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Estrogen Effects on Protein Expressed by Marrow Stromal Osteoblasts

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The aim of this study was to follow the changes of estrogen treatment on osteoblastic MBA-15 cells derived from marrow stromal origin. Following exposure to estrogen, the cells' patterns of protein synthesis and expression were monitored. The proteins synthesized by MBA-15 cells were identified in cell lysate fractionated to soluble proteins (SOL), cytoskeleton (CK), membrane and nuclei, and intermediate filaments (PL) fractions. These cellular fractions of the osteoblastic MBA-15 cell cultures were assayed on SDS-PAGE of total proteins or following radiolabeling of cells by [^{35}-S]-Methionine. Changes in cytoskeletal and membrane proteins of the control and treated cells were monitored by these assays. Reduction in expression of tubulin (TUB) and thropomyosin (TM) were observed by western blot analysis and of actin by fluorescein staining. A reduction in expression of an antigen highly expressed by osteogenic cells and detected by MoAb 85/12 was also observed in these cells. These experiments showed reduction in cytoskeletal and other cellular proteins in the stromal osteoblastic MBA-15 cells treated with 17β-Estradiol in comparison to untreated cells.

Estrogen has been observed to have major effects on cells that function in bone remodeling. Loss in gonadal function leads to decrease of estrogen levels in the blood stream. This reduction of estrogen is speculated to play a major role in the development of osteoporosis, that significantly reduce bone mass. The hallmark of osteoporosis is a reduction in skeletal mass caused by imbalance between bone resorption and bone formation. Ovariectomy (OVX) is also used in animal models to study the metabolic changes following estrogen depletion. In this model, it has been observed changes in the differentiation of functioning cells, the osteoclasts and the osteoblasts. These cells arise from progenitors that are members of the bone marrow; the osteoprogenitors belong to the stromal cellular network and the osteoclasts progenitors arise from the hemopoietic cells. The depletion of estrogen leads to reduction in the stromal progenitor cell pool and to an increase in differentiation of granulocyte/macrophages' cell lineage that are osteoclast progenitors (1). However, the bone loss due to a fault in differentiation regulatory mechanism of these osteoblastic or osteoclastic cell lineage's is needed to further understand their control mechanism.

This study is therefore focused on the osteoprogenic cell population, represented by MBA-15 cells derived from the marrow stromal system. Throughout the past decade, extensive studies on marrow stromal cell subpopulations have been done. These studies looked at the different cell morphology, attachment potential and growth rate. Biochemical activities and mRNA expression were also monitored and when exposed to hormone and growth factors (2-8). The stromal MBA-15 cells were shown to have the potential to mineralize in vitro (2,5) and to form bone in vivo (2,4). The osteogenic potential of MBA-15 cells was demonstrated using the diffusion chamber model (2) and an open ectopic implantation site model (4). Also, the relationship of the osteoblastic MBA-15 cells to hemopoiesis regulation were studied both in vitro (9) and in vivo (4).

The changes in cell function are hormonally controlled. The hormonal modulation leads to changes in cell-cell communication and shape which coordinate the gene expression and cell function. Hormonal modulation may also lead to changes in cell structure that will have a finally effect on cellular metabolism and function. Physical components that make up the ordered structure of cells consist of dynamic linkages between the nucleus (nuclear matrix), the cytoplasm (the cytoskeleton) and the extracellular environment (extracellular matrix). All these components orchestrate cellular response which is necessary to gene expression that maintain the cell specificity and tissue phenotypic properties (10,11).

Herein, we focus on the estrogen's effects on marrow stromal osteoblasts, MBA-15 cells. The MBA-15 cells express the estrogen receptor. Estrogen's effect on

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these cells growth rate and final differentiation was measured by alkaline phosphatase activity and CD-10 expression in a recent study (12). Cell to cell communication via gap junctions and changes of intracellular calcium levels was measured following exposure to estrogen (13). In the following study, the changes of cellular proteins' expression will be evaluated in cells that were estrogen-treated in vitro.

MATERIALS AND METHODS

Culture conditions. In this study we used a mouse stromal osteoblastic cell line MBA-15 (2). The cells were grown in tissue culture plates (Nunc, Denmark) in high glucose Dulbecco's modified Eagle medium (DMEM) and supplemented with 10% fetal calf serum (FCS) (Bet-Haemek, Israel). Cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. When cells were exposed to the hormonal treatment they were transfer into DMEM phenol red-free containing 2% charcoal-stripped serum containing the hormone, 17-β-Estrogen (Sigma) at a concentration of 10^{-8}M. The cultures were exposed to these conditions as indicated for each set of experiments.

Subcellular fractionation. Subcellular fractionation was performed according to Fey and Penman (14). The steps included cell extraction following a wash in PBS. First fractions contain soluble proteins (SOL) that were separated by cytoskeletal buffer (CSK) (10mM PIPS, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM PMSF and 0.5% (v/v) Triton X-100). After 3 min at 4°C the cell's were centrifuged at 600xg. The second fraction containing the cytoskeletal frame work (CK) pellet was extracted in RSB-magik (RSB) (10 mM Tris HCl, pH 7.4, 10 mM $NaCl_2$ 1.2 mM PMSF 1% (v/v) Tween 40 and 0.5% (v/v) sodium deoxycholate) for 5 min at 4°C and pelted. This step striped away the cytoskeleton leaving the pellet containing membrane proteins with nuclei and their attached intermediate filaments. These membrane protein fractions were further extracted in buffer (10mM PIPS, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM PMSF and 0.5% (v/v) Triton X-100). Ammonium sulfate is added to membrane fraction from 1 M stock solution to a final concentration of 0.25 M, and is pelted by centrifuge (PL).

Metabolic labeling and autoradiography. Protein synthesis was detected by incubation of confluent, 3×10^5 cells in methionine-free medium supplemented with 125 μCi L-[^{35}S] methionine (Du Pont, NEN, England) for 2 hours at 37°C. Following labeling, the cells were subcellular fractionated (as describe above). From each fraction the proteins were TCA precipitate and separated by gel electrophoresis as describe below. For gels equal amounts of each sample containing of 50,000 cpm were loaded in each lane. Gels were developed with fluorography (15) and exposed to X-ray film at −70°C.

Gel electrophoresis. The harvested cultured cells were washed twice with PBS, subcellular fractionated and were frozen at −20°C. All samples were prepared for extraction in sodium dodecyl sulfate (SDS), boiled for 3 min and dissolved in a sampling buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, β-Mercapto-ethanol and 0.01% bromophenol blue). The samples were separated by electrophoresis on 1.5 mm-thick of 10% SDS-PAGE polyacrylamide slabs mini-gel with a 2.5% polyacrylamide stacking gel (16). A horizontal electrophoresis apparatus (BioRad Lab. Ltd.) was used and proteins on gels were Coomassie Blue stained.

Western blotting analysis. The gels were electroblotted to a nitrocellulose membrane (Schleicher and Schuell, NH) using a transfer blotting apparatus (BioRad Lab. Ltd.), by a modification of Hurley and Frinkelstein method's (17). Following the protein transfer the nitrocellulose blots were blocked in skim milk and incubated first with MoAb: tubulin (TUB), thropomyosin (TM) (Sigma, Israel) and MMS-85 (18) for over night at 4°C, and then incubated with second antibody, goat anti mouse peroxidase-conjugated affinity purified IgG (BioRad Lab. Ltd.). The blots were incubated with chemilumicence substrate for peroxidase reaction, ECL (Amersham, England) providing high sensitive visualization of the Ag-Ab complex on the blot.

RESULTS

Estrogen-Responsive Modification of Cellular Protein Synthesis

A. Total protein synthesis. A schedule of exposure during 2 to 7 days to 10^{-8}M 17β-Estradiol resulted in changes of protein synthesis. Cell extracts and subcellular fractionation of soluble proteins (SOL), cytoskeleton (CK), membrane and nuclei's fractions with intermediate filaments (PL) were examined. Changes in protein synthesis were observed in 1-D SDS-PAGE gel visualized when stained with Coomassie Blue (Fig. 1) and autoradiography of cell extracts from [^{35}-S]methionine-labeled cells (Fig. 2). Following two days of culture treatment no changes were seen when cell extracts were separated and examined by gel electrophoresis (data not shown). After 4 days of treatment in culture, changes were observed in the cytoskeleton fraction, CK (Fig. 1A). Two bands which appeared in control culture, a 67 Kd and a 55 Kd band were not present in the treated cells. With prolong treatment of cells up to 7 days, visualization of a new band of approximate 37 Kd Mw was seen in CK fraction (Fig. 1B).

Radiolabelling of these cells revealed changes in the pattern of protein synthesis in CK and PL fractions. In each fraction there was noted differences in pattern of radiolabelled newly synthesized proteins between the control and the treated cells (Fig. 2).

B. Specific protein's modulation. Prominent changes in protein synthesis following estrogen treatment and the pattern of distribution mainly at the CK and PL fractions were observed. The nature of these changes in MBA-15 (comparing untreated and treated) cells were analyzed further using specific antibodies by western blot (Fig. 3) and by fluorescence staining (Fig. 4). Monoclonal antibodies against, thropomyosin (TM), tubulin (TUB) and an 85 Kd antigen uniquely expressed by stromal cells and detected by MMS-85 MoAb were used (18). A different localization of subcellular fractions in the MBA-15 cells was demonstrated. The TUB, appearance is correlated with the cytoskeletal fraction (CK) and TM with the membranous fraction and pellet (PL) components. The antigen identified by MMS-85 demonstrated high expression at CK and PL fractions. In cells that were treated with 17-β-Estradiol, a reduction in all three cellular antigens was observed (Fig. 3). An

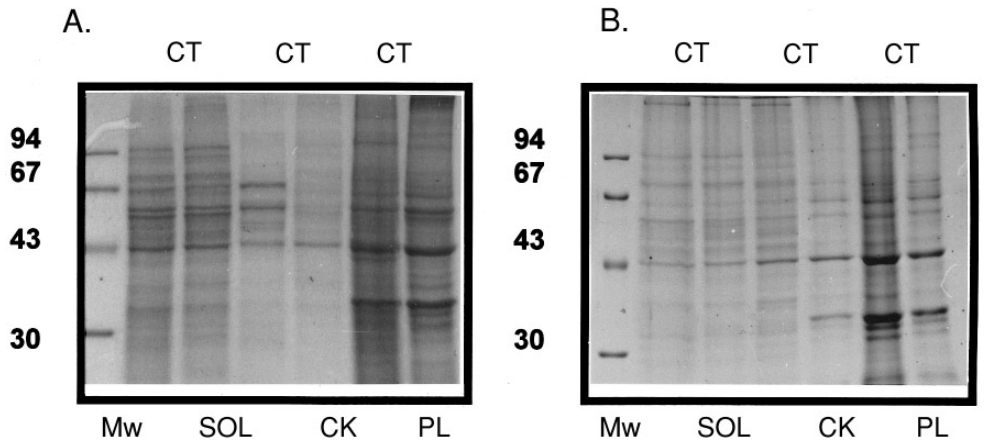

FIG. 1. SDS-PAGE analysis of MBA-15 cell fractions untreated (C) and 17-β-Estradiol, treated cultures (T) for 4 (A) and 7 days (B). The sequential protein fractions (SOL, CK, PL) were visualized by total protein staining with Coomassie Blue. Major changes in protein pattern are noted in CK fraction.

estrogen-modified effect on inhibition of cytoskeletal proteins was detected, specifically a decrease in TUB and TM expression as shown by western blot analysis. Furthermore, cytoskeleton reorganization of actin was demonstrated by fluorescence phallidin-trict staining. A decrease in actin fibers number, density and organization in of the 17-β-Estradiol treated cells (Fig. 4B) in comparison to untreated cells (Fig. 4A) was seen. Previous observation showed that MBA-15 cells constitutively express mRNA for procollagen α2(I) (6). In our study, an addition of 17-β-estradiol to growth medium decreased mRNA for procollagen 2α(I) expression (data not shown).

DISCUSSION

The gonadal hormones are believed to control the osteoblastic cell function. The marrow osteoblasts are mainly functioning in the active formation of endosteal bone surface at the medullary cavity. The loss of gonadal hormone, alter their cellular activity and specifically decreases the ability of progenitors in the marrow

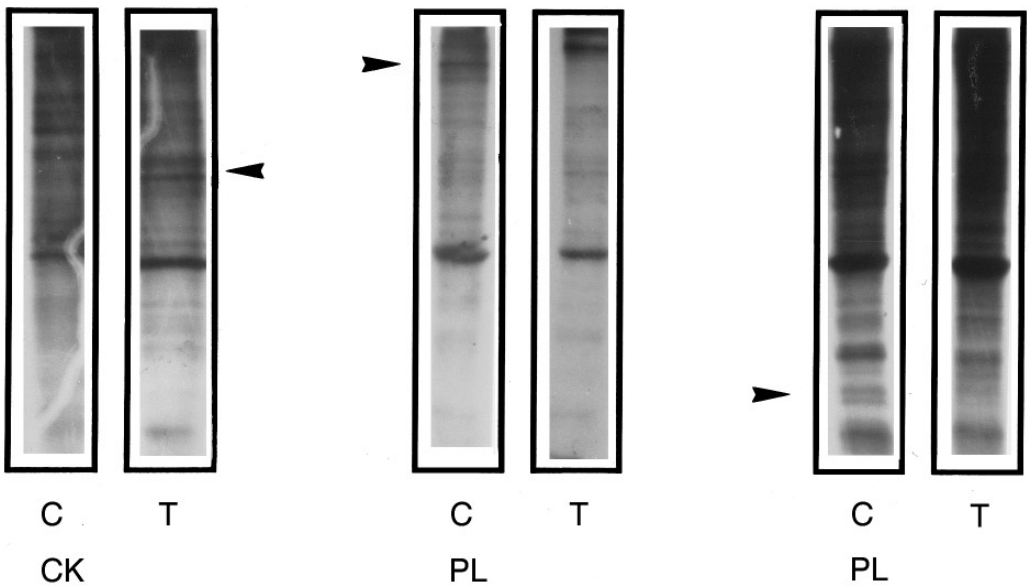

FIG. 2. SDS-PAGE and autoradiography analysis of MBA-15 cell fractions untreated (C) and 17-β-Estradiol treated for 7 days (T). The cultures were [^{35}S]-methionine-labeled for 2 hrs., protein fractions (CK and PL) were further analyzed. Changes are marked with arrowheads comparing control and treated cultures. CK and PL (center panel) were exposed for 4 days PL (right panel) was exposed for 7 days.

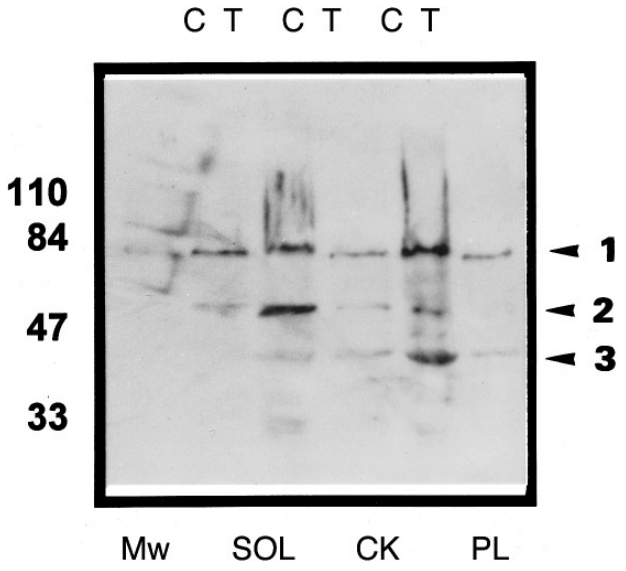

FIG. 3. Western blot analysis was performed to demonstrate different antigens and their subcellular distribution in cellular fractions. MBA-15 untreated (C) and 17-β-estradiol treated for 7 days (T). The protein fractions were extracted (SOL, CK, PL) and further analyzed. Localizations of different cellular proteins in subfractions are identified using MoAb's anti MMS-85 (1), TUB (2) and TM (3). Each protein is marked with arrow and numbers comparing control and treated cultures. Decrease in expression in these antigens in treated cultures is observed.

to differentiate to functional osteoblast. This is believed to contribute to the bone loss that is associated with aging (1,26,27). In view of the changes in estrogen levels in vivo, we were attempting to speculate the nature of changes following an exposure of marrow stromal osteoblasts to estrogen for their activity and function. Thus, to evaluate and characterize the type of changes we used the marrow stromal MBA-15 osteoblastic cells as in vitro model.

Normal osteoblastic cells were shown to express estrogen receptor (12, 21-25). In a previous study, we were able to demonstrated by in situ hybridization and by binding assay that the MBA-15 cells express the estrogen receptor. Furthermore, the presence of the hormone in culture medium modulates their proliferation, and biochemical activities measured by alkaline phosphatase and CD10/NEP (12). In the presence of 17-β-Estradiol an alteration in the number of gap junctions between neighboring cells and fluctuation in levels of intracellular Ca++ (that act as a second messenger) was demonstrated (13). In this study, we further investigated the changes in osteoblasts treated with estrogen. Genomic effects were observed as well as the qualitative changes in cell functions of these cells treated in vitro with 17-β-Estradiol. Specifically, we evaluate the changes in protein synthesis, cytoskeleton protein expression and distribution. Estrogen-induced regulation of the osteoblasts and its correlation to cellular responses was monitored. The alteration in cell function through a concomitant reorganization of cytoskeletal and nuclear protein architecture was noted. It was observed that estrogen causes the structural changes which ultimately altered functional proteins' expression was associated with the hormonal responsiveness.

The cellular architecture and cytoskeleton compo-

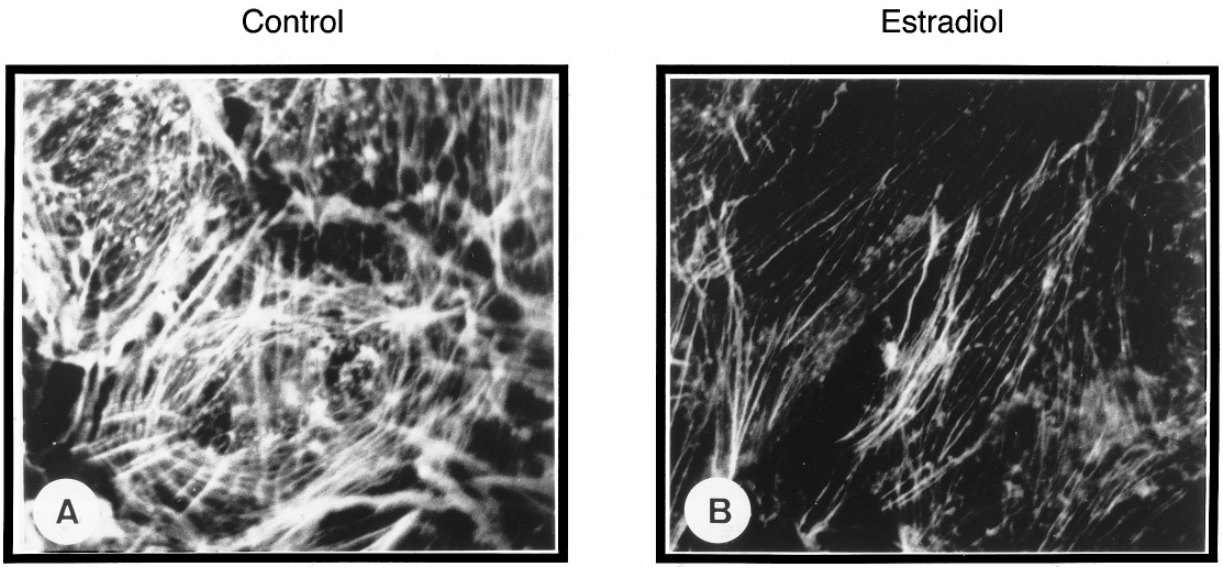

FIG. 4. Phalloidin-labeled actin was identified by fluorescence staining. MBA-15 cells untreated and 17-β-estradiol-treated were stained with fluorescence. The treated cells are expressing decreased numbers of actin fibrils as well as changes in their distribution and orientation in comparison to the untreated control.

nents that maintain cell function are effected by variety modulators. A quick response is need for rapid rearrangement in cytoplasmic microfilaments and microtubular structures for cellular adaptation to intra-extracellular changes following signals. Tubulin, a major protein component of microtubules is intracellular cylindrical filaments which function as a structural and mobile element in mitosis intracellular transport of the cytoskeleton. When MBA-15 cells were exposed to 17-β-Estradiol, a reduction in tubulin was shown. Another component of the cytoskeleton, the tropomyosin (TM) (26) is known for its role in the regulation of microfilament cytoarchitecture. This protein is part of the stress fibers that mediate the mechanism of regulation which stabilizes the changes occurring causing a quick flexible response of cells to its microenvironment. It localization is close to the substratum attached to the plasma membrane (26). There are multiple TM proteins (isoforms) present in cells which regulates the cytoplasm microfilaments on at least two levels: by affecting the polymerization and physical characteristics of F-actin and by modifying the effect of several actin-modulating proteins. As was observed in other cells when treated with estrogen also MBA-15 cells expressed a reduction of tubulin and TM levels. In addition a third cytoskeleton protein, actin was shown to be decreased in levels of expression, fiber's number, density and organization. Interestingly, it was demonstrated in other in vitro system, that the estrogen interferes with the assembly of microtubules polymerization (27). This was demonstrated on the microtubular tau protein in cultured neuroblastoma cells (28). Decrease in expression of microtubules and its organization disruption also has been demonstrated as an estradiol-induced phenomenon as observed in human breast cancer cell lines MCF-7 and MDA-MB-231 (29). It is suggested that the estradiol-induced microtubule network disruption may be independent of estradiol binding to the receptors. The disruption of cellular microtubule-depolymerization by estrogen treatment is believed finally to be caused the cell growth inhibition.

The 17β-Estradiol has a growth inhibition activity on different osteoblastic cell lines (12,24,25). In human osteosarcoma (30), primary cultures of fetal rat calvaria and immortalized clonal rat osteoblastic lines - RCT-1 and RCT-3 (31), an increase of cell proliferation following exposure to estrogen was observed. Thus, this effect may be correlated with stages of cell differentiation. Decrease of cell growth as an effect of estrogen on osteoblastic was accompanied to an increased ALK-P activity (12,24). Other studies have shown more effects on osteoblastic functions such as an increase in the expression of mRNA for type I procollagen and an increase in the production of IGF binding protein (12,31). In SaOS-2, human osteosarcoma cells, estrogen treatment decreased PTH-stimulated adenylate cyclase activity (32). Previous study on gene expression, measured by mRNA, for collagen and noncollagenous proteins in osteoblastic cells that is in response to hormonal stimulation was studied. These matrix proteins have been shown to play an important role in the anchoring of cells to the ECM substrata. The ability of MBA-15 cells to attach to various substrate proteins, which are constituents the bone matrix, was measured (7). The osteoblasts express various cell surface receptors that mediate cell attachment capability (33). The pattern of cytoarchitecture, cell shape, morphology and phenotypic expression when cultured on ECM allowed these cells mRNA expression and biochemical activity (34). In previous study, we had showed that MBA-15 cells express the mRNA for procollagen α2(I) and osteonectin, early markers of osteoblasts differentiation, and that their mRNA expression levels increases under mineralization conditions (5). These mRNA levels of expression were unaffected by retinoic acid treatment. However, mRNA for procollagen 2(α)I was shown to decreased when exposed to 17β-Estradiol. ALK-P enzyme activity in MBA-15 cells was increased with prolong of 17β-Estradiol treatment (12). According to the literature, other osteoblastic cells had variable regulatory responses for ALK-P activity that were accompanied to the cell stage of differentiation and following the estrogen treatment.

The importance of the study of the stromal cell expression and their regulation upon treatment with 17β-Estradiol is a prerequisite for characterizing their function in situ. The understanding of stromal osteoblastic cell differentiation and function assemblies to their specific responses will hopefully lead to their final phenotypic expression. In this study we elaborated on the function of marrow stromal osteoblasts when exposed to estrogen. The marrow stroma is believed to be a major source of the cells responding to this hormone in the modulation and function of endosteal bone formation in situ. These findings offer evidence that marrow osteogenic cells are directly responding to estrogen by synthesizing different proteins profile compare to untreated cells.

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REFERENCES

1. Manolagas, S. C., and Jilka, R. L. (1994) *The New England J Medicin* **332,** 305–311.
2. Benayahu, D., Kletter, Y., Zipori, D., and Wientroub, S. (1989) *J. Cell. Physiol.* **140,** 1–7.
3. Benayahu, D., Fried, A., Zipori, D., and Wientroub, S. (1991) *Tissue Int.* **49,** 202–207.

4. Benayahu, D., Gurevitch, O. A., Zipori, D., and Wientroub, S. (1994) *J. Bone. Miner. Res.* **9,** 1107–1114.
5. Benayahu, D., Kompier, R., Shamay, A., Kadouri, A., Zipori, D., and Wientroub, S. (1994) *Calcif. Tissue Int.* **55,** 120–127.
6. Benayahu, D., Fried, A., Shamay, A., Cunningham, N., Blumberg, S., and Wientroub, S. (1994) *J. Cell. Biochem.* **56,** 62–73.
7. Benayahu, D., Fried, A., Efrati, M., Gehron Robey, P., and Wientroub, S. (1995) *J. Cell. Biochem.* **58,** 151–160.
8. Benayahu, D., Fried, A., and Wientroub, S. (1995) *Biochem. Biophys. Res. Commun.* **210,** 197–204.
9. Benayahu, D., Horowitz, M., Zipori, D., and Wientroub, S. (1992) *Calcif. Tissue Int.* **51,** 195–201.
10. Bidwell, J., van Wijnen, A., Banerjee, C., Fey, E., Merriman, H., Penman, S., Stein, J., Lian, J., and Stein, G. (1994) *Endocrinology* **134**(4), 1738–1744.
11. Bortell, R., Owen, T. A., Shalhoub, V., Heinrichs, A., Aronow, M. A., Rochette-Egly, C., Lutz, Y., Stein, J. L., Lian, J. B., and Stein, G. S. (1993) *Proc. Natl. Acad. Sci.* **15 90,** 2300–2304.
12. Shamay, A., Knopov, V., and Benayahu, D. (1996) *Cell. Biol. Inter.* **20**(6), 401–405.
13. Massess, R., Korenstein, R., and Benayahu, D. (1997) Submitted for publication.
14. Fey, E. C., and Penmen, Y. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 121–125.
15. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341.
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
17. Hurley, W. L., and Frinkelstein, E. (1985) *J. Immunol. Methods* **85,** 195–202.
18. Benayahu, D., Efrati, M., and Wientroub, S. (1995) *J. Bone Miner. Res.* **10**(10), 1496–1503.
19. Kahn, A., Gibbons, R., Perkins, S., and Gazit, D. (1995) *Clinical Orthop. Rel. Res.* **313,** 69–75.
20. Horowitz, M. C. (1993) *Science* **260,** 626–627.
21. Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C., and Riggs, B. L. (1988) *Science* **241,** 84–86.
22. 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. (1988) *Science* **241,** 81–83.
23. Ikegami, A., Inoue, S., Hosoi, T., Mizuno, Y., Nakamura, T., Ouchi, Y., and Orimo, H. (1993) *J. Bone Mineral Res.* **8,** 1103–1109.
24. Gray, T. K., Flynn, S., Gray, K. M., and Nabell, L. M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6267–6271.
25. Gray, T. K., Mohan, S., Linkhart, T. A., and Baylink, D. J. (1989) *Biochem. Biophys. Res. Commun.* **158,** 407–412.
26. Payne, M. R., and Rudinck, S. E. (1984) TIBS pp. 361–363.
27. Metzler, M., and Pfeiffer, E. (1995) *Environ. Health Perspect.* **103** Suppl. 7, 21–2.
28. Lew, G. M. (1993) *Gen. Pharmacol.* **24**(6), 1383–1386.
29. Aizu-Yokota, E., Ichinoseki, K., and Sato, Y. (1994) *Carcinogenesis* **15**(9), 1875–1879.
30. Slootweg, M. C., Ederveen, A. G. H., Schot, L. P. C., Schoonen, W. G. E. J., and Kloosterboer, H. J. (1992) *J. Endocrinol.* **133,** R5–R8.
31. Ernst, M., Heat, J. K., and Rodan, G. A. (1989) *Endocrinol.* **125,** 825–833.
32. Fukayama, S., and Tashjian, A. H. (1989) *Endocrinol.* **124,** 397–401.
33. Grzesik, W. J., and Gehron Robey, P. (1994) *J. Bone Miner. Res.* **9,** 487–496.
34. Fried, A., Shamay, A., Wientroub, S., and Benayahu, D. (1996) *J. Cell. Biochem.* **61,** 246–254.