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The Expression of Transforming Growth Factor-β and Interleukin-1β mRNA and the Response to 1,25(OH)$_2$D$_3$, 17β-Estradiol, and Testosterone Is Age Dependent in Primary Cultures of Mouse-Derived Osteoblasts In Vitro

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The aim of the present study was to examine the hypothesis that primary cultures of osteoblasts obtained from bones of young animals respond to hormones better than cell cultures obtained from old animals. We studied in cultured osteoblastic cells the effects of 1,25(OH)$_2$D$_3$ and sex steroid hormones on several mouse osteoblastic phenotypic expressions including transforming growth factor-β (TGF-β) and interleukin-1β (IL-1β) mRNAs. Second passages of long bone–derived osteoblastic cells from young donors (5–12 wk) and old donors (10–12 mo old) were used for this study. The cells obtained from old animals had decreased ALP activity and cAMP compared with cells obtained from young animals with no change in collagen production and mineralization. The addition of 17β-estradiol and testosterone increased ALP activity and mineralization in the cultured cells from both age groups and collagen production in cells obtained from old mice. Using *in situ* hybridization IL-1β and TGF-β mRNA expression was observed to be higher in the osteoblasts from young than from old donors. 1,25(OH)$_2$D$_3$ increased IL-1β mRNA expression in the cells derived from young mice. Testosterone and 17β-estradiol inhibited IL-1β mRNA expression only in cells derived from young mice. Sex steroid hormones did not change TGF-β mRNA expression in any of the cell lines, but 1,25(OH)$_2$D$_3$ increased its expression in cells derived from old donors. The results of the present study indicate that cells obtained from old mice are generally less active than those obtained from young animals.

Key Words: Osteoblasts; cytokine; hormones; age; expression; *in situ* hybridization.

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Introduction

Bone contains an abundance of locally produced growth factors. Osteoblasts not only respond to these growth factors that exert autocrine/paracrine regulation of osteoblastic phenotype and activity but synthesize many of these regulators. Although bone remodeling is modulated by systemic hormones, there is evidence that the locally produced growth factors play a central role in the focal nature of bone remodeling and may mediate the effects of hormones *(1)*. The effects of age on osteoblastic function have been studied in numerous cell models. In most studies, age-related decreases in the metabolic activity *(2–4)*, proliferative ability *(2,4,5)*, and number of osteoblastic cell lineage *(2,6,7)* have been reported. However, little is known about whether osteoblastic production of growth factors decreases with age. Nicolas et al. *(8)* reported age-related decreases in insulin-like growth factor -1 (IGF-1) and transforming growth factor-β (TGF-β) in femoral cortical bone from both men and women. Kahn et al. *(7)* suggested that age-related diminution in TGF-β is responsible for reduction in the number and function of osteoblastic stem cells.

Interleukin-1β (IL-1β) and TGF-β, both synthesized by osteoblast-like cells, could be involved in the local regulation of bone turnover *(9–12)*. IL-1β is a potent inducer of osteoclast precursor proliferation and differentiation, increasing bone resorption *(13)*. It inhibits the differentiation of osteoblasts and is also a potent mitogen for osteoblast precursors *(14–16)*. Elevated IL-1β was found in peripheral blood monocytes from patients with postmenopausal osteoporosis *(17,18)*; it decreased when patients were treated with estrogen. It is speculated that deregulated IL-1β production may contribute to the onset of postmenopausal osteoporosis *(19,20)*.

TGF-β has potent effects on bone cells. It induces osteoblastic activity and bone formation in vivo *(1,21,22)*. In vitro, TGF-β induces extracellular matrix secretion, inhibits its mineralization, and modulates osteoprogenitor cell proliferation and osteoblastic differentiation *(23)*. The effects

of TGF-β on bone resorption are extremely complex *(24)*. Results may depend on the cell systems and the other cells in the osteoclast microenvironment.

It has been reported that 17β-estradiol stimulates IL-1β mRNA production in human osteoblastic HOBIT cells *(25)*. 17β-Estradiol also stimulated the production of TGF-β in osteoblast-like cells derived from rat osteosarcoma, in mouse calvarial bone cells, and in normal human osteoblastic-like cells *(11,26)*. Testosterone has been reported to enhance the IL-1β mRNA levels in human osteoblastic HOBIT cells and increase the production of TGF-β in human osteosarcoma cells *(27,28)*. However, in human osteoblastic cells, testosterone did not stimulate TGF-β mRNA *(29)*. Ovariectomy selectively reduced the concentration of TGF-β in rat bone, and estrogen eliminated the TGF-β deficit *(26)*. Local and systemic application of TGF-β decreased osteoclastic resorption in ovariectomized rats *(30,31)*. These data indicate that a lack of TGF-β could be responsible for the stimulation of osteoclastic bone resorption secondary to estrogen deficiency.

Ovariectomy in rats increased the production of IL-1β from cultured bone marrow cells and increased bone loss. Treatment with IL-1β receptor antagonist significantly decreased bone resorption *(32,33)*. These findings led us to suggest that IL-1β may mediate the effects of estrogen deficiency on bone resorption. Taken together, cultured osteoblasts derived from old animals seem to respond less well to various hormones than osteoblastic cell cultures obtained from young animals. Moreover, the basal activity and the regulation of either IL-1β or TGF-β production by sex steroid hormones in bone cells may also change with age.

In light of the possibility that cultured osteoblasts derived from old animals have a lower metabolic activity than cells derived from young animals, we were interested in determining whether the expression of TGF-β and IL-1β mRNA and the response to $1,25(OH)_2D_3$, 17β-estradiol, and testosterone in primary cultures of bone-derived osteoblastic cells from mice decrease in old animals in comparison with young ones.

Results

Bone cells started to migrate from the bone chips after 5–10 d. The culture became confluent within approx 4–6 wk. Cells from bone chips of young donors grew to confluency within 3–5 wk whereas cells from old animals grew to confluency within 5 to 6 wk. The confluent cells of the first passage again reached confluency in the culture flasks within 2 to 3 wk. The majority of cells of the first passage had a broad and flattened morphology. Second-passage cells became multilayered; the addition of β-glycerophosphate and ascorbic acid induced the formation of mineralized nodules. These were formed within 2 to 3 wk of confluency, as indicated by strong alizarin red stain.

Each of the cell lines that were used in the present study had detectable basal expression of alkaline phosphatase (ALP) activity. We used 6 batches of cells obtained from young mice and 10 batches of cells obtained from old mice (Table 1). In these cells we examined the increase in cyclic adenosine monophosphate (cAMP) in response to parathyroid hormone (PTH), changes in ALP activity, in collagen synthesis, and the ability to form mineralized extracellular matrix nodules in response to $1,25(OH)_2D_3$ and sex steroid hormones. Each of the primary osteoblastic cells selected in this study showed that at least one of these phenotypic parameters was positive.

Basal Characterization and Effect of Age and $1,25(OH)_2D_3$ on Metabolic Activity

The basal unstimulated levels of ALP activity significantly decreased in cells obtained from old mice (Fig. 1). Stimulation with $1,25(OH)_2D_3$ for 48 h resulted in an increase in ALP activity in the cells of young and old donors (Fig. 1). The basal levels of mineralization in cells obtained from young and old donors were similar. Increased mineralization by $1,25(OH)_2D_3$ was observed in cultures from young and old animals (Fig. 1, Table 1).

Basal levels of cAMP production were higher in cells derived from young mice when compared with old mice (Fig. 2). PTH at 10^{-8} *M* significantly increased cAMP production in cultures from young mice, with an insignificant increase in old mice (Fig. 2). There was no difference in the basal levels of collagen synthesis between old and young animals (Fig. 3). Stimulation with $1,25(OH)_2D_3$ produced a slight but insignificant increase in collagen production in old animals (results not shown).

Effects of Sex Steroid Hormones on Basal Activity

17β-Estradiol stimulated ALP activity in the cultures from most animals (Table 1). Testosterone stimulated ALP activity in the majority of cell lines, but the differences in the various groups were not statistically significant from the basal levels (Table 1). Both 17β-estradiol and testosterone increased collagen production in cells derived from old mice (Fig. 3).

Increased mineralization by 17β-estradiol and by testosterone was observed in cell cultures from young and old mice (Fig. 4). However, there were no statistically significant differences between cultures of young and old animals following stimulation of the various cell batches by any of the hormones used. Table 1 presents the results of ALP activity and mineralization for each individual cell line.

Effect of Age and Hormones on the Expression IL-1β and TGF-β mRNA

Hybridization of cultured cells with the control sense ^{35}S-labeled oligonucleotide probes showed that there are few silver grains randomly distributed across the cultures, demonstrating the specificity of the technique (Fig. 5A). When bone cells were treated with the antisense ^{35}S-labeled oligonucleotide, grains inside the osteoblast-like cells were

Table 1
Summary of Results in Mouse-Derived Osteoblastic Cells Cultured in the Presence of $1,25(OH_2)D_3$, Estradiol, and Testosterone[a]

		ALP					Mineralization				
	Age	D_3^{-8}	E^{-9}	E^{-8}	T^{-9}	T^{-8}	D_3^{-8}	E^{-9}	E^{-8}	T^{-9}	T^{-8}
1	Young	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
2	Young	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
3	Young	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
4	Young	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
5	Young	↑	—	↑	—	—	ND	ND	ND	ND	ND
6	Young	↑	↑	—	—	—	ND	ND	ND	ND	ND
7	Old	↑	↑	↑	↑	—	ND	ND	ND	ND	ND
8	Old	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
9	Old	↑	—	↑	↑	—	↑	↑	↑	↑	↑
10	Old	↑	—	—	—	—	ND	ND	ND	ND	ND
11	Old	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
12	Old	—	—	—	—	—	↑	↑	↑	↑	↑
13	Old	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
14	Old	↑	—	↑	↑	—	↑	↑	↑	↑	↑
15	Old	↑	↑	↑	↑	↑	ND	ND	ND	ND	ND
16	Old	↑	↑	↑	↑	↑	ND	ND	ND	ND	ND

[a]D3, $1,25(OH_2)D_3$; E, estradiol; T, testosterone; ↑, increased activity in response to hormones; —, no change in response to hormones; ND, not done.

detected (Fig. 5B,C). Evidence of IL-1β mRNA expression was observed in young and old donors.

Figure 6 shows the effect of age on IL-1β mRNA levels. IL-1β mRNA levels decreased in old mice (less labeled cells) compared with young ones. Stimulation with $1,25(OH)_2D_3$ increased the expression of IL-1β in cells derived from young but not from old animals (Fig. 7).

With 17β-estradiol and testosterone stimulation, IL-1β mRNA expression was inhibited only in cells derived from young mice (Figs. 8 and 9).

Figures 10 and 11 illustrate the effect of age and $1,25(OH)_2D_3$ on the expression of TGF-β mRNA. Decreased expression of TGF-β was observed in cell cultures from old mice (Fig. 10). About 75% of cells from young donors expressed TGF-β gene (from 1 to 20 grains per cell), whereas only about 55% of cells from old donors were positive (Fig. 10). Cultures from old mice had increased TGF-β levels in response to $1,25(OH)_2D_3$ with no effect on young animals (Figs. 10 and 11). 17β-Estradiol and testosterone did not change the basal expression of TGF-β mRNA in osteoblasts obtained from mice of both ages (data not shown).

TGF-β mRNA and IL-1β expression was observed only in 14 of the 16 cell cultures. The two cell batches that were negative for both TGF-β mRNA and IL-1β were from old mice (results not shown).

Discussion

The present study demonstrates that bone-derived cells from mice that express basal ALP activity also show an increase in cAMP in response to PTH. An increase in ALP activity and mineralization was observed in response to $1,25(OH)_2D_3$ and sex steroid hormones. The basal activity and the response to hormones of 10- to 12-mo-old animals was often lower than that of young, 1- to 3-mo-old animals. We also found that rat and mouse osteoblastic cells in culture express both IL-1β and TGF-β mRNA. The expression is decreased in old animals.

We found previously that estradiol and testosterone enhanced differentiation of rat- derived chondrocytes in culture. They increased in a gender-dependent manner ALP activity and collagen synthesis and decreased mitosis *(34–36)*. In the present study, we demonstrated that sex steroid hormones have similar effects on cultured osteoblast-like cells, i.e. increased ALP activity, increased mineralization, and, in cells derived from old mice increased collagen synthesis.

An age-related decrease in the skeletal content of IGF-1 and TGF-β was observed by Nicolas et al. *(8)* in samples of femoral cortical bone from men and women. This finding is similar to our results of decreased TGF-β mRNA expression in old animals. However, since these growth factors are produced by various cells, it is difficult to know from this study whether the osteoblasts produce fewer growth factors, or whether it is merely a reflection of the decreased production by other cells in the bone marrow since the entire bone and bone marrow were examined. In this study serum levels of IGF-1 were also found to decrease with age, further emphasizing that the decrease in bone IGF-1 and probably TGF-β may be part of a general process of decreased growth factor formation with age *(37,38)*. Our

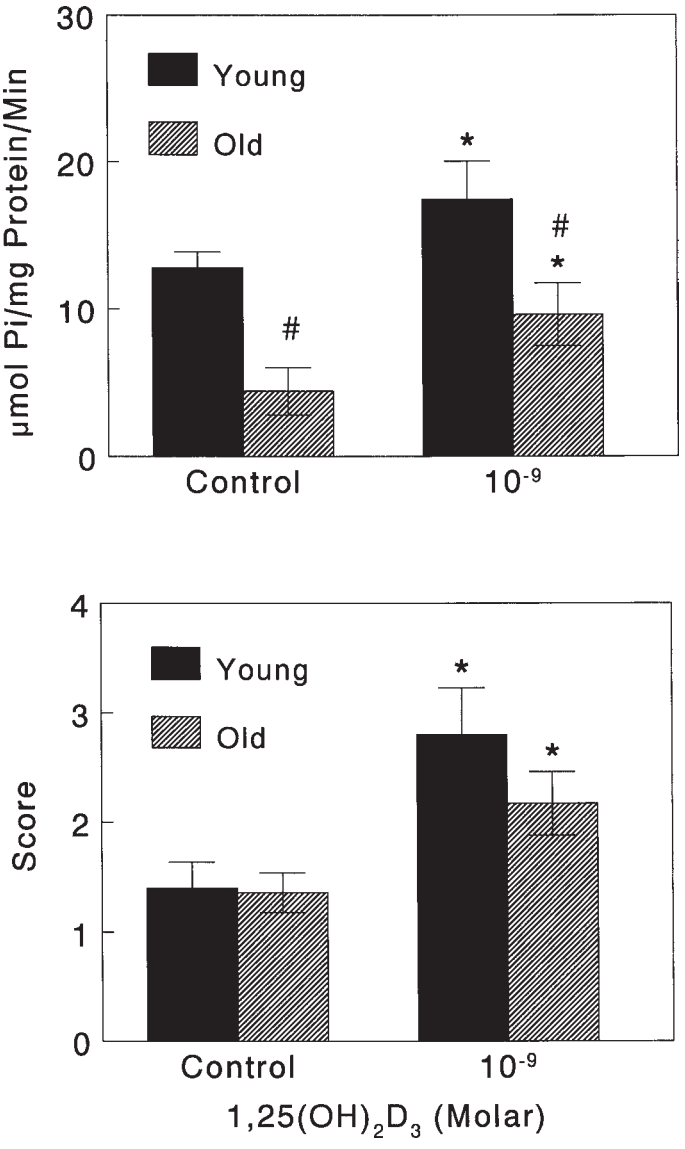

Fig. 1. The effect of age on **(A)** ALP activity and **(B)** mineralization of mouse osteoblastic cells with and without $1,25(OH)_2D_3$ stimulation. Data are shown as mean ± SEM of three to seven samples of each group. Each sample represents four well cultures. $^*p < 0.05$, treatment vs control, in which treatment values are greater than control values.

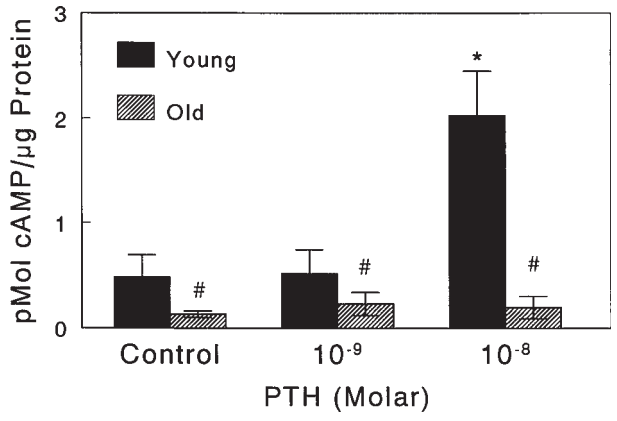

Fig. 2. The effect of PTH on cAMP production in mouse osteoblastic cells. Data are shown as mean ± SEM of three to six samples of each group. Each sample represents the mean of six well cultures. $^{\#}p < 0.05$ values in cells derived from old donors are significantly lower than in cells from young animals. $^*p < 0.05$, treatment vs control, in which treatment values are greater than control values.

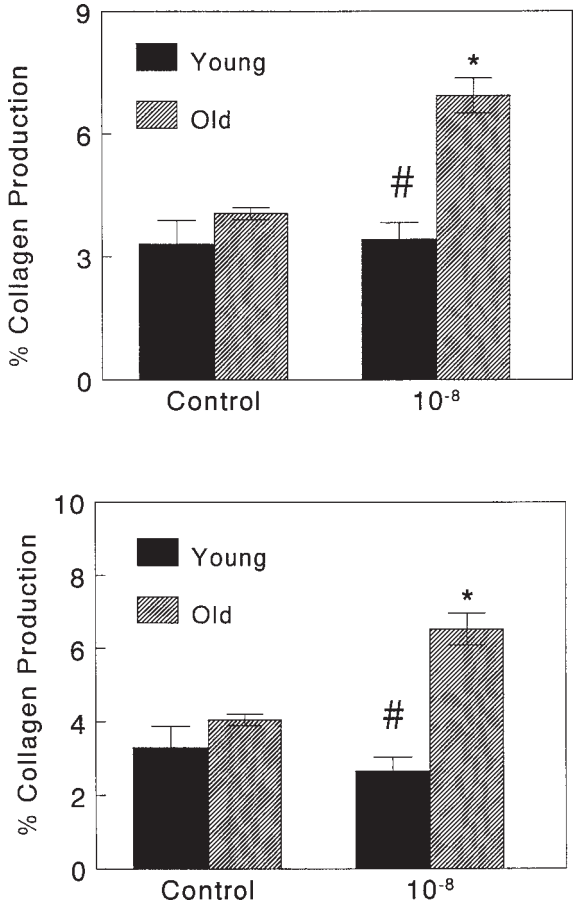

Fig. 3. The effect of **(A)** 17β-estradiol and **(B)** testosterone on collagen synthesis of mouse osteoblastic cells. Data are shown as mean ± SEM of 3–10 samples of each group. Each sample represents the mean of six well cultures. $^{\#}p < 0.05$, collagen synthesis in cells derived from young donors treated with testosterone is significantly lower than in cells derived from old animals; $^*p < 0.05$, treatment vs control, in which treatment values are greater than control values.

results show a direct age-related decrease in TGF-β mRNA expression in the primary osteoblastic cultures.

Generally, the response of human-derived osteoblasts from old donors to bone-seeking hormones was found to be less prominent in comparison with osteoblasts derived from young donors, but these results are inconclusive *(6,7)*. We recently studied primary cultures of human-derived osteoblasts and found an age- and gender-dependent response of these cells to 17β-estradiol as well as testosterone, as expressed by ALP activity, osteocalcin production, and creatine kinase activity, that was generally reduced in cells obtained from old individuals *(39)*.

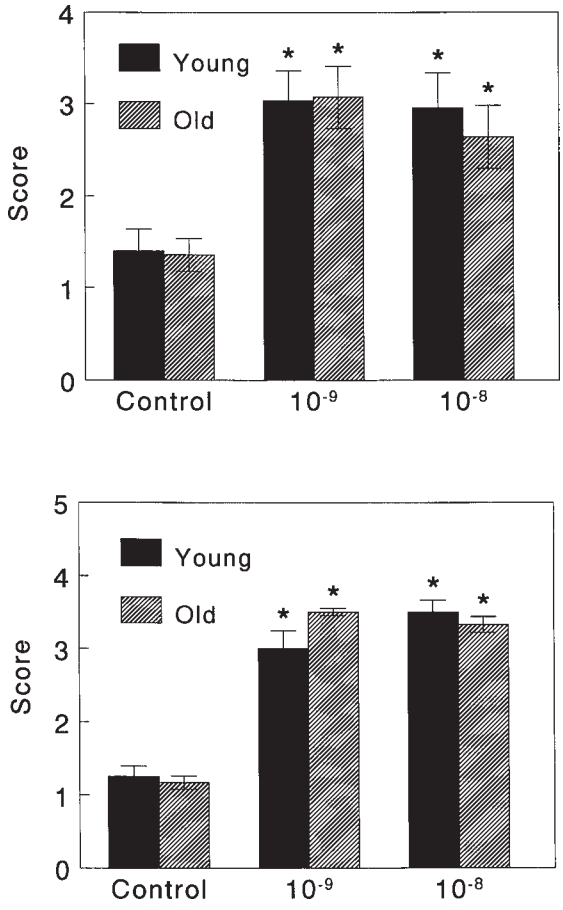

Fig 4. The effect of **(A)** 17β-estradiol and **(B)** testosterone on mineralization of mouse osteoblastic cells treated for 48 h with doses of 10^{-9} and 10^{-8} *M* of 17β-estradiol and testosterone. Data are shown as mean ± SEM of 3–10 samples of each group. Each sample represents the mean of four well cultures. $*p < 0.05$, treatment vs control, in which treatment values are greater than control values.

TGF-β seems to stimulate proliferation of osteoblast precursors, thus increasing the pool of committed osteoblasts. In addition, it decreases bone resorption by increasing the apoptosis of osteoclasts *(1)*. The peak bone mass in mice is reached at 6–12 mo of age *(40)*, an age when gonadal function is still intact. The decrease in TGF-β formation by osteoblasts at 10–12 mo of age in our animals, which is probably more evident at later ages, may gradually lead to bone loss.

$1,25(OH)_2D_3$ increased TGF-β mRNA expression mainly in osteoblasts derived from old animals. Recently we also observed an increased TGF-β mRNA expression induced by $1,25(OH)_2D_3$ in chondrocytes derived from rat growth zone costal cartilage *(41,42)*. In both cases, these findings emphasize that $1,25(OH)_2D_3$ is an important regulator of TGF-β production by mature chondrocytes and osteoblasts, thus directly regulating important phases in cartilage and bone growth.

IL-1β is a known potent bone resorption factor *(16)*. One would expect, therefore, to have an increase in IL-1β pro-

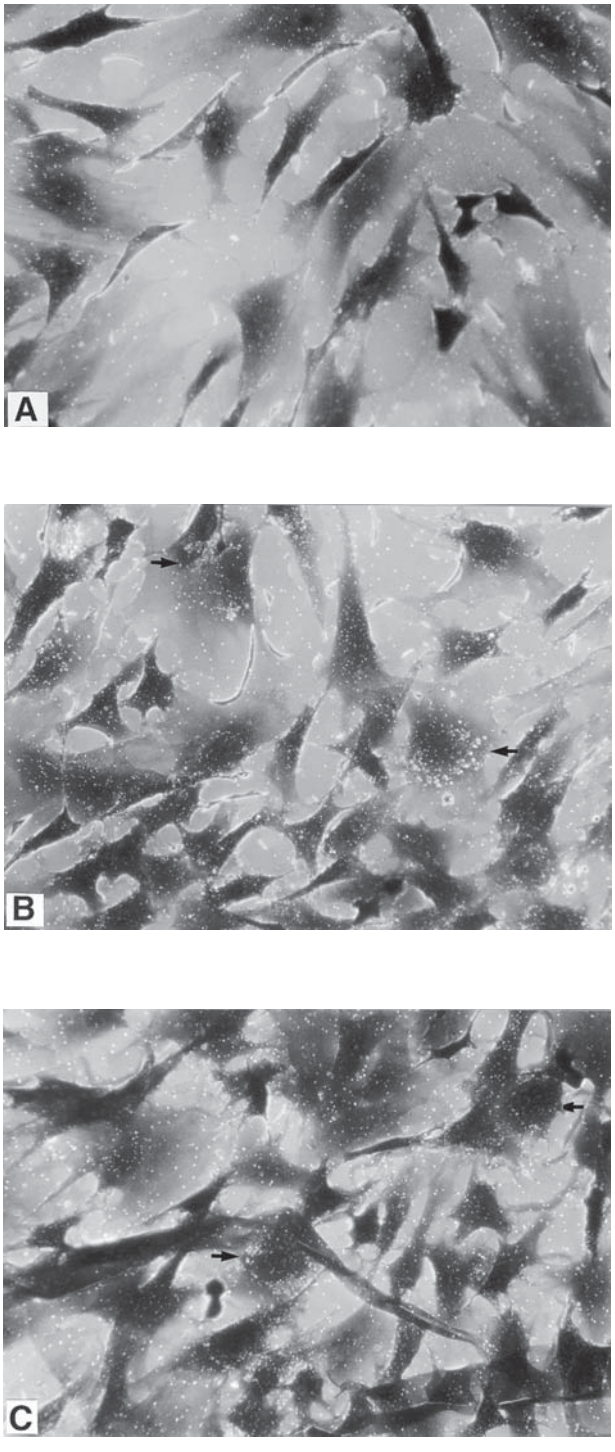

Fig. 5. Dark-field photomicrographs of *in situ* hybridization using IL-1β oligonucleotide probe to mRNA of cultured cells derived form mouse femoral bone. **(A)** Sense IL-1β oligonucleotide probe showing few silver grains randomly distributed across the cultures: ×1000. **(B)** Hybridization with antisense IL-1β probe to cultures from young mouse donors showing specific silver grains accumulated in cells (arrows): ×1200. **(C)** Osteoblastic cell cultures from old mouse donors had decreased silver grains as compared to cells from young ones (B) (arrows): ×1000. Autoradiographs were exposed for 4 weeks and stained with toluidine blue.

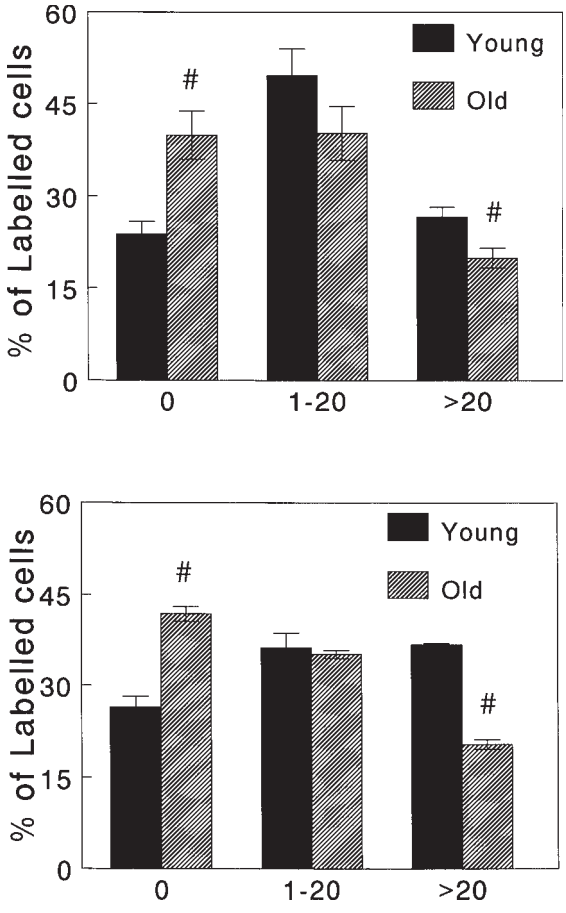

Fig 6. The effect of age on **(A)** IL-1β and **(B)** TGF-β mRNA expression of mouse osteoblastic cells in the absence of $1,25(OH)_2D_3$ and the sex steroid hormones. Data are shown as mean (SEM of four to eight samples. Each sample represents four well cultures. $^{\#}p < 0.05$, old vs young.

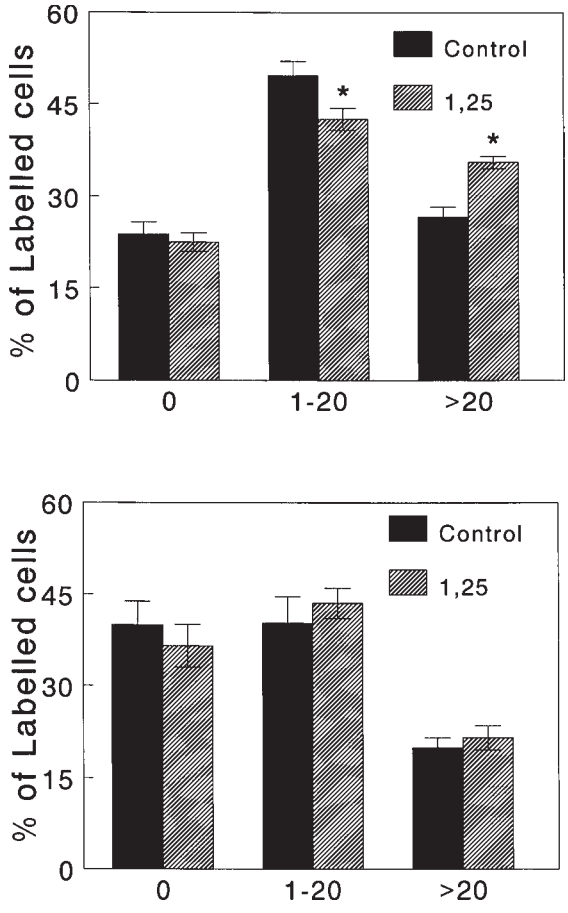

Fig. 7. The effect of 10^{-8} *M* $1,25(OH)_2D_3$ on IL-1β mRNA expression of young and old mouse osteoblastic cells. Data are shown as mean ± SEM of four to eight samples. Each sample represents four well cultures. $^{*}p < 0.05$, treated vs untreated, in which treatment with $1,25(OH)_2D_3$ increased IL-1β mRNA expression in cells from young animals.

duction by osteoblasts with an increase in age or in cases of increased bone loss. Indeed, IL-1β as well as IL-6 production is markedly increased in rats following gonadectomy *(32,33)*. In addition, IL-1β production by human monocytes is increased in women with postmenopausal osteoporosis *(16)*. IL-1β was found also to inhibit bone formation both in vivo and in vitro *(16,20)*, thus adding to the net increase of bone loss. We observed, however, a decrease in IL-1β mRNA expression in old mice. The decrease in IL-1β mRNA expression in old mice can perhaps be explained as a physiological response of the animals to their decreased production of TGF-β (and probably other growth factors as well), in an effort to reduce bone loss, which at that age apparently did not yet begin *(43)*.

It is hypothesized that age-related bone loss is owing to inefficient or impaired skeletal coupling *(43)*. If bone coupling is determined at least in part by bone growth factor production, then the changes in the concentration of bone growth factors could contribute to age-related bone loss.

Gonadectomy is known to induce bone loss in various animals *(43)*. In postmenopausal women an increase in the production of IL-1β was considered to be one of the main mechanisms for postmenopausal osteoporosis *(19,43)*. Ovarian steroid hormones blocked the postmenopausal increase in IL-1β production by peripheral monocytes *(18)*. In our study, testosterone and 17β estradiol were found to reduce and $1,25(OH)_2D_3$ to increase IL-1β mRNA expression only in the cell lines derived from young animals. This implies that young mice are more responsive to the regulation of growth factor secretion by various hormones in comparison with old animals.

In conclusion, we demonstrated a decrease in the activity of primary osteoblastic cell cultures derived from old mice in comparison with cells derived from young animals, and a decreased expression of TGF-β and IL-1β mRNA. This is probably part of a general decrease with age in the formation of growth factors by osteoblasts.

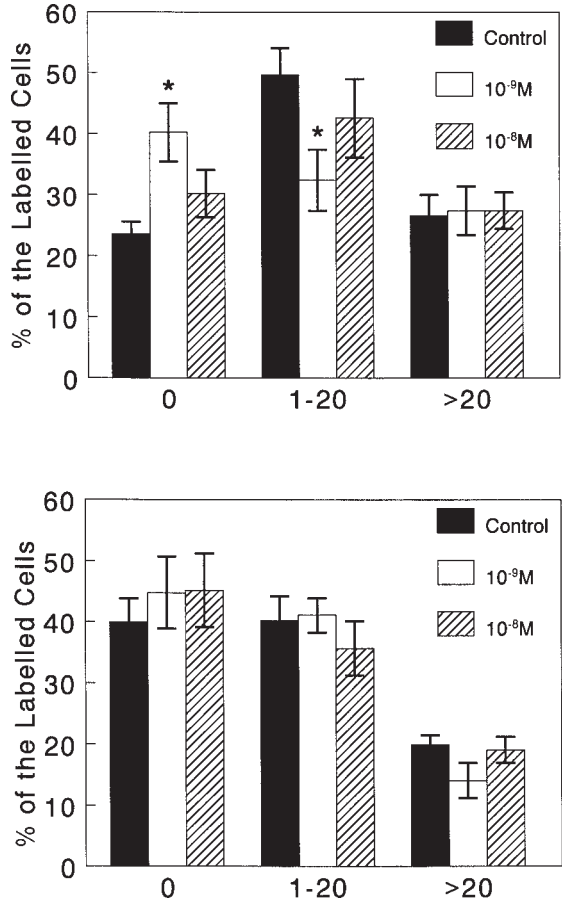

Fig. 8. The effect of 17β-estradiol on IL-1β mRNA expression of young and old mouse osteoblast-like cells treated with 10^{-8} and 10^{-9} *M* 17β-estradiol. Data are shown as mean ± SEM of four to eight samples. Each sample represents four well cultures. $*p < 0.05$, treated vs untreated, showing after treatment an increase in the number of cells with little label and a decrease of those with more than 20 grains/cell.

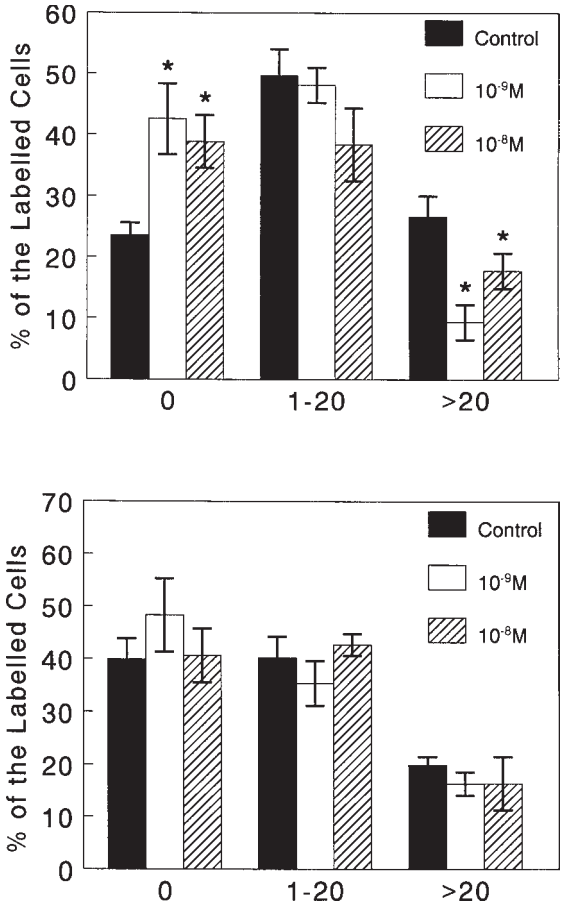

Fig. 9. The effect of testosterone on IL-1β mRNA expression of **(A)** young and **(B)** old mouse osteoblast-like cells treated with 10^{-8} and 10^{-9} *M* testosterone. Data are shown as mean ± SEM of four to eight samples. Each sample represents four well cultures. $*p < 0.05$, treated vs untreated, showing after treatment an increase in the number of cells without label and a decrease of those with more than 20 grains/cell.

Materials and Methods

Animals

Normal mouse bone cells were prepared from the femurs of 5- to 12-wk (young) and 10- to 12-mo-old (old) mice from the Hebrew University Sabra strain (Table 1). We used 16 different cell cultures, 6 from young mice and 10 from old mice. All cells exhibited high ALP activity.

Reagents

$1,25(OH)_2D_3$ was obtained from Teva Pharmaceuticals (Jerusalem, Israel) and human PTH *(1–34)*, 17β-estradiol, and testosterone were obtained from Sigma (St. Louis, MO). BGJ, Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B were purchased from Biological Industries (Migdal Haemek, Israel). Culture flasks and dishes were purchased from Gibco (Gibco-Europe BV, The Netherlands).

Isolation and Culture of Bone Cells

Mouse primary osteoblastic cell cultures were established by a modification of the method described by Beresford et al. *(43)*. Briefly, femurs were obtained at the time of sacrifice, cleaned of the adherent tissue and marrow cells, and were fractured into small pieces 1 × 1 × 2 mm. The pieces of bone further cleaned of any connective tissue, minced, and rinsed several times with DMEM. The pieces of bone were precultured for 48 h in BGJ (Fitton-Jackson modification) without calcium, supplemented with 10% FBS, 2 m*M* glutamine, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 mg/mL amphotericin B). After 48 h of preculture, the medium was replaced by DMEM that included calcium and Ham's F-12 with the supplements as just given. Medium was then changed at 5-d intervals. The confluent cells that grew out of the pieces of bone were harvested by trypsinization and plated at a density of 5×10^4 cells/cm^2 into tissue culture wells with DMEM and F10 medium. Confluent cells were then

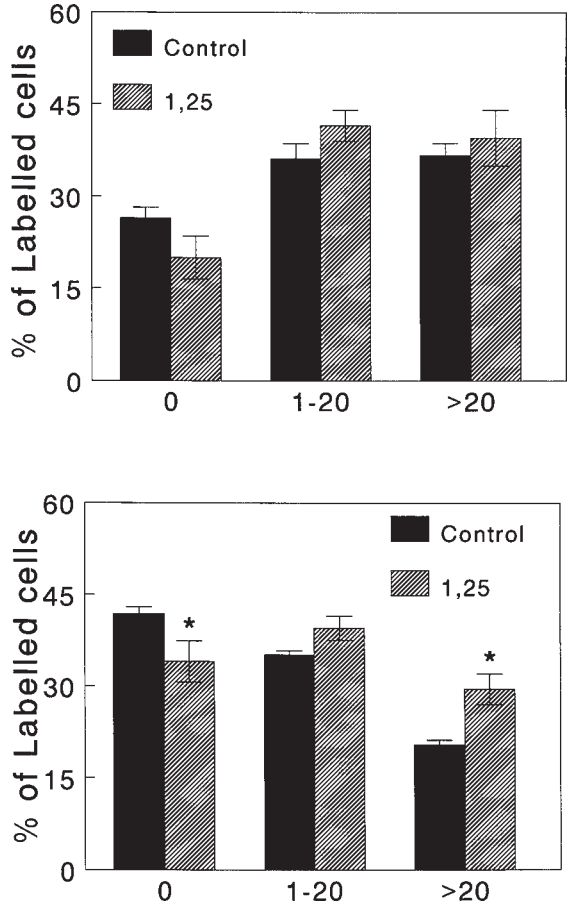

Fig. 10. The effect of $1,25(OH)_2D_3$ on TGF-β mRNA expression of **(A)** young and **(B)** old mouse osteoblast-like cells treated for 48 h with 10^{-9} and 10^{-8} *M* of $1,25(OH)_2D_3$. Data are shown as mean ± SEM of four to eight samples of each group. Each sample represents the mean of four well cultures. $*p < 0.05$, treated vs untreated, showing an increase in the number of cells with more than 20 grains/cell in old mice, and a decrease in the number of unlabeled cells.

transferred to 24-well dishes for subsequent biochemical analysis. All analyses were done on cells from the second passages.

Protein Determination

Protein content was measured using a BCA Protein Assay Reagent Kit (Bio-Rad, Hercules CA) according to the method described by Bradford *(44)*.

ALP Activity

The cells from 24-well plates were harvested after being cultured for 48 h with $1,25(OH)_2D_3$ or sex steroid hormones. Cells are washed within the wells with phosphate-buffered saline (PBS), frozen in liquid nitrogen, defrosted at room temperature three times, and then scraped out of the wells and stored at –20°C. ALP was measured as a function of release of *p*-nitrophenol from *p*-nitrophenol phosphate according to the method described by Bretaudierre and

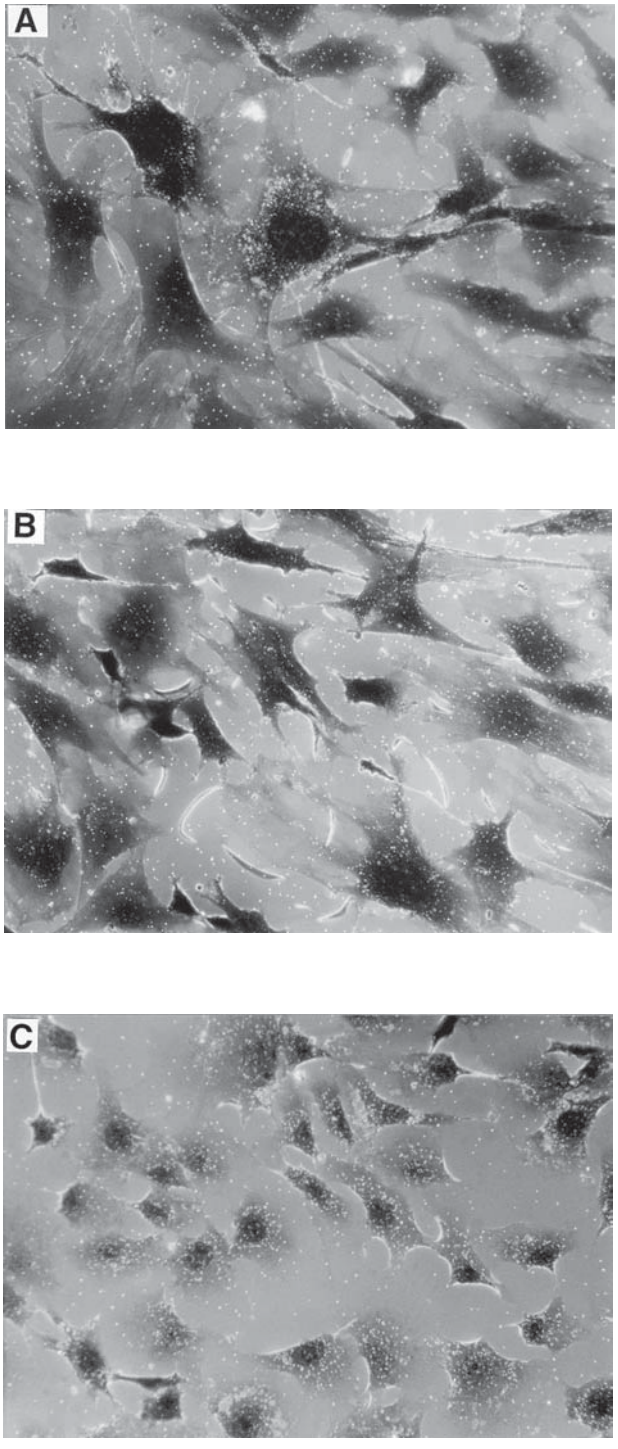

Fig. 11. Dark-field photomicrographs of *in situ* hybridization using TGF-β oligonucleotide probe to mRNA of cultured cells derived from young and old animals. **(A)** Cultures from young mice hybridized with antisense TGF-β probe showing numerous specific silver grains accumulated in cells: ×1200. **(B)** Cultures from old donors with decreased silver grains when compared with cultures from young ones: ×1000. **(C)** Enhanced TGF-β mRNA expression following stimulation with $1,25(OH)_2D_3$ in cultures from old animals: ×800. Autoradiographs were exposed after 4 weeks and stained with toluidine blue.

Spillman *(45)*. ALP activity is expressed as mmol of Pi/(mg of protein · min.

cAMP Response to PTH

Cells in six-well plates from each culture were stimulated for 20 min with PTH 10^{-9}–10^{-8} *M*, and cAMP was measured after trichloroacetic acid (TCA) precipitation of the cell extracts using a competitive protein binding assay as described by Pfeilschifter et al. *(46)*. Results are expressed as pmol of cAMP/μg of protein.

Analysis of Collagen Synthesis

Confluent cells were incubated for 48 h with $1,25(OH)_2D_3$, or sex steroid hormones, and at the last 24 h supplemented by 5 μCi/mL of [^{3}H] proline para aminopropinitole (New England Nuclear, Boston, MA). Medium and cell layer were precipitated with 0.1 μL of 100% TCA containing 10% tannic acid, and were washed three times with 10% TCA plus 1% tannic acid and then twice with ice-cold acetone. The final pellet was dissolved in 500 μL of 0.05 *N* NaOH. The amount of radiolabeled proline incorporated into collagenase digestible protein and noncollagenase-digestible protein was determined according to the method of Pewterkofsky and Diegelmann *(47)*, with the correction by Raisz.

In Vitro Mineralization

Seven d after the addition of the different affectors for 24 h to the cells the medium of the cell cultures was changed to DMEM-F12 + 50 μg/mL ascorbate + 10 m*M* β-glycerol-phosphate + 10 n*M* dexamethasone for 21 d. This medium was changed every 7 d. After 21 d of incubation, cells were rinsed, fixed with methanol, stained with 1% alizarin red, and examined by light microscope for positive stain. The number and size of nodular positive stain (mineralization sites) were examined in each well. A score of 1–4 related to the size and number of mineralization sites was given for each well, with 4 representing the highest number and size of mineralization sites. A number was given for each well, and a mean number of four wells, either controls or cells treated with $1,25(OH)_2D_3$ or sex steroid hormones, was calculated.

In Situ *Hybridization*

In situ hybridization that localizes mRNA expression at the single-cell level was performed as a modification of the method described by Wilkinson *(48)*. Second-passage cells were grown on cover slips. (Sub)confluent cells from second passage were rinsed twice in 0.1 *M* PBS, fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, and stored in 70% ethanol at –20°C.

For *in situ* hybridization, the following 30 mer antisense and sense (used as negative control) oligonucleotide probes were synthesized by 394 Synthesis Setup Listing (Version 2.01): the antisense 30 mer oligonucleotide for mouse IL-1β probe *(49)* (nucleotides from 196 to 225), and the 30 mer oligonucleotide probe for mouse TGF-β *(50)* (nucleotides from 471 to 500). The oligonucleotide probes were checked for the absence of palindromes by Gene Bank data. Sense probes also similarly synthesized were used for negative control. [^{35}S] dATP labeled oligonucleotide probes were prepared using the DNA 3'-end labeling system (Promega, Madison, WI). The probes were purified using Bio-Spin 6 chromatography columns (Bio-Rad). The specific activity was 4–5 × 10 cpm/μg.

The cover slips were rehydrated for 10 min in 0.1 *M* PBS, pretreated with 1 μg/mL of proteinase K, and diluted in 0.1 *M* Tris buffer, pH 8.0, and 50 m*M* EDTA, pH 8.0 (Sigma) at 37°C for 10 min. The cover slips were subsequently refixed in 4% paraformaldehyde, acetylated with 0.25% acetic anhydrite in 0.1 *M* triethanolamine (Sigma), and rinsed in 10% SSC for 10 min. One hundred percent SSC (or 20X SSC) is composed of 3 *M* NaCl, 0.3 *M* sodium citrate, adjusted to pH 7.0 by HCl, with 1 mL/L of diethylpyrocarbonate (DEPC). The cover slips were hybridized in a buffer solution containing 50% formamide, 20% SSC, 10% dextran sulfate, 10 m*M* dithiothreitol, 500 μg/mL tRNA, 1X Dendardt's, and 100 μg/mL salmon sperm DNA (SSDN). Thirty microliters of hybridization buffer was added to each cover slip, which contained the probe with a specific activity of 1×10^6 cpm/μg (all from Sigma). Hybridization was performed in a humidified environment in 24 wells overnight at 37°C. All the solutions used before the process of washing the cover slips were pretreated with 0.1% DEPC (Sigma) to destroy RNase activity.

After hybridization the cover slips were washed with SSC containing 0.1 *M* β-mercaptoethanol as follows: 10% SSC for 10 min at room temperature; 1% SSC twice for 30 min at 50°C; 0.5% SSC twice for 30 min at 52°C; 5% SSC for 10 min at room temperature. Finally, the cover slips were air-dried and then mounted face up on slides.

The cover slips were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with water, dried at room temperature overnight, and exposed at 4°C in desiccated slide boxes for 4 wk. The exposed cover slips were developed in D-19 developer at 24°C for 4 min, fixed in Unifix for 5 min, and finally washed with water for 10 min. Cover slips were counterstained with 0.5% toluidine blue and mounted using ENTELLAN (Merck, Darkstadt, Germany).

Morphometric analysis was assessed by counting the silver grains in each cell as described by Watson et al. *(51)*. One hundred cells per cover slip were counted under a high-power objective that gave a 1000-fold magnification in random fields. Data were accumulated as the number of grains per osteoblast after correction for the background. The background hybridization signal was assessed on every slide, and this value was subtracted before calculation of the data. All observations and photographs were made on an Aristoplan photomicroscope (Leitz, Wetzlar, Germany) using Kodak Ektar 100 film.

Statistics

Silver grain count data from the *in situ* hybridization were calculated as an average grain density per osteoblast for each cover slip, expressed as a mean ± SEM. The frequency of distribution of silver grain density per osteoblast in each treatment group was calculated for graphical presentation. Nonparametric Mann-Whitney tests were used to determine the statistical significance between young and old donors. Differences between control and $1,25(OH)_2D_3$, 17β-estradiol, or testosterone treatment were analyzed using the Wilcoxon test.

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