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*J Clin Invest.* 1986;78(3):637-642. <https://doi.org/10.1172/JCI112621>.

### Research Article

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# Bone Deficit in Ovariectomized Rats

## Functional Contribution of the Marrow Stromal Cell Population and the Effect of Oral Dihydroxycholesterol Treatment

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### Abstract

This study investigates the proliferative and osteogenic role of marrow stromal/osteoprogenitor cells in the development of the cortical bone deficit in ovariectomized (OVX) female rats. In vitro, clonal growth of marrow stromal cells from OVX rats was significantly impaired (vs. sham-operated controls). Yet in vivo, cells from sham-operated and OVX rats had equal osteogenic potential in several in vivo experimental situations, such as in intraperitoneally implanted millipore diffusion chambers and in intramuscular implants of marrow plus osteoinductive bone matrix (composite grafts). Long-term (6 mo) dihydroxycholesterol (DHT) treatment of OVX rats enhanced their in vitro proliferative potential and clonal growth, as well as their osteogenic expression in composite grafts. The observation that the in vivo osteogenic performance of OVX rat marrow stromal cells was normal at extraosseous sites suggests that the mechanisms leading to osteopenia may involve an abnormality in cell-matrix interactions.

### Introduction

It is well known that rats develop osteopenia after ovariectomy (OVX),<sup>1</sup> and the OVX rat has been used as a model for postmenopausal osteoporosis (1–3). Our studies have shown that bone density in rats is reduced 6 mo post-OVX, and that the ratio of femoral cortical thickness to midshaft diameter was significantly lower than normal (x-ray femoral score; Table I). This suggested that the osteopenia might be due in part to deficient endosteal bone formation, and, by extension, either to a deficit in the proliferative capacity of osteoprogenitor cells (OPC) and/or to their capacity to modulate to functional osteoblasts. Marrow might serve as the source of the OPCs for endosteal osteoblasts since the stromal cells within marrow grafts form bone in ex-

traosseous sites (4), within millipore diffusion chambers (5), and in culture (6). In vitro, marrow stromal cells form clones known as fibroblast colony forming units (FCFU) (7), and, in rats, the FCFU census appears to be closely associated with the in vivo osteogenic performance of marrow biopsies combined with osteoinductive demineralized bone matrices (8). If the in vitro FCFU census could be used as an indirect osteogenic bioassay, and it might prove to be a system which could be applied in animal model systems and patients to develop a fuller understanding of mechanisms involved in the development of the osteoporoses.

In this study, we focus on the relationships between the in vivo patterns of FCFU development from, and proliferation kinetics of isolated marrow cell preparations derived from, age-matched intact and OVX rats, and the in vivo capacities of these cells to form bone in several animal model systems. We also tested the effect of protracted oral administration of dihydroxycholesterol, a 1,25(OH)<sub>2</sub>D<sub>3</sub> analogue, on these parameters since vitamin D therapy has resulted in improved calcium balance and bone mass/density scores in OVX rats (9, 10) and postmenopausal osteoporotic women (11–14). The data indicate that the cortical bone deficit in OVX rat is associated with an impaired proliferative capacity of marrow stromal osteoprogenitor cells rather than with the inability of these cells to differentiate/mature into functional osteoblasts. Dihydroxycholesterol treatment improved FCFU proliferation as well as their osteogenic potential in demineralized bone-marrow composite grafts.

### Methods

#### Animals

Female rats (Sprague-Dawley strain, 50–60 d old, 200 g body weight) were surgically ovariectomized (OVX), and they and their age-matched intact controls were maintained in a vivarium under conditions of ad lib. feeding (Purina Lab Chow; Ralston Purina Co., St. Louis, MO). Tap water was freely available. The OVX rats were significantly heavier (20%) than their age-matched intact controls at 1.5 mo postoperatively when, in several studies, OVX animals began to be treated for 1, 3.5, or 6 mo with dihydroxycholesterol (1.0 or 2.0 µg/100 g body weight, three times per week; Table I). 6 mo postoperatively, the femurs from untreated rats also demonstrated thinner than normal cortices and they were undermineralized (ash weight and bone density; Table I).

1.5–6.0 mo postoperatively, smears of intact plugs of marrow were dried and fixed in methanol for cytologic examination, and marrow was expressed from the femurs of 3–5 control and OVX animals into chilled alpha minimal essential medium (MEM). The marrow samples from each group were pooled.

#### Marrow preparation

A single cell suspension was prepared by passing the marrow plugs through a fine metal mesh, and cell counts were performed with a hemocytometer

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Received for publication 2 January 1986 and in revised form 9 April 1986.

1. *Abbreviations used in this paper:* DBM, demineralized allogeneic bone matrix; DHT, dihydroxycholesterol; FCFU, fibroblast colony forming units; OPC, osteoprogenitor cells; OVX, ovariectomy (ovariectomized); PE, FCFU plating efficiency.

J. Clin. Invest.

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0021-9738/86/09/0637/06 \$1.00

Volume 78, September 1986, 637–642

Table I. Effect of Ovariectomy on Bone Density Parameters in the Rat (6 mo Postoperatively)

	Sham-op rats	OVX rats	
Parameter	n = 8	n = 5	P Value
Femoral score*	46.61±7.0	40.93±2.0	<0.05
Density (mg/ml)	1.62±0.01	1.55±0.02	<0.01
Percent Ash Wt.	74.92±0.29	72.52±1.02	<0.05

\* The Femoral Score is calculated from radiographs, and represents the ratio between the sum of the widths of the cortical bone "walls" to the midshaft femoral bone diameter (29).

using the Trypan blue exclusion technique. Viable cells were plated-out in 25 cm<sup>2</sup> T-flasks at a density of 10<sup>7</sup> cells, or they were seeded into multiwell plates (Costar, Cambridge, MA) at a concentration of 8 × 10<sup>5</sup> cells per well. The cells were grown in alpha-MEM supplemented with 15% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) and a 1% penicillin-streptomycin-Fungizone mixture (Whittaker; MA Bio-products, Walkersville, MD). The cells were incubated at 37°C in a tissue culture incubator under an atmosphere of 5% CO<sub>2</sub>. The medium was usually changed completely after the first 24 h and every 2–3 d thereafter.

#### T-flask cultures

The percentages of the cell inocula which failed to attach after 24 and 72 h were calculated by counting the cells per milliliter recovered at the times the media were changed (Coulter Counter; Coulter Electronics, Inc., Hialeah, FL). The cells which attached and grew to form FCFU colonies were fixed in 60% citrate buffered acetone, stained for alkaline phosphatase activity (Sigma Kit No. 85; Sigma Chemical Co., St. Louis, MO) after 7, 10, and 12 d, and counterstained with Mayer's hematoxylin. The numbers and areas of the FCFU which contained a minimum of 50 cells were counted under a dissecting microscope. We also tested the effect of delaying the first medium change until the second and third days of culture on the FCFU census.

#### Costar cultures

These cultures were pulsed with <sup>3</sup>H-thymidine (<sup>3</sup>H-Tdr) for 1.0 h on the fifth, seventh, 10th, and 12th days of culture to study the kinetics of DNA synthesis. The cells were washed twice in 10% TCA to remove thymidine that was not incorporated into DNA, digested in 0.5 ml 0.2 N NaOH, and the <sup>3</sup>H-activity in 0.2-ml aliquots was counted in an automated Packard scintillation counter (Packard Instrument Co., Downers Grove, IL) using an internal standard. The total <sup>3</sup>H-activity measured in the cells was expressed as the fractional retention of the <sup>3</sup>H-activity introduced into each of the wells.

#### Osteogenic potential of marrow stromal cells

**Millipore diffusion chambers.** Aliquots of 3 × 10<sup>7</sup> freshly isolated marrow cells (pooled samples) from sham-operated and 7-wk-postoperative OVX rat donors were loaded into Algire-type millipore diffusion chambers (0.45-μm pore size). Two chambers bearing one or the other of the different classes of cells were implanted into the peritoneal cavities of 4-mo-old intact and OVX (7-wk-postoperative) host rats. Preliminary histologic investigations showed that bone was the only skeletal tissue formed within such chambers. The chambers from the animals were recovered at 3 wk postimplantation. Each of the animals had been injected i.p. 24 h before these times with 5.0 μCi <sup>45</sup>Ca to permit incorporation of the isotope into newly forming bone matrix, i.e., as an index to the osteogenic performance of the marrow stromal cells (8). At recovery, the chambers were stripped of adhering soft tissues, placed in several changes of 0.1 M CaCl<sub>2</sub> to dispose of any unincorporated radiocalcium, and extracted for 18 h in 0.6 N HCl at 110°C. An aliquot of the solution was counted in an automated scintillation counter (Packard Instrument Co., Downers

Grove, IL) equipped with an external standard. The data were expressed as cpm per chamber.

**Composite and demineralized bone grafts.** 8 and 20 wk postoperatively, intact rats and OVX rats were anesthetized with sodium pentobarbital, and their left hind limbs were amputated before marrow biopsy. A third group of OVX rats (20 wk postoperatively) was given dihydro-tachysterol orally (2.0 μg/100 g body weight [three times per week]; Winthrop Laboratories, New York, NY) 1 wk before biopsy. Fresh marrow was obtained from the femurs and combined with 10–15 mg demineralized allogeneic bone matrix (DBM) that had been prepared according to the recommendations of Urist (15) to preserve the osteoinductive bone morphogenetic protein. The bone-autologous marrow composite grafts were implanted into the paraspinous muscle (right side), and DBM grafts (10–15 mg) were implanted in a similar site on the contralateral side. Dihydro-tachysterol (DHT) was administered at the above schedule to the DHT-pretreated group until the grafts were recovered. The rats that had been OVX 8 and 20 wk before grafting were killed at 2 and 3 wk postimplantation, respectively, 24 h after receiving an intraperitoneal injection of 5.0 μCi <sup>45</sup>Ca to permit incorporation of isotope into the mineral fraction of newly forming bone and into any recalcifying sites in the original graft tissue. At recovery, the grafts were stripped of adhering soft tissues, placed in several changes of 0.1 M CaCl<sub>2</sub>, and they were demineralized in 0.6 N HCl for radioactivity measurements (see above). The amount of radiocalcium incorporated into the grafts was expressed as cpm per milligram weight of the original osseous component of the grafts (8).

#### Bone mineralization rates

To determine whether the changes in the marrow FCFU census (number of colonies) and their osteogenic expression was associated with changes in the endosteal bone mineralization/formation rate(s), the rats (sham-operative group and 20-wk post-OVX group) grafted with composite bone marrow and DBM were doubly labeled with tetracycline (10 mg/kg Achromycin, Lederle, Pearl River, NY) at 4 and 18 d postimplantation. The animals were killed 3 d after the second injection. At time of death, the femurs were recovered, stripped of soft tissues, defatted in acetone, and embedded undecalcified in methylmethacrylate. The diaphyses of the bones were serially sectioned (transversely at 75–100 μm), and examined by ultraviolet microscopy to identify the regions of active bone formation/mineralization and to measure the separation of the fluorescent labels. When possible, the data were recorded in terms of the daily rate of endosteal bone growth (micrometers per day).

#### Statistics

The differential performances of the in vitro marrow cell cultures (FCFU formation) and the osteogenic potential of the marrow cells was assessed by the Student *t* test when the variances of the data were equal, and by analysis of variance (ANOVA) (*F* test) and the Whitney-Mann *U* Test for ranked observations when the variances were unequal (16).

## Results

#### Marrow FCFU growth

Differential cell counts from the marrow smears failed to show significant intergroup differences in marrow cell populations. In both groups, 80% of the initial inocula remained unattached to the flask after 24 h, and 3–4% floating cells were detected in all cultures at 72 h. Yet the marrow from OVX rats (2.5–4.5 mo postoperatively) grew 30–55% fewer fibroblast colonies than normal (Fig. 1). The differences in growth were highly reproducible and were apparent as early as the seventh day of culture (Fig. 2). The normal FCFU plating efficiency (PE) was ~1/10<sup>5</sup> cells; in different experiments, the PE of OVX rats was always reduced by 20–50% after 7–12 d of culture.

The FCFU that grew in the control and OVX cell cultures

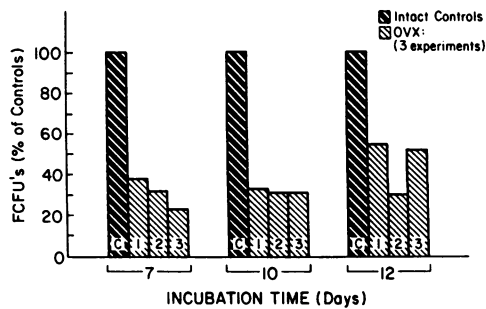


Figure 1. Bar graphs showing that the numbers of fibroblast colony forming units (FCFU) which develop in isolated marrow cell cultures from ovariectomized rats are fewer than those which develop in control cultures. The FCFU census in the control cultures in three separate experiments is expressed as 100%.

were similar in terms of their size distributions (control,  $1.65 \pm 0.19 \text{ mm}^2$  OVX,  $1.47 \pm 0.87 \text{ mm}^2$ ) and in the proportion of clones that were alkaline phosphatase-positive (7 d, 15%; 12 d, 40%).

#### Cell proliferation studies

The fractional retention of  $^3\text{H}$ -activity was significantly greater in the cultures of cells harvested from the control animals than from OVX rats (Fig. 3). Differences in cell proliferation were evident by the seventh day of culture. The activity incorporated by the cells in control cultures was four to five times that of the OVX cultures by the 12th day.

#### Effect of dihydrotachysterol treatment on marrow cells in vitro

Table II shows that in three of four experiments, marrow cultures from 5-9-mo-old OVX rats that had been treated with 1.0 and 2  $\mu\text{g}$ , respectively, for 1 or 6 mo postoperatively grew a significantly greater number of FCFU than marrow from untreated OVX rats ( $P < 0.05$ - $<0.001$ ). At the lower dose level and short treatment time, the DHT-FCFU census was slightly lower than that observed in cultures of marrow from untreated sham-operative rats. At the higher dose with the longer treatment time, DHT markedly increased the FCFU census above the values recorded for the sham-operative and untreated OVX rats ( $P < 0.01$ ). The benefit of DHT treatment was not observed in a single experiment of 3.5-mo duration.

#### In vivo animal models

**Millipore diffusion chambers.** The radioactivity incorporated into the contents of the diffusion chambers varied over a wide range

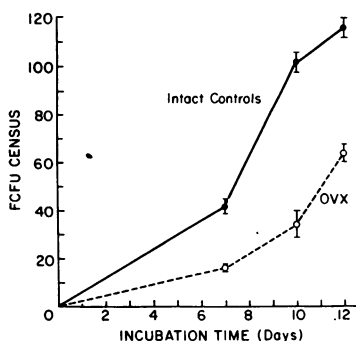


Figure 2. Graph comparing the census of fibroblast colony forming units (FCFU) which develop during culture of single cell suspensions of marrow ( $10^7$  cells) from intact and ovariectomized female rats.

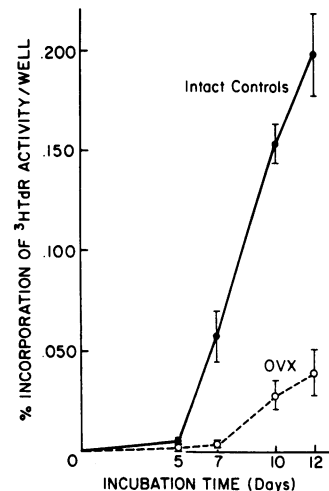


Figure 3. Graph comparing the fractional  $^3\text{H}$ -activity (SEM) incorporated by proliferating cells in cultures (Costar plates) of marrow from intact control female rats and ovariectomized rats.

in each group (cpm,  $<100$ - $\rightarrow$ 15,000). ANOVA indicated that the osteogenic performance of the stromal cells derived from marrows of sham-operative and OVX rats were not statistically different in the different types of host rats. However, rank-order analyses (Mann-Whitney  $U$  Test) indicated that the stromal cells from OVX rats formed significantly more bone in sham-operative hosts than in OVX hosts, but that OVX-stromal cells did not outperform the sham-operative stromal cells in the sham-operative hosts (Table III).

**Composite bone grafts (Table IV).** Radiocalcium incorporation by composite grafts of allogeneic bone-autologous marrow was not quantitatively different 2-3 wk after intramuscular implantation, irrespective of the ages of the rats from which the marrow was obtained. However, isotope incorporation was markedly increased two- to threefold in the grafts implanted in 20 wk post-OVX DHT-treated rats.

#### Demineralized allogeneic bone implants (Table IV)

The patterns of radiocalcium incorporation by DBM grafts in sham-operative and OVX rats mirrored the results observed for the composite bone grafts. Isotope retention was nearly identical in DBM grafts implanted in sham-operative and OVX hosts, but it was significantly increased after DHT treatment. In the same animal, DBM grafts always incorporated two- to threefold less  $^{45}\text{Ca}$  than the marrow-replete composite grafts ( $P < 0.01$ ).

#### Endosteal bone formation/mineralization

The radiologic bone score measurements for ovariectomized (OVX) rats 6.0 mo postoperatively were significantly lower than normal (Table I). Ultraviolet microscopy of diaphyseal bone sections from the femurs of sham-operative, OVX and 6-mo DHT-treated OVX rats revealed double tetracycline labels on periosteal surfaces, and only single labels on endosteal surfaces. The rate of periosteal appositional bone growth/mineralization in these very mature rats was in the order of  $1.0 \mu\text{m/d}$ .

#### Discussion

It has long been recognized that marrow stromal cells have osteogenic potential (5, 7, 8), but there has been no direct evidence, in lieu of pathology or of fracture healing, that they ever express this potential in situ. We attempted to develop such data using

Table II. Effect of Oral DHT Treatment on the In Vitro Development of Marrow Stromal Cell Fibroblast Colony Formation Units (FCFU) in Ovariectomized Rats

Age	Post-op	Group	DHT dose (three times weekly)	Treatment time	No. of FCFUs (Duration of culture)	
					7-d T-flasks	10-d T-flasks
5	3	Control	—	—	—	180.6±5.1 (5)
		OVX	—	—	—	54.4±8.9 (5) <sup>  </sup>
		OVX + DHT	1.0 µg	4 wk	—	110.4±18.3 (5) <sup>  ,¶</sup>
					7-d T-flasks	10-d T-flasks
7	5	Control	—	—	—	76.6±5.8 (5)
		OVX	—	—	—	37.8±6.8 (5) <sup>§</sup>
		OVX + DHT	1.0 µg	3.5 mo	—	19.4±5.4 (5) <sup>  </sup>
9	7	Control	—	—	7-d*	9-d*
		OVX	—	—	14.2±0.9 (6)	33.7±3.7 (6)
		OVX + DHT	2.0 µg	6.0 mo	2.9±0.6 (6) <sup>  </sup>	7.0±0.6 (6) <sup>  </sup>
(Expt. A)		OVX + DHT	2.0 µg	6.0 mo	99.3±2.5 (6) <sup>  ,***</sup>	(‡)
9	7	Control	—	—	—	16.3±1.9 (6)
		OVX	—	—	—	5.4±1.1 (6) <sup>  </sup>
(Expt. B)		OVX + DHT	2.0 µg	6.0 mo	—	46.8±1.0 (6) <sup>  ,***</sup>

Numbers in parentheses are the number of cultures prepared from 3–5 sham-operative and OVX rats. \* cultures in 35-mm petri dishes. ‡ cultures are confluent at 9–12 d. § *P* < 0.01 vs. sham-op control. || *P* < 0.001 vs. sham-op control. ¶ *P* < 0.05 vs. OVX. \*\*\* *P* < 0.001 vs. OVX.

the OVX rat as an osteopenic model. The experiments were designed to explore the possibility that the thinning of diaphyseal bone cortices in rats after ovariectomy was due, at least in part, to a deficit in the proliferation and/or the osteogenic expression of the putative endosteal osteoprogenitor marrow stromal cell population. This validity of the thesis was tested by comparing the growth of marrow stromal cells in vitro to their ability to sustain osteogenesis in different kinds of bone grafts in vivo. Parallel studies were conducted in OVX rats treated in vivo with DHT; these studies were predicated upon reports that DHT and the active vitamin D metabolite, 1,25(OH)<sub>2</sub>D<sub>3</sub>, are structurally similar and have stereospecific receptors on bone cells (17). 1,25-Dihydroxyvitamin-D<sub>3</sub> is therapeutically effective in the OVX rat (1, 3, 10, 18), improving both calcium balance and bone density.

*Marrow cell growth in vitro.* The marrow stromal cells from OVX osteopenic rats proved to be impaired in terms of their

proliferative potential (radiothymidine incorporation) and ability to sustain clonal growth (FCFU) in culture. These differences did not appear to be associated with an altered marrow cytology. Nor were we able to attribute the differences in FCFU development to the failure of cells from OVX rats to attach to the flasks. The percent of the inocula which were found floating in the media throughout the first 3 d of culture were similar in sham-operative and OVX rats. However, because the size distribution of the FCFU which grew in all cultures was nearly identical, the postattachment rate of growth for individual stromal cells was probably identical in both groups. In vivo DHT treatment was salutary in most experiments, such that it stimulated marrow stromal cell proliferation, thereby increasing the FCFU census. DHT treatment also resulted in the normalization of the femoral midshaft thickness (radiologic bone score) and bone density.

While the work of Osdoby and Caplan (19) and Ashton et al. (20) emphasizes that an important fraction of fibroblast-like cells in isolated mesenchymal cell cultures are not osteoprogenitor cells, our findings intimate that the etiology of the osteopenia in OVX rats probably involves a primary failure of the proliferation of the mesenchymal cells resident at the endosteum. The data do not exclude the event of a similar effect on periosteal mesenchymal cells, and the tetracycline labeling studies pursued herein indicated that the changes could affect the levels of bone formation on all surfaces. Similarly, the data do not exclude the possibility that the development of post-OVX osteopenia is also due to a failure of maturation of the putative osteoprogenitor cells in the stromal cell population to the osteoblast class.

*Osteogenic role of marrow stromal cells.* In our in vivo models (Millipore diffusion chambers and composite grafts), the osteogenic potential of marrow stromal cells from OVX rats appeared

Table III. 24-h Radiocalcium (<sup>45</sup>Ca) Incorporation into Calcifying Bone Formed by Marrow Stromal Cells Loaded within Diffusion Chambers, 3 wk Postimplantation

Host rat	CPM/diffusion chamber (SEM)*		<i>P</i> Value
	Control cells	Ovariectomized cells	
Sham-op	634.43±206.90 (24)	1,946.92±850.96 (24)	NS
OVX	400.95±109.82 (24)	451.41±147.66 (23)	NS
<i>P</i> value	NS	<0.001	

Numbers in parentheses are no. of Millipore chambers implanted in 12 sham-operative and 12 OVX host rats.

\* Mann-Whitney *U* Test

Table IV. Osteogenic Response to Intramuscular Implants of Demineralized Allogeneic Bone Matrix and Composite Grafts of Demineralized Allogeneic Bone Combined with Autologous Marrow Cells

Hosts	Time of grafting post-OVX	Implant time	24-h <sup>45</sup> Ca incorporation (SEM) (cpm/mg implanted bone matrix)		P value
			Demineralized bone matrix	Composite graft	
	wk	wk			
Sham-op	8	2	865.1±129.6 (14)	1,711.5±241.7 (13)	<0.01
OVX	8	2	550.7±62.9 (16)	1,583.7±187.8 (16)	<0.001
Sham-op	20	3	516.1±57.4 (10)	1,878.7±282.2 (8)	<0.001
OVX	20	3	430.6±80.9 (9)	1,672.2±267.8 (10)	<0.001
OVX + DHT	20	3	1168.9±180.2 (8)*	3,264.6±610.2 (8)‡	<0.001

( ) = No. of grafts. \* *P* < 0.01 vs. sham-op/<0.005 vs. OVX. ‡ *P* < 0.05 vs. sham-op/<0.05 vs. OVX.

to be equal to that from the sham-operative controls. Those outcomes suggested that OVX rat marrow has a normal complement of stromal osteoprogenitor cells—cells that one would otherwise expect to be competent to proliferate and to modulate to osteoblasts under the inductive influences of a bone morphogenetic protein (BMP)-rich demineralized bone matrix (15, 21). From the standpoint of cell-matrix interactions, the apparently normal performance of these cells in vivo at sites away from bone suggests that OVX rat marrow could be deficient in one or more of the noncollagenous proteins that constitute the biologically active BMP (21, 22). Other mechanisms such as immunologically based cellular interactions or “systemic changes” in endocrine sensitivity of bone cells (e.g., parathyroid hormone) could also be involved, but if present, these are probably of lesser importance (23). Here, cells removed from the constraint of a hypothetically unfavorable local intraosseous microenvironment rapidly recovered and proved superior to cells biopsied from normal rats in their ability to proliferate and mature (*viz* Millipore Diffusion Chambers); the outcome resembled a catch-up growth phenomenon (24–26). Vitamin D metabolites and analogues may operate at these different levels in bone. However, when our findings that DHT treatment enhanced the osteoinductive potential of allogeneic DBM are viewed from the perspective of the recent work by Canalis (27), it would appear that the primacy of DHT action was to stimulate osteoprogenitor cell proliferation. In this particular experimental model, the secondary consequences of DHT treatment would be expressed as enhanced osteoblast maturation and new bone formation (28). In other situations such as in osteoblast cultures, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment appears to enhance those activities or processes that denote cell maturation/differentiation (alkaline phosphatase and collagen synthesis), and has no effect on cell proliferation (28).

We conclude that the bone mass deficit which develops in rats after ovariectomy can be corrected by oral DHT administration, and that the mechanism of hormone action involves a primary stimulus to mesenchymal osteoprogenitor cell proliferation (endosteal marrow stromal cells and periosteum).

### Acknowledgment

This work was partially supported by U.S. Public Health Grant (National Institutes of Health) AM16391.

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