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Thrombospondin co-localises with TGFβ and IGF-I in the extracellular matrix of human osteoblast-like cells and is modulated by 17β estradiol

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Abstract. Thrombospondin (TSP) is a multifunctional glycoprotein which is synthesised by several cell types including osteoblasts, and incorporated into the extracellular matrix (ECM) of these cells. The function and regulation of TSP in bone is not clear. In this study, using a long term culture model of human osteoblast-like cells, we examined the distribution of TSP in the ECM and its modulation by added estradiol. In this model the osteoblast-like cells form a regular multilayer which continues to increase in depth up to 50 days post confluence. In the ECM of these cultures and in 19-week fetal bone, the bone markers osteocalcin and alkaline phosphatase were diffusely distributed in the matrix. In contrast, labelling for TSP was concentrated, confined to the banded collagen and its immediately adjacent ECM. This pattern of labelling resembled that of the growth factors transforming growth factor β-I (TGFβ), and insulin-like growth factor-I (IGF-I), with which TSP label co-localised. Labelling intensities were comparable between fetal bone and the in vitro material for TSP, TGFβ and IGF-I. TSP label was present by 10 days post confluence, reached a maximum by 20 days, and declined slowly thereafter, a time course which was similar to that of IGF-I. Incubation of osteoblast-like cell cultures with 17β estradiol resulted in an increase in multilayer depth and a maximal 3-fold increase in TSP labeling at 30 days as well as approximately 2-fold increases for TGFβ and IGF-I. The dose-response relationship for these responses to estradiol treatment was biphasic with maximal increases at 10^{-10} M–10^{-11} M of added estradiol. Treatment with 17α estradiol produced labelling intensities that were not significantly different from controls. Studies with other cell types have suggested that TSP may be involved in modulation of growth factor activity. The similarities between TSP, TGFβ and IGF-I, in terms of their distribution and regulation by 17β estradiol treatment, may indicate a role for TSP in modulating bone cell proliferation and function through interaction with local growth factors.
Key words. Human; bone; thrombospondin; growth factors.

Thrombospondin (TSP) is a multidomain multifunctional glycoprotein. In its native state it has a molecular weight of 450 kilodaltons (kD) and consists of three chains with globular regions at each terminus[1–3]. At least four genes encoding for thrombospondin have been described[4–7]. TSP has a large number of binding sites for heparan sulfate and other sulfated glycosaminoglycans, sulfated glycolipids, collagen types I to V, von Willebrand factor, thrombin, fibrinogen, fibronectin, laminin, plasminogen, plasminogen activator, osteonectin, histidine-rich glycoproteins and platelets[1–3,8–13]. The carboxy globular domain contains a calcium binding site and also an RGD sequence, implicated in cell attachment[3].

TSP is produced by a variety of cells including human osteoblasts, osteosarcoma cells, fibroblasts, monocytes and macrophages, vascular endothelial cells, granular type II pneumocytes, keratinocytes, glial cells, and aortic smooth muscle cells[1,3,9,14–17]. TSP is also the most abundant protein in platelet α granules and is released during platelet activation in response to thrombin[11,12]. TSP binds to osteonectin, fibrinogen, transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and insulin-like growth factor I (IGF-I) and 2 (IGF-II) in the α granules of platelets. These substances are released together during degranulation[3,13,18–23]. In wounds, TSP binds platelets to fibrinogen[10], causes rapid binding and spreading of platelets on the endothelial substratum[24] and promotes the attachment of osteoblasts[14].

The role of thrombospondin (TSP) in bone is not clear[3,12]. It may influence both proliferation and differentiation in osteoblasts. Several studies have shown that TSP promotes osteoblast proliferation and cell attachment to the ECM[12,16,25,26] probably via its carboxy globular domain[2,3]. In MC3T3 osteoblast-like cells, TSP mRNA levels increase during osteoblastic differentiation[4] whereas in other cell types, TSP expression appears to be increased during proliferative phases[27].

Bone cell ECM components may play a significant role in the shutdown of osteoblast proliferation and subsequent differentiation into the mature osteoblast phenotype. TSP may influence this process by modulating cell shape and cell-matrix interactions[4].

Osteoblast-like cell responses to estradiol may depend on the state of differentiation of the cell[28]. The anabolic actions of estrogen on bone may be mediated by alterations in the levels of growth factors produced by the

osteoblast in response to estrogens. Cultured bone cells have both estrogen and androgen receptors. Estrogen increases the secretion of IGF-I in UMR106 osteosarcoma cells[29,30] and bone cells in vitro[31,32] while both estrogen and androgen increase transforming growth factor beta (TGFβ) mRNA[33,34]. Growth hormone and 17β estradiol increase IGF-I and collagen type I production in cultures of human bone cells[31,35]. Gray et al.[29,30] reported increases in the production of both IGF-I and II in response to estradiol by the osteosarcoma-derived clonal cell line UMR106.

Studies on other clonal osteosarcoma cell lines and primary cultures of osteoblast-like cells, both rodent and human, have shown that basal levels of mRNA for both IGF-I[35] and TGFβ[33] are stimulated by estradiol. In addition to their mitogenic actions on cells of the osteoblast lineage[23,32,35–37] growth factors may mediate the actions of estrogen on collagen synthesis as indicated by an increase in pro α1(I) collagen mRNA levels in response to TGFβ and IGF-I[38]. As well as the estrogenic effect on local growth factor production, the responses of bone cells to growth factors may also be modulated by estrogenic effects on growth factor binding sites as reported for androgens[34], or through altered bioavailability via changes in carrier protein concentration, as reported for IGF-I in rat calvarial osteoblast-like cells[32,39]. PDGF and TGFβ have been shown to significantly increase TSP mRNA in both rat vascular smooth muscle and human mesangial cells[40]. The modulation of TSP by growth factors, or vice versa, have not been demonstrated in human osteoblast-like cells however. Since TSP has also been shown to modulate TGFβ activity[13], we sought to examine the distribution and labelling intensity of TSP in bone and in a long term human osteoblast-like formation model[41] under basal conditions and after estrogen treatment.

Materials and methods

All chemicals including culture media were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Salt solutions and fetal calf serum (FCS) were obtained from Cytosystems Pty Ltd (Sydney, Australia). Cell culture medium, BGJb Fitton Jackson Modification, was obtained from Gibco (Sydney, Australia). Ethanol, acetone, sodium dihydrogen ortho-phosphate dihydrate and disodium hydrogen orthophosphate dodecahydrate were obtained from Ajax Chemicals (Sydney, Australia). Sodium hydrogen carbonate was obtained from BDH Chemicals (Kilsythe, Australia). Cacodylate buffer, osmium tetroxide, Spurr's resin, LR White resin, glutaraldehyde and BEEM capsules were all obtained from Alltech (Sydney, Australia). 1,25 dihydroxyvitamin D_3 was kindly provided by Roche (Sydney, Australia). 17β and α estradiol were purchased from Sigma (St. Louis, MO, USA). Human parathyroid hormone (PTH) was purchased from Peninsula Labs (Belmont, CA, USA).

Tissue culture materials. Plastic culture flasks and multiwell plates were obtained from Nunc Inter Med (Roskilde, Denmark) and Becton Dickinson Labware (NJ, USA). Thermanox® coverslips were obtained from Nunc Inc. (IL, USA).

Cell culture. The trabecular ends of long bones were dissected from 5 donors of human fetal tissue* estimated to be of 17–20 weeks gestation. Some bone material was kept and processed for electron microscopy intact, as a control. After removal of the periosteum, the whole bone end was minced and the resulting fragments were washed extensively before plating onto 25 cm^2 flasks (Nunc Inter Med, Roskilde, Denmark) in BGJ medium[42] containing 10% (v/v) FCS and supplemented with penicillin 30 mg/ml and streptomycin 40 mg/ml (Commonwealth Serum Laboratories, Parkville, Australia). After 2–4 weeks incubation the cells growing out from the explanted bone chips reached confluence and were subcultured into 24-well plates (Becton Dickinson Labware, NJ, USA) on Thermanox® coverslips (Nunc Inc., IL, USA). Cells were plated at a concentration of 50,000 cells/cm^2 and maintained in BGJ without antibiotics and supplemented with 1.97 mM phosphate, 10% FCS, and 30 µg/ml ascorbic acid, which was added every other day. Following overnight incubation, media was aspirated and experimental media was added. The cells were maintained for the duration of the study with media changes every other day. Long term cell culture media containing additives or vehicle were supplemented with ascorbic acid 30 mg/ml at each media change.

The 17β estradiol stock solution was added in spectroscopic grade ethanol so that final concentration of vehicle did not exceed 0.1%. In previous studies this concentration of ethanol had been shown not to affect normal osteoblast-like cell function or proliferation. Dilutions of 10^{-9} M, 10^{-10} M and 10^{-11} M 17β or α estradiol were prepared and added to separate cultures.

Biochemical analyses. Cultures were analysed for their response to PTH as previously described[43]. Osteocalcin measurements were made by radioimmunoassay of serum-free conditioned medium using a commercially available kit as described previously[44]. Alkaline phosphatase was measured in the culture wells using a modification of the method of Lowry et al.[45] with p-nitrophenol phosphate as a substrate. Enzyme activity was expressed as units/well where 1 unit represented the

* The experimental protocol conformed to the guidelines of the National Health and Medical Research Council of Australia for the use of human fetal tissue and was approved by the Sydney University Medical Ethics Review Committee.

amount of enzyme activity that liberated 1 μM of p-nitrophenyl at 37 °C in 1 min.

Preparation of multilayers for ultrastructural examination and immuno electronmicroscopy. Three sets of long term cultures were processed for ultrastructural examination and immuno electronmicroscopy as previously described[41].

Measurement of multilayer depth. To standardise the measurements, only one brand of 300 mesh copper grids was used (Alltech, Australia). The interior aspect of each single grid square bar was 63 μm ± 2.0 (information supplied by manufacturer). By dividing this length visually into 10 subdivisions, multilayer depth measurements were made along the length of each multilayer.

Cell to ECM ratio. The ratio of cell area to extracellular matrix area was measured by image analysis using a Noran TN-8502.

Antibodies. The monoclonal anti-thrombospondin antibody from Sigma did not differentiate between TSP 1 and TSP 2. Monoclonal anti-thrombospondin (Sigma) does not cross-react immunologically with human thrombin, fibrinogen, fibronectin, laminin or serum albumin[51-54]. The TGFβ (monoclonal) antibody was from British Biotechnology (Oxford, UK); the IGF-I (polyclonal) antibody was from Chemicon (Temecula, USA); the IGF-II (polyclonal) antibody was from Research and Diagnostic Antibodies (Berkley, USA); and the bFGF (polyclonal) antibody was from Biomedical Technologies (Stoughton, USA). The TGFβ antibody has no cross-reactivity with acidic or basic FGF, human PDGF, human IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, TNFα, TNFβ, GM-CSF or G-CSF as determined by ELISA. The IGF-I antibody shows only 8% cross-reactivity with IGF-II. The IGF-II antibody exhibits 1% cross-reactivity with IGF-I, <1% cross-reactivity with human insulin, and no cross-reactivity with atrial natriuretic polypeptide, ACTH, calcitonin, somatostatin 28 or vasoactive polypeptide.

Controls. Tissue that was previously known to stain positively for each respective growth factor was used as a positive control. Negative controls for each labelling parameter were established by the substitution of the specific antibody with normal rabbit serum (1:2 dilution), drops of BSA/PBS and omission of the primary antibody. This resulted in the presence of only occasional particles of gold across the entire ultrathin section. Any such background labelling was deducted from the gold probe count data (per square micron).

As an additional control, micrographs of the resin adjacent to the cell multilayers were also produced for each labelling parameter. Any labelling of the resin per se recorded in these micrographs was quantified and also deducted from the data prior to statistical analysis.

The sections were also incubated with a mouse mono-

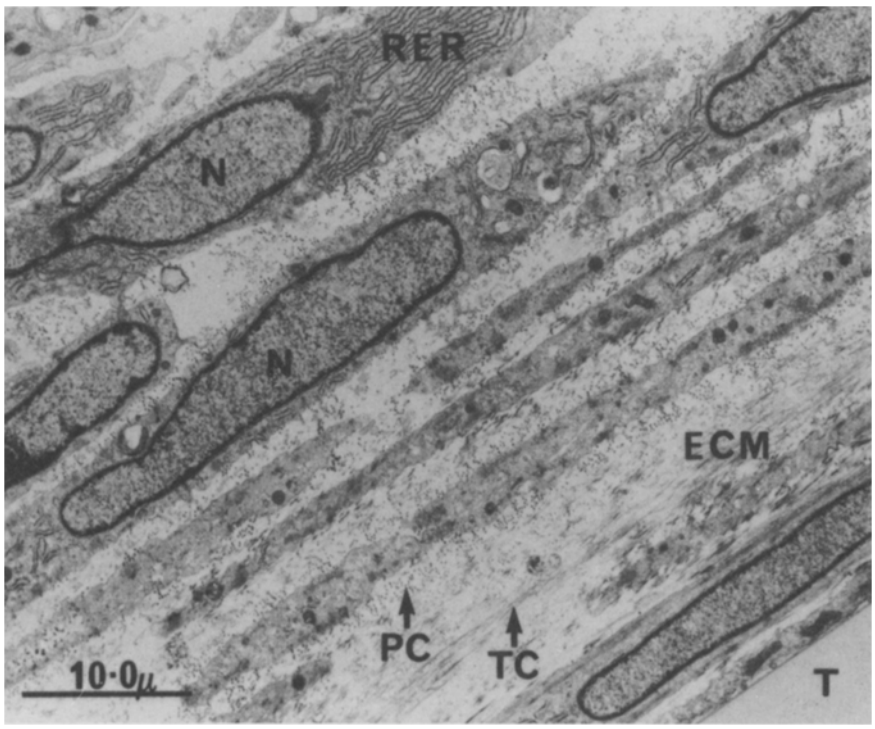

Figure 1. Transmission electron micrograph (TEM) of human fetal osteoblast-like cell multilayer at 20 days post confluence. Letters indicate Thermanox coverslip (T), collagen fibres transverse to plane of section (TC arrow), collagen fibres perpendicular to plane of section (PC arrow), extracellular matrix (ECM), cell nucleus (N), rough endoplasmic reticulum (RER). Fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, uranyl acetate and lead stain. Original magnification 8,000×. Bar 10 μm.

clonal antibody of the IgM isotype, which does not react with any known human protein (Silenus). IgM aggregates may sometimes cause non-specific labelling. This procedure resulted in no apparent labelling in this case, however.

Immunoabsorption studies. A solution of human osteocalcin containing 20 µg of osteocalcin in 200 µl of distilled water was prepared. 20 µl of this solution was thoroughly mixed with 20 µl of a 1 in 50 dilution of osteocalcin antibody, and incubated with the osteoblast-like cell multilayers as previously described. No immunogold labelling resulted. As a positive control, a dilution of 1 in 50 osteocalcin antibody was mixed with 20 µl of distilled water and simultaneously incubated. This resulted in positive labelling of an intensity similar to that previously described.

Similarly, 1 µg of human transforming growth factor beta (TGFβ) was mixed with 30 µl of distilled water. This was thoroughly mixed with 30 µl of a 1 in 50 dilution of TGFβ antibody, and incubated with the osteoblast-like cell multilayers as previously described. No immunogold labelling resulted. A positive control comprising 30 µl of a 1 in 50 dilution of the TGFβ antibody was mixed with 30 µl of distilled water and also simultaneously incubated. This resulted in positive labelling of an intensity similar to that previously described. Similarly, immunoabsorption of both the bFGF antibody with bFGF and IGF-I with the IGF-I antibody resulted in no labelling.

Quantitation. The electron microscope was calibrated using a grating replica of known dimensions. The correct magnification value for each print was determined from these data. Each block from the 5 donors was sectioned and each section fully scanned. Representative micrographs were taken from all parts of the multilayer. The quantitation protocol chosen was the direct particle counting method[46]. For each print magnification value, a square corresponding to one square micron was cut in a piece of white card. Immunogold labelling probe counts per square micron were made using these cards. At least 5 labelling probe counts were made for each print. For each labelling parameter at least 10 different

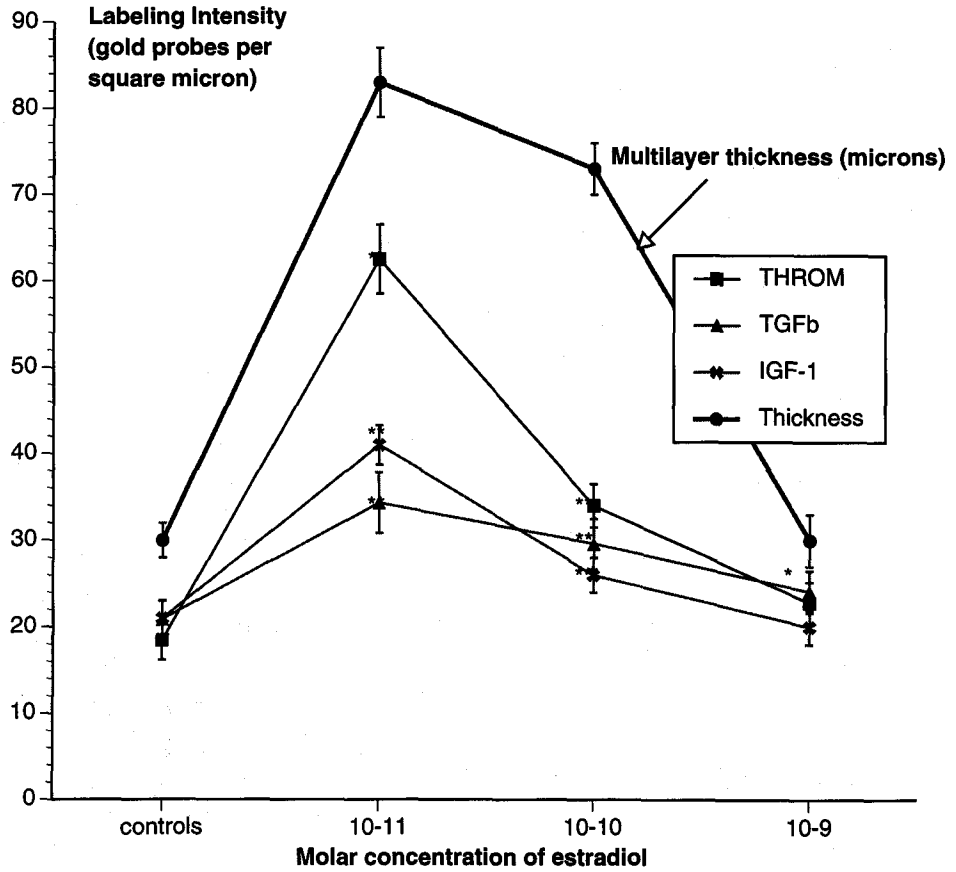

Figure 2. Labelling intensity in mean gold probes per square micron of thrombospondin, transforming growth factor beta and insulin-like growth factor 1, with the addition of 10^{-11} M, 10^{-10} M and 10^{-9} M exogenous 17β estradiol. Also, increases in multilayer thickness after addition of 10^{-11} M, 10^{-10} M and 10^{-9} M exogenous 17β estradiol. Cultures maintained on Thermanox coverslips were examined by immuno electronmicroscopy at 30 days post confluence. Points indicated by asterisks were significantly different from controls, $^{**}p < 0.0001$ and $^{*}p < 0.05$.

prints per donor were examined. Use of this number of prints per donor had previously allowed statistically significant variations in culture labelling to be detected under different conditions[41]. Results were expressed as the mean $\pm$SD. The statistical results were calculated using either Student's t test for unpaired samples, or a one way analysis of variance coupled with a Ryan's Q test.

Electron dispersive spectroscopy. The multilayers were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for one hour. No osmium or heavy metal block stains were used. After TEM processing and embedding in LR White resin, in the manner previously described, one micron sections were cut but not grid stained with heavy metals. These multilayers were examined at 120 kv using a Phillips EM 12 equipped with an EDAX 9900 system.

Results

The explanted bone chips were seen to produce cells within 24 hours of seeding. Bone cultures were established from several donors with similar results seen in each case. The cells displayed several osteoblast-like features.

Radioimmunoassay of the supernatants of 10^{-8} M $1,25(OH)_2D_3$-treated cells cultured in serum-free BGJ media[42] demonstrated a dose-dependent increase in osteocalcin secretion to a maximum of 15.4 fold. The osteocalcin response was specific for $1,25(OH)_2D_3$ since treatment with 17β estradiol and insulin-like growth factor-1, and a variety of other agents, caused no increase in osteocalcin secretion. Similarly, the cells responded to 10^{-8} M $1,25(OH)_2D_3$ with a 1.9-fold increase in alkaline phosphatase activity. When cultures were incubated in the presence of hPTH 1-34 at 50 ng/ml, there was a 2.5-fold increase in intracellular cAMP[41].

Cells cultured for periods of up to 50 days on Thermanox coverslips formed a relatively ordered structure. This consisted of layers of cells with a well developed rough endoplasmic reticulum separated by extracellular matrix. This matrix was collagen-based with fibres arranged in layers which tended to run alternatively parallel to and perpendicular to the plane of the section (fig. 1). The cell multilayer continued to increase in depth up to 50 days post confluence, the latest time point examined. The cells did not form nodules when multilayering occurred, rather the multilayer was fairly uniform over the whole well. 17β estradiol treatment resulted in significant increases in the depth of the multilayered culture. The dose response was biphasic. The mean multilayer depth at 20 days, expressed as a percentage of vehicle treatment controls which measured 29–30 µm, in cultures with 3 donors was 178% $\pm$ 10% (SEM) for cultures treated with 10^{-11} M 17β estradiol, 144% $\pm$ 8% with 10^{-10} M 17β estradiol and 148% $\pm$ 8%

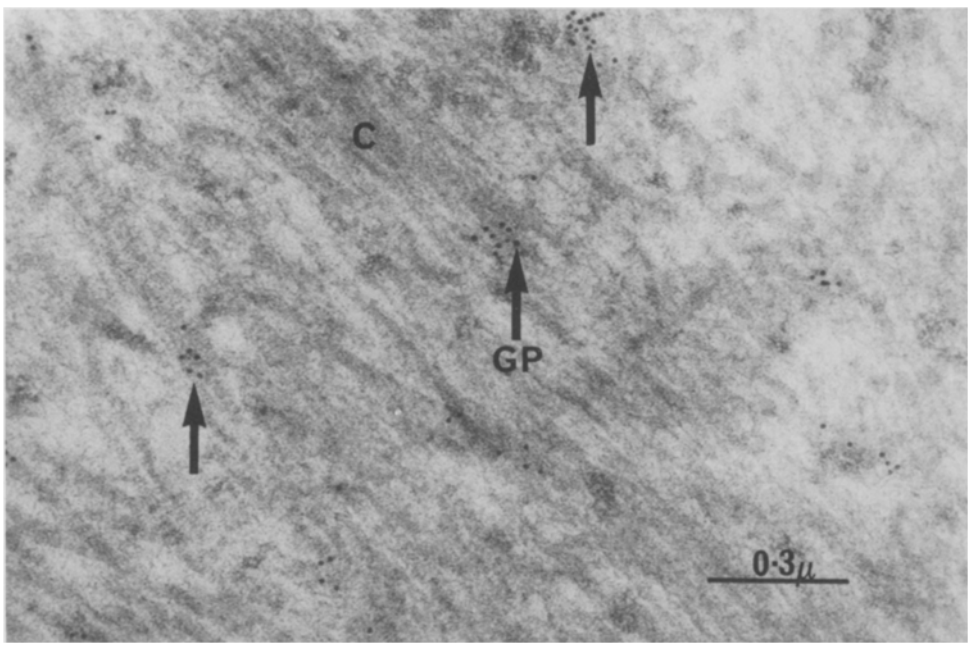

Figure 3. Transmission electron micrograph of banded collagen and its associated extracellular matrix in a 30-day post-confluence culture of osteoblast-like cells. Thrombospondin is labelled with a monoclonal anti-thrombospondin antibody coupled to a 10 nm goat anti-mouse IgG + IgM gold probe (GP, arrows). The label is focally restricted to electron dense areas of the extracellular matrix and its associated banded collagen. The tissue was sub-optimally fixed using 1% glutaraldehyde in 0.1 M cacodylate bufler pH 7.3, in order to preserve antigenicity. Uranyl acetate and lead stain. Original magnification 70,000 ×. Bar 0.3 µm.

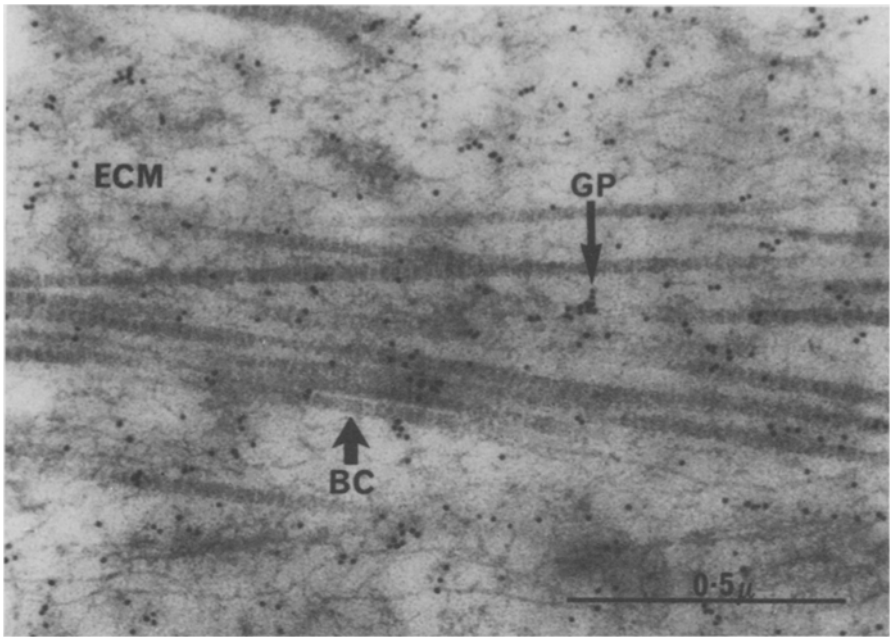

Figure 4. Transmission electron micrograph of the ECM of a 30-day post-confluence culture of osteoblast-like cells showing immunogold labelling for osteocalcin (GP). This marker was not focally incorporated in discrete areas but was distributed diffusely throughout the extracellular matrix (ECM) and its associated banded collagen (BC). Anti-osteocalcin polyclonal antibody conjugated to Protein A, conjugated to 10 nm colloidal gold. The tissue was sub-optimally fixed using 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, in order to preserve antigenicity. Uranyl acetate and lead stain. Original magnification 50,000×. Bar 0.5 μm.

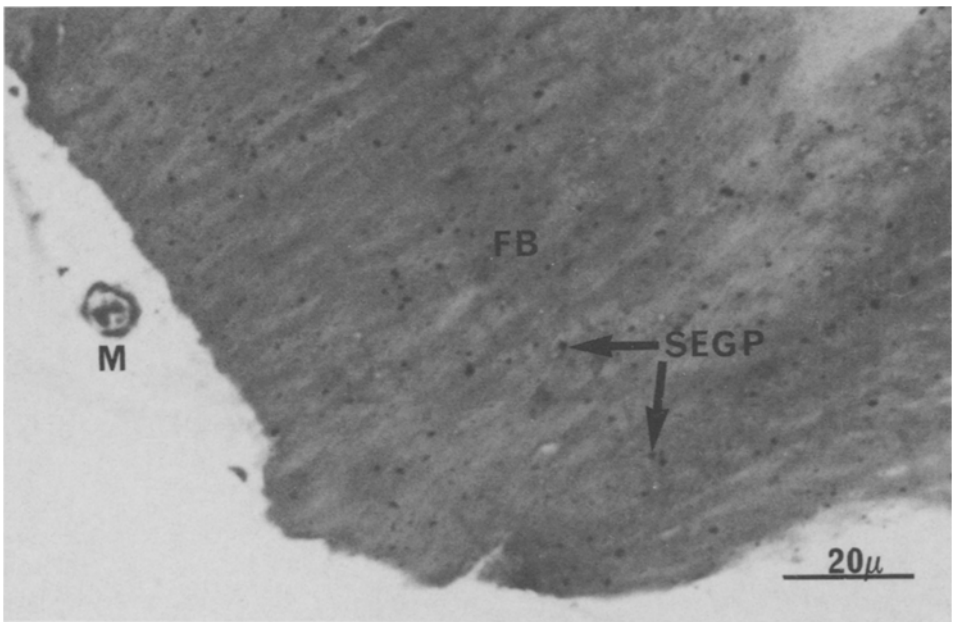

Figure 5. Light micrograph of 19-week calcified fetal bone showing silver enhanced immunogold labelling for thrombospondin. Letters indicate fetal bone (FB), silver enhanced gold probe (SEGP arrow) and marrow cell remnant (M). Fixation in 1.0% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, uranyl acetate and lead stain. Original magnification 1,000×. Bar 20 μm.

with 10^{-9} M 17β estradiol (fig. 2). No significant increase in multilayer depth was seen in the presence of 10^{-9} M 17α estradiol. Image analysis showed these changes to be proportional with similar increases in both cell and matrix areas.

At 30 days post confluence, electron dense areas were noted in the extracellular matrix. Energy dispersive spectroscopy (EDS) studies showed this material to be a poorly crystallised form of hydroxyapatite, similar to the material present in fetal bone. The calcium to phos-

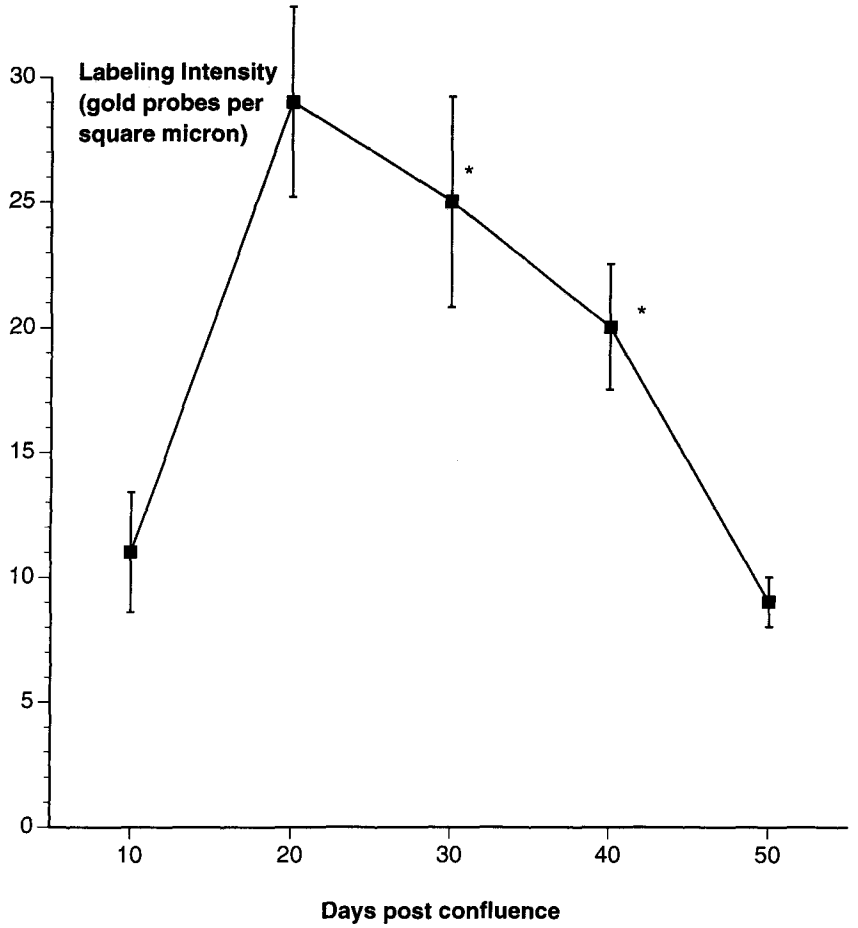

Figure 6. Labelling intensity, in mean gold probes per square micron, of thrombospondin over 50 days post confluence. Cultures maintained on Thermanox coverslips are examined by immuno electronmicroscopy between 10 and 50 days post confluence. Points indicated by asterisks were significantly different from controls, $^{**}p < 0.0001$ and $^{*}p < 0.05$.

phate ratios for these electron dense areas, as measured by EDS were: synthetic hydroxyapatite 1:1.74 (control); fully calcified fetal bone 1:1.62; and multilayer electron dense areas 1:1.41.

Immunoelectron microscopy. Labelling for thrombospondin was concentrated and confined to banded collagen and its immediately adjacent extracellular matrix (ECM) between the osteoblast-like cells. Note, however, that only some areas of collagen were labelled. This concentrated distribution of thrombospondin (fig. 3) contrasted with the diffuse distribution of osteocalcin and alkaline phosphatase in all areas of the multilayers (fig. 4). In 19-week fetal bone, light microscopic silver enhanced immunogold labelling indicated that thrombospondin in the mineralized matrix was distributed in clusters 0.5 μm–20 μm apart (fig. 5). Ultrastructurally, as seen in the ECM of the multilayers (fig. 1), these clusters appeared to have a fairly regular size indicating that the variation in apparent cluster size seen at light microscopic level was likely to be the result of overlapping silver enhanced clusters. By immunoelectron microscopy TSP had a labelling intensity of 19 ± 3 gold probes per square micron (gpm) in 19-week fetal bone, similar to its labelling intensity in the multilayers (fig. 5).

In the ECM of the osteoblast-like cell multilayers, average labelling for thrombospondin increased significantly from 10 to 29 days post confluence and thereafter slowly decreased (fig. 6). Labelling for thrombospondin was significantly increased by treatment with 17β estradiol in a biphasic manner with 10^{-11} M resulting in the largest increase compared to controls (figs 2, 7). Labelling for TGFβ and IGF-I was similarly increased (fig. 2). In double labelling studies, thrombospondin co-localised with both TGFβ (fig. 8) and IGF-I (fig. 9) in both the multilayer ECM and 19-week fetal bone. In 19-week fetal bone TGFβ labelled with an intensity of 22 ± 5 gpm while that for IGF-I was 16 ± 4 gpm, again similar to their labelling intensities in the multilayers. The bone cell marker osteocalcin was not significantly affected by 17β estradiol treatment. No significant differences in labelling intensities for any parameters were noted between controls and cultures treated with 17α estradiol.

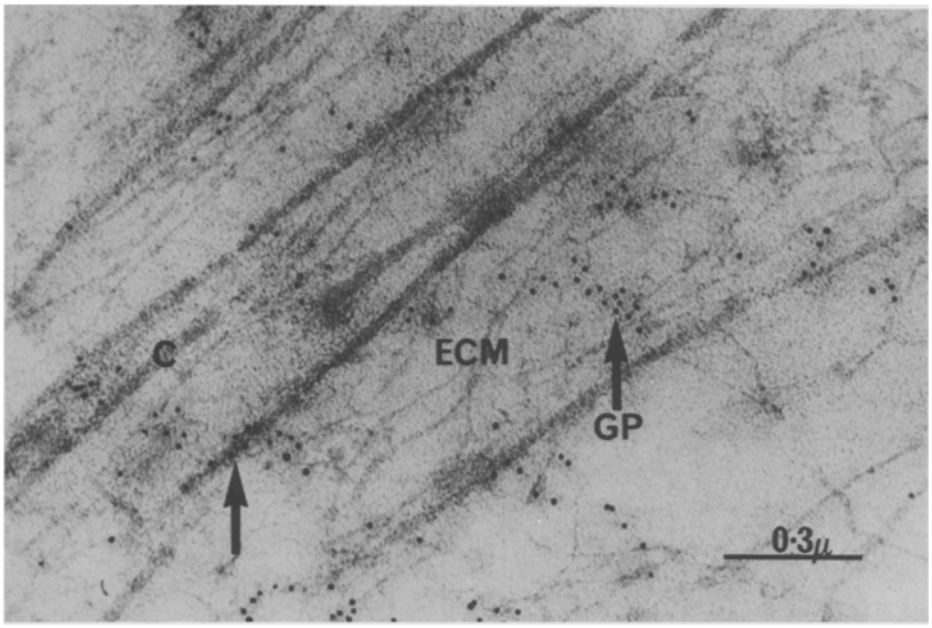

Figure 7. Transmission electron micrograph of banded collagen (C) and its associated extracellular matrix (ECM) in a non-mineralised section of a 30-day post-confluence culture of osteoblast-like cells in the presence of 10^{-11} M 17β estradiol. The addition of 17β estradiol to the culture medium resulted in an increase in labelling for thrombospondin by a factor of 3.4 ($p < 0.0001$) in comparison to controls (fig. 2). Thrombospondin is labelled with a monoclonal anti-thrombospondin antibody coupled to a 10 nm goat anti-mouse IgG + IgM gold probe (GP, arrows). The tissue was sub-optimally fixed using 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, in order to preserve antigenicity. Uranyl acetate and lead stain. Original magnification 70,000×. Bars 0.3 μm.

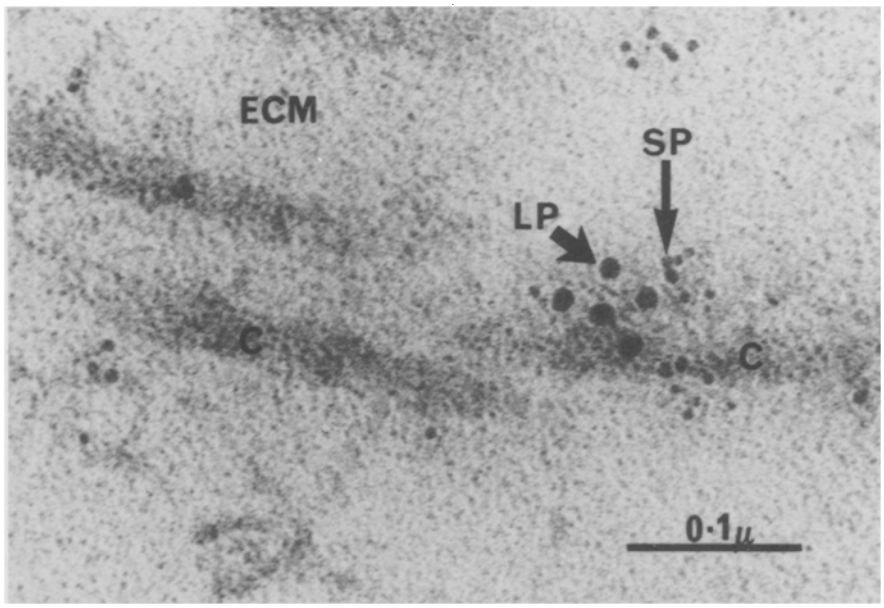

Figure 8. Transmission electron micrograph of the extracellular matrix (ECM) in a 30-day post-confluence culture of osteoblast-like cells. Thrombospondin is labelled with a monoclonal anti-thrombospondin antibody coupled to a 10 nm goat anti-mouse IgG + IgM gold probe (LP = large probe: arrow). Transforming growth factor beta (TGFβ) was labelled with 5 nm goat anti-mouse IgG + IgM conjugated to colloidal gold (SP = small probe: arrow). Collagen fibres (C). Indirect two-sided double labelling procedure. The tissue was sub-optimally fixed using 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, in order to preserve antigenicity. Uranyl acetate and lead stain. Original magnification 300,000×. Bar 0.1 μm.

Discussion

It has previously been reported that thrombospondin (TSP) is synthesized by osteoblasts and secreted into the extracellular matrix (ECM)[3,15]. The concentrated incorporation of TSP in human osteoblast-like cells matrix or the co-localisation of TSP with TGFβ and IGF-I has

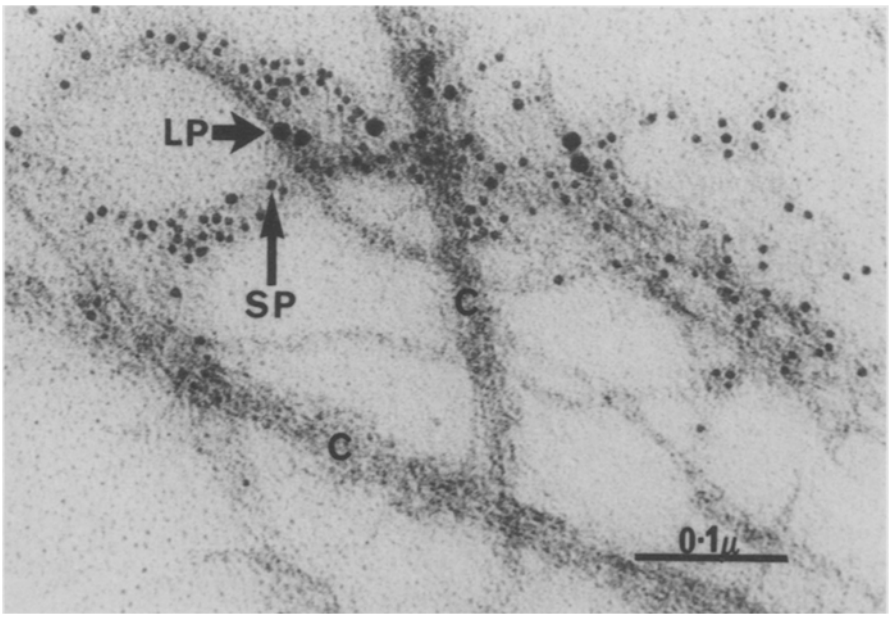

Figure 9. Transmission electron micrograph of the extracellular matrix (ECM) in a 30-day post-confluence culture of osteoblast-like cells. Thrombospondin is labelled with a monoclonal anti-thrombospondin antibody coupled to a 5 nm goat anti-mouse IgG + IgM gold probe (LP = large probe: arrow). Insulin-like growth factor-1 growth factor (IGF-1) was labelled with 10 nm protein A gold conjugated to colloidal gold (SP = small probe: arrow). Collagen fibre (C). Indirect two-sided double labelling procedure. This tissue was sub-optimally fixed using 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, in order to preserve antigenicity. Uranyl acetate and lead stain. Original magnification 250,000×. Bar 0.1 μm.

not, however, been previously described. Furthermore, we have extended these static observations to show that the incorporation of TSP into the ECM is coordinately increased with the growth factors TGFβ and IGF-I after treatment with 17β estradiol.

TSP binds to a variety of ECM factors and may play a role in the organisation and formation of the ECM[1,11,17,47]. Although TSP label was present in the vicinity of the collagen, not all the collagen was labelled. TSP may be binding directly to the collagen and/or extracellular matrix (ECM) or to TGFβ or IGF-I that is itself bound to the banded collagen and/or ECM of the osteoblast-like cells. The reason for the non-universal labelling of TSP in these areas is not understood. Part of the problem may relate to the relative accessibility of the protein epitopes embedded in the resin. In the ECM, the long term mean labelling intensities for TSP and the growth factors rose to 30 days and thereafter slowly declined. One explanation for these findings may be that prolonged exposure of cells or tissues to a hormone or growth factor can lead to de-sensitisation[48]. Another explanation may be the onset of the mineralisation process in the multilayer which may alter the composition of the extracellular matrix and thus the immunoreactivity of its constituent proteins. Leaching of growth factors and other materials into the culture medium may also account for decreasing labelling densities. This may occur via openings seen in the multilayer surface in contact with the culture medium[41].

There have been relatively few reports of modulation of TSP expression by hormones, although one report, using cultures of rat granulosa cells, found that production of TSP was markedly decreased by insulin, and suggested that TSP production was inversely correlated with differentiation in these cells[49]. There is a body of evidence that suggests TSP is markedly affected by growth factors, however. At least one growth factor (TGFβ) has been shown to increase the retention of TSP in the cell layer/matrix of human adult and fetal bone cultures[3]. TSP production is also regulated by growth factors in rat aortic smooth muscle cells[50], and 3T3 cells[51] and has an autocrine role in the growth regulation of these cells[3,52]. TGFβ, PDGF, and epidermal growth factor (EGF) stimulate the synthesis of thrombospondin into rat aortic smooth muscle cell ECM[53] and human mesangial cells[39], whereas heparan sulphate glycosaminoglycans inhibit the incorporation of newly synthesised thrombospondin into the ECM of smooth muscle[50].

Secretion of growth factors, in turn, may be affected by hormones. It has been suggested that estradiol exerts its anabolic effect, seen here as an increase in multilayer depth, through the increased production of bone growth factors such as TGFβ and IGF-I[32,33,35,37]. Estradiol increased TGFβ, IGF-I and TSP incorporation into the matrix in the current study. The coordinate behaviour of thrombospondin in the presence of the growth factors TGFβ and IGF-I may identify it as a

growth factor inducible protein in bone as it is in aortic smooth muscle and possibly also other tissues[2,3,50]. It is therefore possible that the observed increase in TSP in the current study resulted from estradiol-induced increases in TGFβ and IGF-I rather than the direct effect of estradiol treatment on TSP itself. The similar modulation of TSP, TGFβ and IGF-I when stimulated by 17β estradiol suggests a coordinate role for these proteins in modulating bone cell proliferation and function.

In conclusion, there is evidence that many of the effects of agents which modify bone formation, including sex steroids, are mediated through changes in the local synthesis of growth factors such as TGFβ and IGF-I[54]. The similarities between TSP, TGFβ and IGF-I, in terms of their distribution and modulation by 17β estradiol treatments, may indicate a role for TSP in modulating bone cell proliferation and function through interaction with local growth factors.

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1 Lawler, J., Blood *67* (1986) 1197.
2 Hennessy, S. W., Frazier, B. A., Kim, D. D., Deckwerth, T. L., Baumgartel, D. M., Rotwein, P., and Frazier, W. A., J. Cell Biol. *108* (1989) 729.
3 Robey, P. G., Young, M. F., Fisher, L. W., and McClain, T. D., J. Cell Biol. *108* (1989) 719.
4 Sherbina, N. V., and Bornstein, P., Bone *13* (1992) 197.
5 Vos, H. L., Devarayalu, S., de Vries, Y., and Bornstein, P., J. Cell Biol. *267* (1991) 12192.
6 Clezardin, P., Frappart, L., Clerget, M., Pechoux, C., and Delmas, P. D., Cancer Res. *53* (1993) 1421.
7 Lawler, J., Duquette, M., Whittaker, C. A., Adams., J. C., McHenry, K., and DeSimone, D. W., J. Cell Biol. *120* (1993) 1059.
8 Lahav, J., Schwartz, M. A., and Hynes, R. O., Cell *31* (1982) 253.
9 Mumby, S. M., Raugi, G. J., and Bornstein, P., J. Cell Biol. *98* (1984) 646.
10 Galvin, N. J., Vance, P. M., Dixit, V. M., Fink, B., and Frazier, W. A., J. Cell Biol. *104* (1987) 1413.
11 Mosher, D. F., Sun, X., Sottile, J., and Hogg, P. J., Adv. molec. cell. Biol. *7* (1992) 1–33.
12 Clezardin, P., Malaval, L., Morel, M. C., Guichard, J., Lecompte, T., Trzeciak, M. C., Dechavanne, M., Breton-Gorius, J., Delmas, P. D., and Kaplan, C., J. Bone Min. Res. *6* (1991) 1059.
13 Murphy-Ullrich, J. E., Schultz-Cherry, S., and Hook, M., Molec. Biol. Cell *3* (1992) 181.
14 Clezardin, P., Serre, C. M., Trzeciak, M. C., Drouin, J., and Delmas, P. D., Cancer Res. *51* (1991) 2621
15 Hott, M., Clezardin, P., and Marie, P. J., J. Bone Min. Res. *7 (Suppl. 1)* (1992) S213.
16 McKeown-Longo, P. J., Hanning, R., and Mosher, D. F., J. Cell Biol. *98* (1984) 22.
17 Hogg, P. J., Stenflo, J., and Mosher, D. F., Biochemistry *31* (1992) 265.
18 Stenner, D. D., Tracy, R. P., Riggs, B. L., and Mann, K. C., Proc. natl Acad. Sci. USA *83* (1986) 6892.
19 Miyazono, K., and Takaku, F., Blood Rev. *3* (1989) 269.
20 Canalis, E. S., in: Regulation of Bone Remodeling, p. 23. Ed. M. J. Favus. American Society for Bone and Mineral Research, Kelseyville, California, 1991.
21 Mohan, S., and Baylink, D. J., Clin. Orthop. rel. Res. *263* (1991) 30.
22 Breton-Gorius, J., Clezardin, P., Guichard, J., Debili, N., Malaval, L., Vainchenker, W., Cramer, E. M., and Delmas, P. D., Blood *79* (1992) 936.
23 Martin, P., Hopkinson-Woolley, J., and McCluskey, J., Prog. Growth Factor Res. *4* (1992) 25.
24 Mosher, D. F., A. Rev. Med. *41* (1990) 85.
25 Ernst, M., Schmid, C., and Froesch, E. R., Proc. natl Acad. Sci. USA *85* (1988) 2307.
26 Abbadia, Z., Clezardin, P., Serre, C., Maillard, C., and Delmas, P. D., J. Bone Min. Res. *7 (Suppl. 1)* (1992) S223.
27 Castle, V. P., Ou, X., O'Rourke, K., and Dixit, V. M., J. biol. Chem. *268* (1992) 2899.
28 Ederveen, A. G. H., and Kloosterboer, H. J., J. Bone Min. Res. *7 (Suppl. 1)* (1992) S200.
29 Gray, T. K., Flynn, T. C., Gray, K. M., and Nabell, L. M. Proc. natl Acad. Sci. USA *84* (1987) 6267.
30 Gray, T. K., Mohan, S., Linkhart, T. A., and Baylink, D. J. Biochem. biophys. Res. Commun. *158* (1989) 407.
31 Rodan, G., J. Bone Min. Res. *6* (1991) 527.
32 Schmid, C., Ernst, M., Zapf, J., and Froesch, E. R., Biochem. biophys. Res. Commun. *160* (1989) 788.
33 Komm, B. S., Terpening, C. M., Benz, D. J., Graeme, K. A., Gallegos, A., Korc, M., Greene, G. L., O'Malley, B. W., and Haussler, M. R., Science *241* (1988) 81.
34 Kasperk, C., Fitzsimmons, R., Strong, D., Mohan, S., Jennings, J., Wergedal, J., and Baylink, D., J. clin. Endocrin. Metab. *71* (1990) 1322.
35 Ernst, M., Heath, J. K., and Rodan, G. A., Endocrinology *125* (1989) 825.
36 Canalis, E., McCarthy, T. L., and Centrella, M., J. clin. Invest. *81* (1988) 277.
37 Elford, P. R., and Lamberts, S. W. J., Endocrinology *127* (1990) 1635.
38 Thiebaud, D., Ng, K. W., Findlay, D. M., Harker, M., and Martin, T. J., J. Bone Min. Res. *5* (1990) 761.
39 Chen, T. L., Liu, F., Bates, R. L., and Hintz, R. L., Endocrinology *128* (1991) 2489.
40 Kobayashi, S., and Yamamoto, T., J. diabet. Comp. *5* (1991) 121.
41 Slater, M., Patava, J., and Mason, R. S., J. Bone Min. Res. *9* (1994) 161.
42 Gronowicz, G., Woodiel, F. N., McCarthy, M. B., and Raisz, L. G., J. Bone Min. Res. *4* (1989) 313.
43 Seshadri, M. S., Mason, R. S., Cornish, C. J., Pryke, A. M., and Posen, S., Clin. Sci. *71* (1986) 261.
44 Wilkinson, M. R., Wagstaffe, C., Delbridge, L., Wiseman, J., and Posen, S., Archs intern. Med. *146* (1986) 268.
45 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
46 Slater, M., Micron *24* (1993) 661.
47 Kramer, R. H., Fuh, G. M., Bensch, K. G., and Karasek, M. A., J. Cell Physiol. *123* (1985) 1.
48 Heersche, J. N. M., Aubin, J. E., Grigoriadis, A. E., and Moriya, Y. S., in: Hormone Responsiveness of Bone Cell Populations. Searching for Answers in Vivo and in Vitro, p. 286. Ed. W. T. Butler. Ebsco, Birmingham, Alabama, 1985.
49 Dreyfus, M., Dardik, R., Suh, B. S., Amsterdam, A., and Lahav, J., Endocrinology *130* (1992) 2565.
50 Majack, R. A., Cook, S. C., and Bornstein, P., J. Cell Biol. *101* (1985) 1059.
51 Donoviel, D. B., Amacher, S. L., Judge, K. W., and Bornstein, P., J. Cell Physiol. *145* (190) 16.
52 Majack, R. A., Cook, S. C., and Bornstein, P., Proc. natl Acad. Sci. USA *83* (1986) 9050.
53 Majack, R. A., Mildbrandt, J., and Dixit, V. M., J. biol. Chem. *262* (1987) 8821.
54 Rodan, G. A., and Noda, M., Crit. Rev. Eukaryot Gene Expr. *1* (1991) 85.