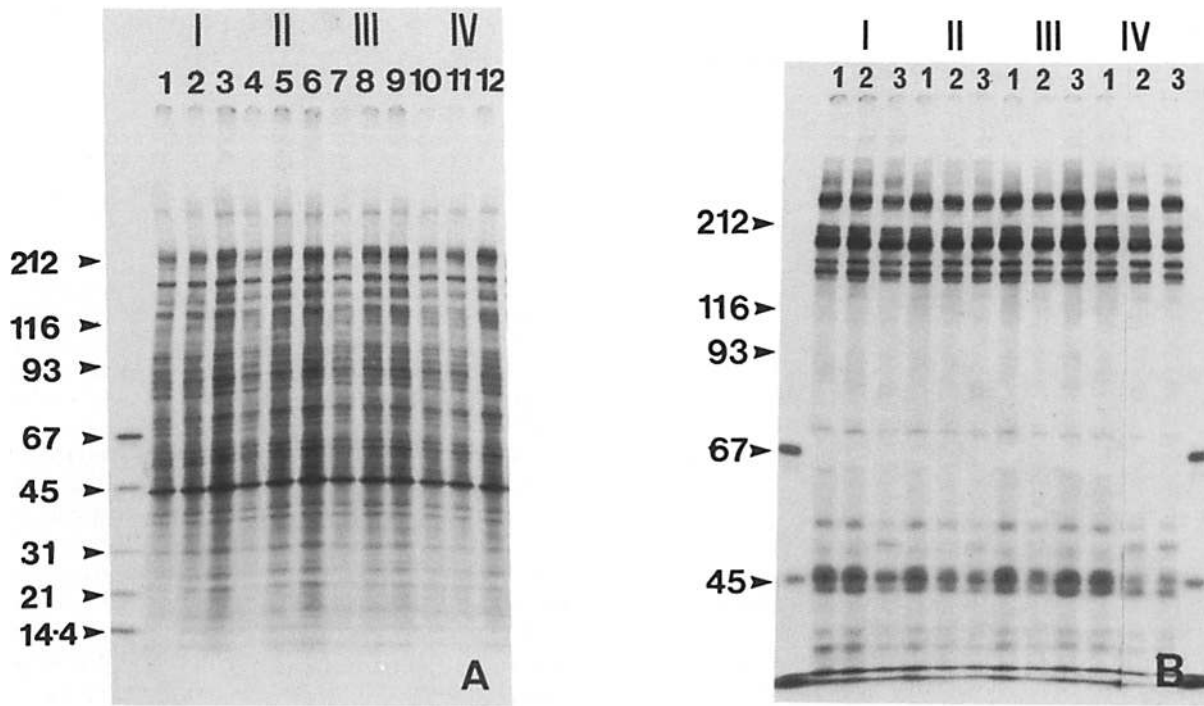


Figure 3. Analysis of [³⁵S]methionine-labeled proteins by SDS-PAGE and fluorography. Aliquots containing approximately equal amounts of radioactivity from the cell layer (A) and chase medium (B) from each of the four bone cell populations (I-IV) maintained for 24 h in the absence or presence of TGF-β were analyzed by SDS-PAGE on 5–20% gradient (cell layer proteins) and 7.5% linear (medium proteins) gels under reducing conditions. (A) Lanes 1, 4, 7, and 10, controls; lanes 2, 5, 8, and 11, 0.1 ng/ml TGF-β; lanes 3, 6, 9, and 12, 1.0 ng/ml TGF-β. Note the similar profiles for the radiolabeled proteins in the cell layer samples with increased band intensities in the position of fibronectin in the TGF-β-stimulated cells. (B) Lane 1, control; lane 2, 0.1 ng/ml TGF-β; lane 3, 1.0 ng/ml TGF-β.

sized (shown in Fig. 2 d) parallel the results obtained for secreted proteins shown in Fig. 2 b. Thus, stimulation of collagen synthesis ranged from 1.74-fold in population IV, with 0.1 ng/ml TGF-β, to four-fold in population I, at 1.0 ng/ml TGF-β.

To determine whether or not TGF-β has a selective effect on the type of collagen synthesized, media samples from populations I and IV were digested with pepsin and the α chains of collagen types I, III, and V were separated by SDS-PAGE (Fig. 4) and quantitated by densitometric analysis of fluorographs. Type I collagen was the major collagen with type III and V collagens comprising $16.8 \pm 2.5\%$ ($\bar{X} \pm \text{SEM}$) and $\sim 1\%$, respectively, in population I and $10.8 \pm 0.5\%$ and $2.3 \pm 0.6\%$, respectively, in population IV. As shown in Fig. 4, several high and low relative molecular mass pepsin-resistant proteins that have not been characterized also appear to be differentially affected by TGF-β. In the

presence of 1.0 ng/ml TGF-β, the proportion of type III collagen synthesized was decreased by 50% to $8.1 \pm 0.3\%$ in population I and by 35% to $7.0 \pm 0.5\%$ in population IV.

Fibronectin secreted into the culture medium was isolated by affinity chromatography on gelatin-Sepharose and then analyzed by SDS-PAGE and fluorography. A major protein band migrating in the position expected for fibronectin was observed with a minor band at 70 kD, demonstrating the effectiveness of the affinity chromatography (Fig. 5). Quantitation of the radioactivity eluted from the affinity column by direct counting and by densitometric analysis of the protein on fluorographs revealed that similar amounts of radiolabeled fibronectin were secreted by each population and this represented $\sim 5\%$ of the secreted proteins (Fig. 6 a). However, because the amount of total secreted proteins is much less in population I (see Fig. 2), fibronectin is proportionately higher in this population. TGF-β at 0.1 ng/ml only in-

Table II. Percentage of [³⁵S]Methionine Incorporated into Collagens

Treatment	Bone cell population			
	I	II	III	IV
Control	20.4 ± 0.8	22.9 ± 0.8	24.0 ± 1.1	24.2 ± 0.3
TGF-β (0.1 ng/ml)	23.8 ± 0.3*	26.9 ± 0.5‡	27.1 ± 0.2*	24.4 ± 0.4
TGF-β (1.0 ng/ml)	25.0 ± 0.7‡	27.6 ± 0.1‡	25.1 ± 1.4	22.8 ± 1.1

* Significantly different from control ($P < 0.01$).
 ‡ Significantly different from control ($P < 0.005$).

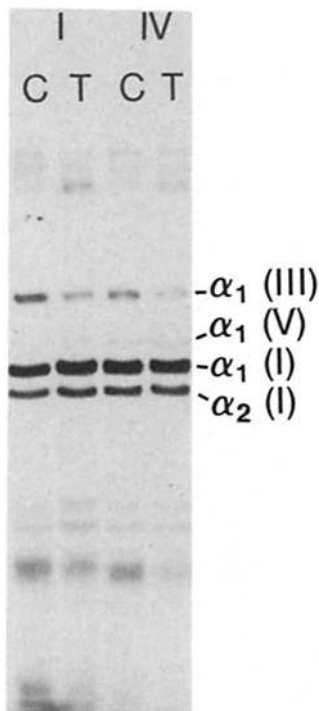


Figure 4. Analysis of collagen types by SDS-PAGE and fluorography. Aliquots of culture medium containing 10,000 dpm radioactive protein were digested with pepsin at pH 2.2 for 4 h at 15°C. The pepsin-resistant collagens were denatured and the constituent α chains separated by SDS-PAGE on 7.5% cross-linked gels using delayed reduction. The relative amounts of each α chain was determined by densitometric scans of the fluorographs. Samples of control (C) and TGF- β (1.0 ng/ml)-treated (T) medium from cell populations I and IV are shown.

creased fibronectin significantly in population IV, but at 1.0 ng/ml fibronectin was increased in populations I, III, and IV, with a 3.5-fold increase in population IV. Since large amounts of fibronectin are also found associated with cell surfaces, the radiolabeled fibronectin in the cell layers was also quantitated. TGF- β increased the amount of radiolabeled fibro-

nectin in the cell layers of each population; in the presence of 1.0 ng/ml TGF- β the degree of stimulation varied from threefold for population IV to eightfold for population III (Fig. 6 b).

When specific immunoprecipitates, prepared with anti-osteonectin antibody, were analyzed by SDS-PAGE the major protein migrated as a distinct doublet with a relative molecular mass of 40 kD (Fig. 7). Small amounts of radiolabeled protein in the procollagen region were the only contaminants. These proteins were not enriched and were selectively removed during repeated washings, indicating that they were not specifically associated with the antibody. A similar pattern for the specific immunoprecipitate, including the presence of a doublet for osteonectin, was seen in earlier studies of porcine bone cells (20). Under the culture conditions used, SPARC/osteonectin isolated by immunoprecipitation was synthesized in appreciable amounts by all cell populations and represented $\sim 2\%$ of the radiolabeled proteins secreted. In the presence of TGF- β at both concentrations, the synthesis of SPARC/osteonectin was markedly stimulated in populations I and II, with little or no stimulation in populations III and IV (Fig. 8). In most instances maximal or near maximal stimulation was observed with the lower level of TGF- β .

Since alkaline phosphatase activity is characteristically high in osteoblastic cells, the basal levels of this enzyme were measured together with changes effected by TGF- β in population I and IV cells. As expected, the basal enzyme levels were higher in the osteoblast-enriched populations (2.4 ± 0.2 μ moles substrate hydrolyzed per hour per 10^6 cells) compared with the fibroblastic cells (1.2 ± 0.1 μ moles/h/ 10^6 cells). In the presence of TGF- β , a reduction in activity was

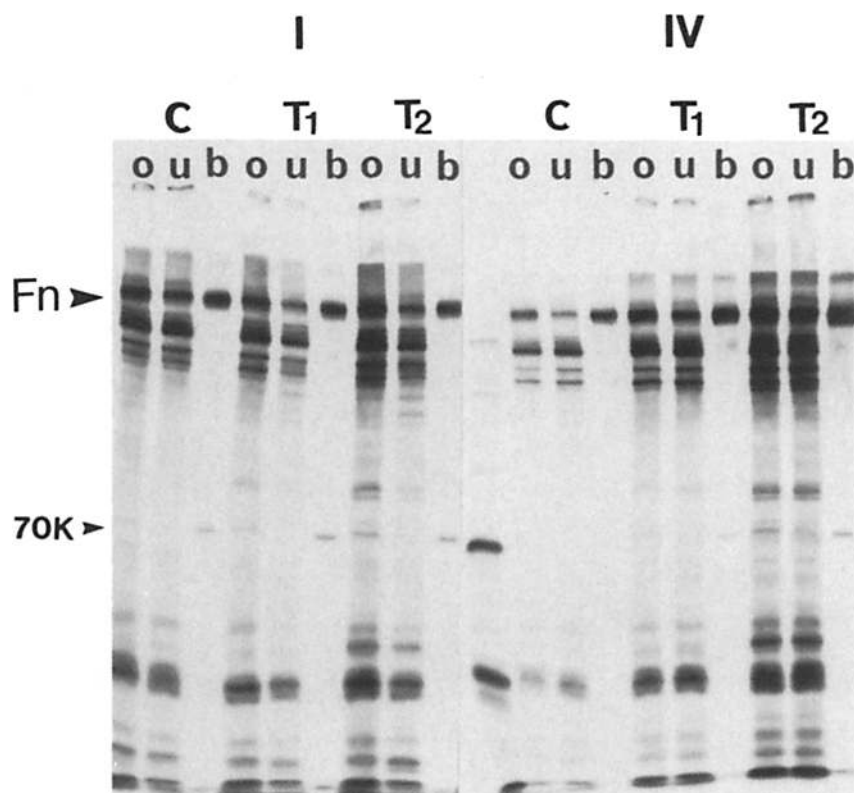


Figure 5. Isolation of fibronectin. Radiolabeled fibronectin from equal aliquots of culture medium was quantitatively absorbed to gelatin-Sephadex. The radiolabeled proteins in various fractions of the isolation were analyzed by SDS-PAGE on 7.5% gels. Control cultures (C) and cells treated with 0.1 (T_1) and 1.0 ng/ml TGF- β (T_2) from populations I and IV are shown. o, original sample of radiolabeled medium proteins; u, unbound radiolabeled proteins; b, specifically bound radiolabeled proteins. Some 70-kD, gelatin-binding protein (70K) was also specifically bound in the fibronectin (Fn) preparation.

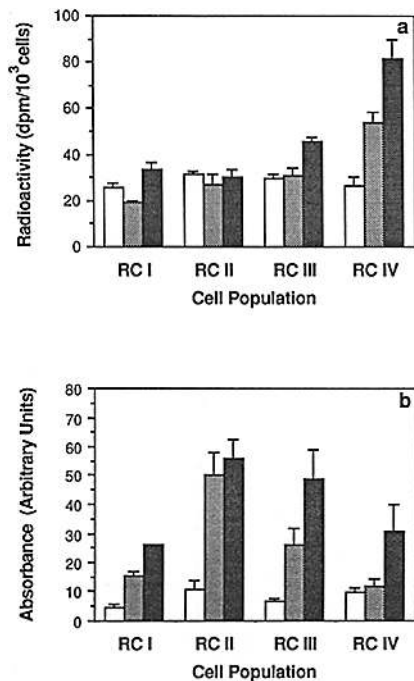


Figure 6. Stimulation of fibronectin synthesis by TGF- β . Radiolabeled culture medium was incubated with gelatin-Sepharose as shown in Fig. 5 and the bound fibronectin (*Fn*) quantitated by scintillation counting (a). Since the fibronectin in the cell layer was not sufficiently purified by gelatin-affinity chromatography, quantitation was achieved by densitometric analysis from fluorographs (b). (Open bars) Controls; (lightly stippled bars) 0.1 ng/ml TGF- β ; (heavily stippled bars) 1.0 ng/ml TGF- β . Increases were significant according to Student's *t*-test for 1.0 ng/ml TGF- β in all ($P > 0.01$ – 0.001) except RC II (a) and for 0.1 ng/ml TGF- β , except RC I and RC III (a), and RC IV (b).

observed in both populations (0.6 ± 0.1 μ moles/h/ 10^6 cells, a 50% reduction in population I; 1.0 ± 0.1 μ moles/h/ 10^6 cells, a 58% reduction in population IV).

Discussion

Because bone cells are highly heterogeneous (2), discrete populations of cells enriched in fibroblastic or osteoblastic cells were prepared to study the effects of TGF- β . Population IV cells were polygonal in morphology, showed a good cAMP response to parathormone (23), had relatively high alkaline phosphatase activity, and synthesized type I collagen almost exclusively; these are features that are characteristic of osteoblastic cells. In contrast, population I cells were mostly spindle shaped, synthesized higher amounts of type III collagen, and expressed lower levels of alkaline phosphatase. Despite these distinct phenotypic differences, the radiolabeled proteins synthesized by these different populations were qualitatively similar (Fig. 2).

Since TGF- β at 0.1 and 1.0 ng/ml gives half maximal and maximal responses, respectively, in anchorage-independent growth assays (24) and in stimulating protein synthesis by human gingival fibroblasts *in vitro* (39), these concentrations were chosen to study effects on the calvarial cell populations. Despite the stimulation of cellular protein synthesis (Fig. 2 a), TGF- β did not demonstrate a mitogenic effect on popula-

tions I, II, and IV. These results are consistent with previous studies of TGF- β effects on human gingival (39) and lung (7) fibroblasts, and on rat calvarial cells (4). However, the lack of response in the osteoblastic population differs from the observations of Centrella, McCarthy, and Canalis (4) who reported a mitogenic effect of TGF- β on a fetal rat calvarial cell population comprising population III and IV cells. Notably, the mitogenic effects were cell density dependent and were more pronounced at levels of TGF- β that were higher than used in this study. Although we observed a slight mitogenic effect on population III cells, this was not evident in the most osteoblastic population (IV) which, after prolonged incubation, often showed reduced cell numbers (results not shown). It is possible that TGF- β has a mitogenic effect at higher concentrations, or it may exert a mitogenic effect on a subpopulation of bone cells that elute earlier than most of the osteoblastic cells.

The TGF- β -induced stimulation of collagen and fibronectin synthesis, which may influence the change in the osteoblast morphology (Fig. 1), has been suggested to be responsible for the induction of anchorage-independent growth (9). However, TGF- β alone, or in combination with epidermal growth factor, was unable to cause anchorage-independent growth of the calvarial cells, as observed previously with other freshly isolated normal cells in which matrix protein synthesis is stimulated by TGF- β (39). Thus, the induction of anchorage-independent growth that has been used to characterize TGF- β appears to be a phenomenon exhibited typically by established, nontumorigenic cells, and is unlikely, therefore, to have physiological significance.

The two- to threefold stimulation of protein synthesis observed in cell layers in the presence of 1.0 ng/ml TGF- β (Fig. 2 a) was similar in all the populations and is also consistent with results obtained previously with human gingival fibroblasts (39). The majority of these proteins are cellular proteins that appear to be stimulated collectively by the action of TGF- β . TGF- β also stimulated the synthesis of the total secreted proteins to a similar degree, as observed previously for fibroblasts (7, 39). However, the stimulation was more selective with the greatest increase observed in plasminogen activator inhibitor synthesis by cell populations I and IV (Fig. 3). Notably, plasminogen activator inhibitor has also been shown to be stimulated preferentially by TGF- β in several epithelial (34) and human lung fibroblast lines (11, 13).

Since the majority of collagen synthesized by cells in culture is secreted into the culture medium within 2 h (10, 36), radiolabeled collagen was measured in 4-h chase medium after a 30-min pulse with [³⁵S]methionine. In each population collagen synthesis was stimulated by TGF- β ; in most cases the stimulation was slightly greater than that observed for the total secreted proteins, resulting in a higher percentage of radiolabel being incorporated into collagenous proteins (Table II). A preferential stimulation of collagen has been observed in embryonic lung fibroblasts (7) but not in adult gingival fibroblasts (39), indicating possible age related differences in the response of fibroblasts to TGF- β . The stimulation of collagen synthesis by TGF- β in the absence of significant cell division supports the concept that cell growth and matrix synthesis are unrelated (4, 7, 39). In fetal bone cells TGF- β appears to act preferentially on type I collagen synthesis since little, if any, stimulation of type III collagen synthesis occurred in population I cells. Similarly, in popula-

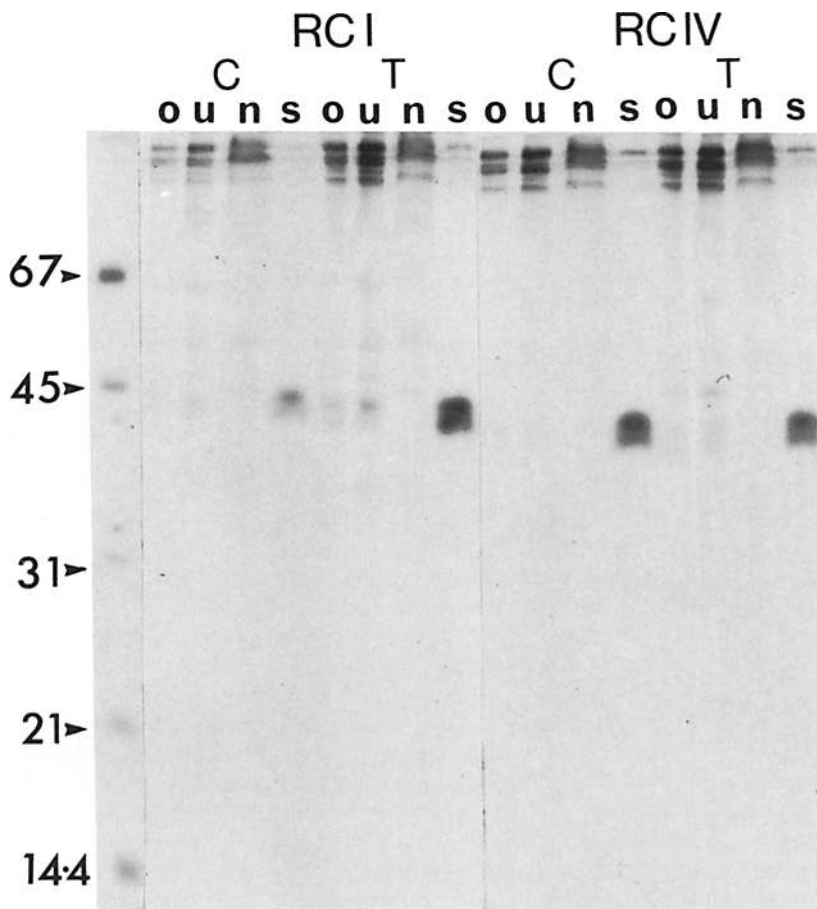


Figure 7. Immunoprecipitation of SPARC/osteonectin. Rabbit anti-porcine osteonectin antibody coupled to protein A-Sepharose was used to immunoprecipitate SPARC/osteonectin from equal aliquots of culture media. Samples from control (C) and 1.0 ng/ml TGF- β -treated (T) culture medium for rat calvarial populations I (RC I) and IV (RC IV) are shown. o, original medium sample; u, unbound material; n, radiolabeled protein bound nonspecifically to protein A-Sepharose; s, radiolabeled protein bound specifically to osteonectin antibody.

tion IV cells, type III collagen was stimulated less than two-fold compared with a threefold stimulation of total collagen. As there was no significant cell growth in the presence of TGF- β , these results are not due to preferential proliferation of cells that constitutively produce lower amounts of type III collagen, but indicate a differential effect on collagen gene

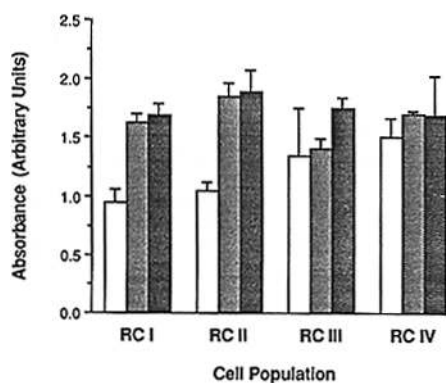


Figure 8. Stimulation of SPARC/osteonectin synthesis. SPARC/osteonectin specifically immunoprecipitated and analyzed by SDS-PAGE as shown in Fig. 7 was quantitated by densitometric scanning of fluorographs. (Open bars) Controls; (lightly stippled bars) 0.1 ng/ml; (heavily stippled bars) 1.0 ng/ml TGF- β ; Increases at both levels of TGF- β for populations RC I and II were significant ($P > 0.001$) whereas the differences in RC III and RC IV were not significant.

expression. This contrasts the observations of Fine and Goldstein (7) who found no selectivity in the action of TGF- β on type I and III collagen synthesis by human lung fibroblasts.

TGF- β also stimulated the synthesis and secretion of fibronectin in each of the populations. Radiolabeled fibronectin was much greater in the medium of osteoblast-enriched population whereas the greatest increase in cell-associated fibronectin was observed in population I. These differences in stimulation of secreted versus cell-associated fibronectin may simply reflect a differential distribution of the protein. However, since fibronectin is known to be synthesized in various forms as a result of differential mRNA splicing (19), it is possible that the various forms of fibronectin may distribute differently and may be regulated independently.

The effects of TGF- β on SPARC/osteonectin synthesis appeared to be more selective for the different cell types. Whereas TGF- β stimulated SPARC/osteonectin synthesis in the fibroblast-enriched populations, which synthesize lower basal levels of this protein, TGF- β had little effect on SPARC/osteonectin synthesis by the osteoblast-enriched population IV cells. Since the osteoblast-enriched populations would include some fibroblastic cells, in which SPARC/osteonectin is stimulated, the net effect may reflect an inhibition of SPARC/osteonectin synthesis by osteoblasts. However, as the function of SPARC/osteonectin is unknown, the significance of this result is unclear. Nevertheless, SPARC/osteonectin is a major protein secreted by fibroblastic (37)

and osteoblastic (20) cells as well as endodermal (15, 16), endothelial (26, 27), and epithelial (14) cells, and a potential role for this calcium-binding protein has been indicated in both cellular differentiation (16) and in wound healing (27).

This study has shown some differences in the expression of matrix proteins by osteoblast- and fibroblast-enriched bone cell populations and a differential modulation of these proteins by TGF- β . The strong stimulation of protein synthesis by TGF- β in all populations indicates a general increase in cell activity and connective tissue formation that could be important both in bone remodeling and fracture healing. The quantitative differences in the stimulation of matrix proteins is likely to alter the nature of the matrix formed and consequently influence cell shape and behavior. The decrease in alkaline phosphatase activity in osteoblastic cells caused by TGF- β is consistent with TGF- β effects on a mouse osteoblastic cell line MC 3T3-E1 (18) and suggests a suppression of the osteoblast phenotype, as indicated also by the morphological changes. However, the decrease in type III collagen synthesis and the stimulation of bone phosphoprotein (Kubota, T., J. L. Wrana, C. Domenicucci, W. T. Butler, and J. Sodek, unpublished data) by TGF- β indicate that not all osteoblastic characteristics are suppressed.

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