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Number and Proliferative Capacity of Osteogenic Stem Cells Are Maintained During Aging and in Patients with Osteoporosis*

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ABSTRACT

Decreased bone formation is an important pathophysiological mechanism responsible for bone loss associated with aging and osteoporosis. Osteoblasts (OBs), originate from mesenchymal stem cells (MSCs) that are present in the bone marrow and form colonies (termed colony-forming units-fibroblastic [CFU-Fs]) when cultured in vitro. To examine the effect of aging and osteoporosis on the MSC population, we quantified the number of MSCs and their proliferative capacity in vitro. Fifty-one individuals were studied: 38 normal volunteers (23 young individuals [age, 22–44 years] and 15 old individuals [age, 66–74 years]) and 13 patients with osteoporosis (age, 58–83 years). Bone marrow was aspirated from iliac crest; mononuclear cells were enriched in MSCs by magnetic activated cell sorting (MACS) using STRO-1 antibody. Total CFU-F number, size distribution, cell density per CFU-F, number of alkaline phosphatase positive (ALP^+) CFU-Fs, and the total ALP^+ cells were determined. In addition, matrix mineralization as estimated by alizarin red S (AR-S) staining was quantified. No significant difference in colony-forming efficiency between young individuals (mean ± SEM; 87 ± 12 CFU-Fs/culture), old individuals (99 ± 19 CFU-Fs/culture), and patients with osteoporosis (129 ± 13 CFU-Fs/culture; $p = 0.20$) was found. Average CFU-F size and cell density per colony were similar in the three groups. Neither the percentage of ALP^+ CFU-Fs (66 ± 6%, 65 ± 7%, and 72 ± 4% for young individuals, old individuals, and patients with osteoporosis, respectively) nor the percentage of ALP^+ cells per culture (34 ± 5%, 40 ± 6%, and 41 ± 4%) differed between groups. Finally, mineralized matrix formation was similar in young individuals, old individuals, and patients with osteoporosis. Our study shows that the number and proliferative capacity of osteoprogenitor cells are maintained during aging and in patients with osteoporosis and that other mechanisms must be responsible for the defective osteoblast (OB) functions observed in these conditions. (J Bone Miner Res 2001;16:1120–1129)

Key words: human bone marrow, mesenchymal stem cells, colony-forming unit-fibroblastic, aging, STRO-1

INTRODUCTION

DECREASED BONE formation is a major cause of bone loss during aging and in patients with osteoporosis.[1–4] The rate-limiting step during the bone formation phase of the skeletal remodeling cycles seems to involve osteoblast (OB) recruitment and differentiation.[5] OBs originate from osteoprogenitor cells in bone marrow stroma termed mesenchymal stem cells (MSCs) or bone marrow stromal cells.[6–8] Human MSCs can give rise to several cell types including fibroblasts, adipocytes, chondrocytes, and OBs.[9–12] An age-related atrophy of MSCs has been suggested as a cause of decreased number of osteoprogenitor cells and decreased bone formation capacity because of a lack of mature matrix-forming OBs.

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During in vitro culture, MSCs form distinct colonies of cells with fibroblast morphology, hence the term colony-forming units-fibroblastic (CFU-Fs). Each CFU-F represents the descendants of a single MSC and thus the number of CFU-Fs is a direct estimate of the number of MSCs present at culture start.[13,14] Previous studies that quantified changes in the number of MSCs with age in animal models showed variable results. Egrise et al.[15] found that the total number of CFU-Fs, but not the proportion of alkaline phosphatase positive (ALP^+) CFU-Fs, was significantly decreased in bone marrow cultures obtained from old rats. Quarto et al.[16] found a decrease in the total number of CFU-Fs together with a decrease in ALP^+ CFU-Fs in aged rats. In mice, some studies reported an age-related decrease in CFU-F number,[17,18] while others found either an increase or no effect of aging on CFU-F population.[19,20] Similarly, no consistent changes in the number of CFU-Fs with age were observed in human studies.[21–25]

The aim of this study was to examine the influence of age and osteoporosis on CFU-F population in the bone marrow obtained from healthy individuals of different ages and from patients with osteoporosis. We established cell cultures that were enriched in MSCs using magnetic activated cell sorting (MACS) using the STRO-1 antibody. STRO-1 is a monoclonal antibody that was shown to recognize all the clonogenic MSCs in human bone marrow.[26,27] The antigenic epitope of STRO-1 antibody has not been identified yet; however, STRO-1 antibody does not bind to T and B cells, myeloid cells, megakaryocytes, macrophages, and their precursors.[26,27] Although nucleated erythroid cells show some reactivity with STRO-1 antibody,[27] most of these cells are removed on medium change.

MATERIALS AND METHODS

Heparin, Hank's buffer, bovine serum albumin (BSA), alizarin red S (AR-S), and cetylpyridinium chloride were purchased from Sigma-Aldrich (Copenhagen, Denmark); phosphate-buffered saline (PBS), PBS^{2+} (PBS containing Ca^{2+} and Mg^{2+}), penicillin, streptomycin, and modified essential medium (MEM) were purchased from GIBCO/BRL (Copenhagen, Denmark); fetal calf serum (FCS) was from BioWhittaker (Brussels, Belgium); Lymphoprep was from Nycomed Pharma (Oslo, Norway); tissue culture plasticware was from NUNC (Copenhagen, Denmark); L-ascorbic acid 2-phosphate was from Wako Chemicals (Neuss, Germany); variamine Blue B salt was from Fluka Chemika (Buchs, Switzerland); 1-naphthyl-phosphate sodium salt was from Merck (Aarhus, Denmark); antibodies X 0934, X 0903, and E 0465 were from DAKO (Glostrup, Denmark); and MiniMACS cell isolation unit, streptavidin microbeads, and MACS MS^+-separation column were from Miltenyi Biotech (Bergisch Gladbach, Germany). Hybridoma cells secreting STRO-1 antibody were purchased from Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

Study population

Fifty-one individuals participated in the study. Twenty-three healthy young individuals aged 22–44 years (10 women, aged 22–44 years [mean, 25 years], and 13 men, aged 22–43 years [mean, 28 years]) and 15 healthy postmenopausal elderly women (aged 66–74 years [mean 69 years]) were recruited from the local community. Twelve female patients with osteoporosis, aged 58–83 years (mean 72 years), and one male patient with osteoporosis, aged 70 years, were included in the study and were studied before the initiation of treatment. Osteoporosis was defined as low bone mass determined by bone mineral density (BMD) measurements (T score of femoral neck or spine of < -2.5) and/or the presence of one or more low-energy fractures in the spine. The participants had no evidence of any concurrent illness or took any medication that could affect bone metabolism. The study was conducted according to the ethical guidelines of the Helsinki II Declaration and was approved by the Regional Ethical Committee.

Bone marrow cultures

Bone marrow was obtained by aspiration from the iliac crest after infiltration of the area with local anesthetic (lidocaine, 10 mg/ml). Ten milliliters of bone marrow were aspirated in a 20-ml syringe and mixed with 10 ml of heparin (100 U/ml). Low-density mononuclear cells were isolated by centrifugation over a Lymphoprep density gradient (density = 1.077 ± 0.001 g/cm^3) at 1500*g* for 25 minutes at room temperature (RT). Cells were washed once in MEM containing 1% penicillin/streptomycin (P/S) and 10% (vol/vol) heat-inactivated FCS. The FCS batch was tested for its ability to support stromal cell growth. Viable cells were counted after methylene blue staining in a Bürker-Türk counting chamber.

Preparation of the STRO-1 monoclonal antibody

The STRO-1 secreting hybridoma cells were cultured in MEM containing 10% FCS. The supernatant containing STRO-1 antibodies was harvested from cultured hybridoma cells (2×10^6 cells) in 20 ml of medium (MEM containing 10% FCS) grown for 7 days. Aliquots from the supernatants were frozen at −80°C and used undiluted in subsequent experiments. The presence of STRO-1 antibody in the supernatant was determined by testing its ability to identify the STRO-1 antigen present on osteosarcoma cells MG63[28] using fluorescence-activated cell sorting (FACS). MG63 cells were incubated with the supernatants and STRO-1 bound to MG63 cells was detected by fluorescein isothiocyanate (FITC) conjugated secondary antibody (data not shown). Also, a supernatant known to contain the STRO-1 antibody (kindly provided by Dr. J.N. Beresford, Bath, UK) was included as a positive control in these experiments. An isotype immunoglobulin M (IgM) antibody (X 0934) was used as negative control.

MACS

$STRO\text{-}1^+$ cells were isolated from the low-density mononuclear cell fraction using the MiniMACS cell isolation unit following the manufacturer's recommendations with modifications. Briefly, mononuclear cells were washed twice in

Hank's buffer and incubated with rabbit immunoglobulin fraction (normal; X 0903), 10 μl/10^7 cells for 10 minutes at RT. Supernatants containing the STRO-1 antibody (0.5–1 ml/10^7 mononuclear cells) were added and the cell suspension was left at 4°C for 1 h. Cells were washed in MACS column buffer (1× PBS, 2 mM EDTA, 0.5% BSA, and degassed for 1 h). Afterward, the cells were incubated on ice for 1 h with biotinylated rabbit anti-mouse IgM (E 0465) diluted 1:125 in Hank's. After washing in MACS column buffer, streptavidin-conjugated microbeads were added (10 μl/10^7 cells) and incubated for 15 minutes at 4°C. Cells were washed in MACS column buffer and loaded onto a MACS MS^+-separation column positioned in a high-magnetic field. The STRO-1^- cells were collected as column eluate while STRO-1^+ cells were retained. STRO-1^+ cells were eluted by removing the column from the magnetic field and flushing it with 1 ml of MACS column buffer. Both STRO-1^+ and STRO-1^- cell fractions were centrifuged for 10 minutes at 700*g* and resuspended in medium containing 10% FCS in MEM supplemented with 1% P/S and the cells were counted. The STRO-1^+ cells were seeded into chamber slides at 1×10^5 cells/cm^2 (1×10^6 cells per chamber slide). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C and the cells were fed by completely replacing the medium once a week. The STRO-1^- cells were always seeded in a parallel chamber slide to monitor the efficiency of the isolation procedure.

Cytochemical staining

ALP staining was performed by a standard procedure after 13 days of culture. Cells were washed in PBS, fixed in methanol/formalin (9:1) for 15 s, and incubated for 60 minutes in a buffer containing variamine Blue B salt and 1-naphthyl-phosphate sodium salt mixed in concentrated propanediol. Mayers-Hematoxylin was used as a counterstain. The number of CFU-Fs and cells staining positive for ALP was counted using inverted light microscopy (Olympus BH2; Olympus Denmark, Albertslund, Denmark).

Determination of CFU-F number, CFU-F size, and cell density per CFU-F

When mononuclear bone marrow cells are seeded in vitro, they adhere to the plastic surface and form CFU-Fs.[9,14] A CFU-F was defined as a group of at least 16 cells within a circular area.[6] We have performed extensive preliminary studies to determine the optimal seeding density and the time in culture needed to obtain clear distinct colonies in a reproducible fashion. A range of plating densities were examined: 3×10^4, 6×10^4, 1×10^5, and 3×10^5 cells/cm^2. Parallel experiments were terminated after 6, 9, 13, 16, and 21 days. Also, the number of chamber slides (i.e., independent cell cultures) needed per participant to obtain a stable CFU-F number was determined using calculations from six chamber slides seeded with 1×10^5 cells/cm^2. CFU-F number and CFU-F sizes were determined using a light microscope (Olympus BH2) together with a Scriptel digitizer table (Berendtsen Components, Soeborg, Denmark) coupled to SIGMA SCAN software program version 3.90 (Dyrberg Trading, Karlslunde, Denmark). Each CFU-F was traced and its size was determined. To determine the relationship between CFU-F size and cell number, CFU-Fs were divided into three different categories depending on cell number per CFU-F: low density (<5 cells/mm^2), medium density (5–25 cells/mm^2), and high density (>25 cells/mm^2). All counts were performed by a single observer without knowledge of the donor status. Intraobserver and interobserver variation for CFU-F number determination were 14% and 18%, for CFU-F size were 11% and 13%, and for cell density per CFU-F were 13% and 14%, respectively.

Quantification of ALP$^+$ stained CFU-Fs and cells

The extent of ALP staining was determined by two different methods. First, the percentage of ALP^+ CFU-Fs per total CFU-F number was determined. A CFU-F was considered ALP^+ if it contained at least one cell positively stained for ALP.[24,25] Second, percentage of ALP^+ cells per culture was determined by counting all ALP^+ cells in several randomly assigned microscopic fields. Preliminary experiments were performed to determine both the number of slides needed to be counted for each single donor to obtain a stable ALP^+ CFU-F number and the total number of cells needed to be counted to get a stable percentage of the ALP^+ cells per culture. Intraobserver and interobserver variation in percentage of ALP^+ CFU-F counting were 4% and 14% and for percentage of ALP^+ cells were 11% and 12%, respectively.

AR-S assay for in vitro mineralization

STRO-1^+ cells were cultured for 9–10 weeks with or without L-ascorbic acid 2-phosphate (85 μg/ml). The cells were washed in PBS, fixed in 70% ethanol at −20°C for 60 minutes, and rinsed in distilled water. AR-S (40 mM, pH 4.2) was added for 10 minutes at RT. The cells were rinsed in distilled water and PBS to reduce nonspecific staining. The red dye was eluted by adding 10% cetylpyridinium chloride in sodium phosphate (10 mM, pH 7.0) for 15 minutes[29] and measured in a spectrophotometer at absorbance of 570 nm. The amount of deposited Ca^{2+} was determined by using an AR-S standard curve measured at 570 nm with AR-S concentration range of 0–400 μM (1 mol of AR-S binds 1 mol of Ca^{2+}).

BMD measurements

BMD was measured in the lumbar spine (L2–L4) and the femoral neck by a Hologic DXA scanner (Hologic, Inc., Waltham, MA, USA) in all old donors and patients with osteoporosis. Precision for BMD was 1.5% for the lumbar spine and 2.1% for the femoral neck. Long-term stability was high, with changes of less than 0.2% per year.[30]

Statistics

Intraobserver and interobserver CVs were determined from the formula $CV = SD/X_{mean}$, where $SD = [\Sigma(X_i -$

TABLE 1. BASELINE CHARACTERISTICS OF STUDY GROUP

	Old	*OP*	p *Value*
Number	15	13	
Age (years)	66–74	58–83	NS
Height (cm)	161.9 ± 1.3	161.4 ± 1.9	NS
Weight (kg)	72.7 ± 3.4	70.5 ± 3.2	NS
BMI (kg/m^2)	27.6 ± 1.2	27.2 ± 1.4	NS
BMD spine (g/cm^2)	0.924 ± 0.016	0.820 ± 0.035	<0.01
BMD femur (g/cm^2)	0.733 ± 0.016	0.605 ± 0.020	<0.01

OP, patients with osteoporosis.

$Y_i)^2/(2N - 1)]^{1/2}$ (X_i and Y_i are pairs of measurement for i = 1 to N; n = number of repeated counts) and $X_{mean} = (\Sigma X_i + \Sigma Y_i)/2N$. All values are expressed as mean ± SEM. Comparing means between groups was performed using one-way analysis of variance (ANOVA) and unpaired *t*-test (two-tailed). Relationships between different variables were determined by simple linear regression analysis. A value of $p < 0.05$ was considered significant.

The study power was determined using the following formula: $n = (C_{2\alpha} + C_\beta)^2 \times S^2/M^2$, where n = number of donors required, $C_{2\alpha} = 1.96$ ($\alpha = 0.05$, type I error, *t*-distribution), $C_\beta = 0.841$ ($\beta = 0.20$, type II error, *t*-distribution), $S^2 = 2 \times SD^2$ (unpaired *t*-test), and M = minimal biological difference of significance.

RESULTS

Study subjects

Baseline characteristics of the study group are shown in Table 1 and Table 2. There were no significant differences between elderly healthy donors and patients with osteoporosis in their age, height, weight, and body mass index (BMI). Spine and femur BMD measurements were significantly lowered in patients with osteoporosis compared with age-matched controls.

Cell morphology

The initially adherent stromal cells obtained from STRO-1$^+$ cells appeared as widely scattered elongated and fusiform cells. They divided and formed CFU-Fs with a morphologically homogenous population of cells (Fig. 1A). The fraction of STRO-1$^-$ cells was composed of mainly hematopoietic mononuclear cells (Fig. 1B). CFU-F growth was observed consistently only in STRO-1$^+$ cell cultures.

Cultures of STRO-1$^+$ cells give better estimate of the CFU-F number

We enriched the mononuclear bone marrow cells in MSCs using MACS employing the STRO-1 antibody. A comparison between the number of CFU-Fs obtained from cultures of STRO-1$^+$ cells and cultures established from total bone marrow mononuclear cells seeded at a wide range of cell densities was performed (Figs. 2A and 2B). The one MSC to one CFU-F relationship between the number of initially adherent cells counted after 5 days in culture and the number of CFU-Fs obtained was better in STRO-1$^+$ cell cultures than in total bone marrow mononuclear cell cultures ($\alpha = 0.81$ for STRO-1$^+$ cells vs. $\alpha = 0.37$ for mononuclear cells). The slope for mononuclear cells was significantly different from 1 ($p < 0.05$) whereas the slope for STRO-1$^+$ cells was not. This finding was consistent in six cultures from six different donors tested indicating that some of the initially plastic adherent cells from the mononuclear cell population are not true CFU-F–forming cells.

Changes in the number of STRO-1$^+$ cells with age

Immunopurification using MACS resulted in a variable loss of mononuclear marrow cells but there was no significant difference in the degree of cellular loss among groups (34 ± 3% young donors, 37 ± 3% old donors, and 30 ± 3% patients with osteoporosis). The percentage of STRO-1$^+$ cells per total output cells (STRO-1$^+$ + STRO-1$^-$ cells) was calculated for each donor. There were no statistically significant age-related changes in the percentage of STRO-1$^+$ cells (27 ± 7% young and 25 ± 6% old). In addition, STRO-1$^+$ cells in patients with osteoporosis (24 ± 7%) were similar to those of age-matched controls.

Colony-forming efficiency

In a large number of preliminary experiments, we found that the optimal protocol for obtaining distinct CFU-Fs in a reproducible fashion was to culture the STRO-1$^+$ cells at a cell density of 1×10^5 cells/cm^2 for 13 days.[31] Also, to obtain a stable CFU-F number, four chamber slides were counted for each donor.[31]

The mean colony-forming efficiency for the whole donor group (51 individuals; age, 51 ± 3 years) was 104 ± 8/10^6 STRO-1$^+$ cells giving 1 CFU-F per 10,000 STRO-1$^+$ cells, which is around 10 times enrichment in MSCs compared with previous studies performed on total marrow mononuclear cells.[11,25]

Effect of donor age on CFU-F number and CFU-F size distribution

The number of CFU-Fs obtained in the young donor group was 87 ± 12 ($n = 23$), which was not statistically different from CFU-Fs obtained in cultures of old donors (99 ± 19; $n = 15$), and in patients with osteoporosis (129 ± 11, $n = 13$) (Fig. 3). Considering the whole group of donors (young, old, and patients with osteoporosis), we found no correlation between age of the donor and the number of CFU-Fs obtained ($r = 0.18$; $p = 0.20$).

CFU-F sizes were determined as an estimation of the proliferative potential of the MSCs. Young donors, old donors, and patients with osteoporosis showed mean CFU-F sizes of 7.2 ± 0.5 mm^2, 6.1 ± 0.5 mm^2, and 7.8 ± 0.4 mm^2, respectively, which was not significantly different. We further determined CFU-F size distribution by classifying the CFU-Fs into three groups: 1 mm^2 < x < 5 mm^2, 5 mm^2 <

TABLE 2. EFFECT OF SEX, AGE, AND DISEASE ON MEASURED PARAMETERS

Effect of sex	*Women (n = 10)*	*Men (n = 13)*	p *Value*
Number	10	13	
Age (years)	22–44	22–43	
STRO-1$^+$ cells (%)	27 ± 7	27 ± 8	NS
CFU-F number/culture	85 ± 17	89 ± 18	NS
Mean CFU-F size (mm^2)	8.1 ± 0.8	6.5 ± 0.8	NS
CFU-F size distribution			
1 mm^2 < x < 5 mm^2 (%)	31 ± 7	44 ± 7	NS
5 mm^2 < x < 10 mm^2 (%)	39 ± 4	37 ± 4	NS
>10 mm^2 (%)	30 ± 6	19 ± 4	NS
ALP positive CFU-Fs (%)	71 ± 7	62 ± 9	NS
ALP positive cells (%)	32 ± 6	35 ± 8	NS
Effect of age	*Young*	*Old*	p *Value*
Number	23	15	
Age (years)	22–44	66–74	
STRO-1$^+$ cells (%)	27 ± 7	25 ± 6	NS
Mean CFU-F size (mm^2)	7.2 ± 0.5	6.1 ± 0.5	NS
Effect of disease	*Old*	*OP*	p *Value*
Number	15	13	
Age (years)	66–74	58–83	
STRO-1$^+$ cells (%)	25 ± 6	24 ± 7	NS
Mean CFU-F size (mm^2)	6.1 ± 0.5	7.8 ± 0.4	<0.05

Low-density mononuclear cells were enriched in MSCs using STRO-1 antibody. STRO-1$^+$ cells were seeded (1×10^5 cells/cm^2) into chamber slides.

OP, patients with osteoporosis.

$x < 10$ mm^2, >10 mm^2 (CFU-Fs < 1 mm^2 contained less than 16 cells and were not included). We found, no age-related differences in CFU-F size distribution between young and old donors (Fig. 4). However, there was a shift toward an increased number of larger CFU-Fs (>10 mm^2) in cell cultures from patients with osteoporosis (Fig. 4).

To validate that CFU-F sizes reflect true cell density and proliferation potential per CFU-Fs, CFU-Fs were classified as low, medium, and high density CFU-Fs depending on cell number (low density [<5 cells/mm^2], medium density [5–25 cells/mm^2], and high density [>25 cells/mm^2]). We found a positive correlation between CFU-F size determination and cell density per CFU-F ($r = 0.36$; $p < 0.05$; $n =$ 51 individuals) confirming that CFU-F sizes reflect true cell growth.

Effect of donor age on the expression of ALP by CFU-Fs

STRO-1$^+$ cultures were stained for ALP as a marker for osteogenic commitment. Cultures contained variable number of ALP$^+$ and ALP$^-$ CFU-Fs. In our preliminary experiments, we found that it is necessary to determine the number of ALP$^+$ CFU-Fs in at least four chamber slides per donor and to count at least 200 cells per chamber slide to obtain a stable and reproducible number of ALP$^+$ CFU-Fs and ALP$^+$ cells, respectively.[31] The values of ALP$^+$ CFU-Fs were 66 ± 6%, 65 ± 7%, and 72 ± 4%, and values for ALP$^+$ cells were 34 ± 5%, 40 ± 6%, and 41 ± 4% for young, old, and patients with osteoporosis, respectively (Fig. 5). We found no statistically significant differences among the groups in any of these parameters. In addition, we found no statistically significant differences among these groups when the absolute number of ALP$^+$ CFU-Fs and ALP$^+$ cells were compared (data not shown).

Difference between young female and male donors

We found no difference between young female and male donors in any of the parameters measured (Table 2). Therefore, both groups were considered as one young group when comparing with old donors and patients with osteoporosis.

Differences between young and old donors irrespective of disease

To study the effect of age per se irrespective of disease state, we combined the results of patients with osteoporosis and healthy old donors and compared them to young donors (Table 3). We found no statistically significant difference between the two groups in any of the parameters measured.

In vitro matrix mineralization

The ability of the MSCs to generate fully differentiated OBs with the capacity to form a mineralized matrix in vitro

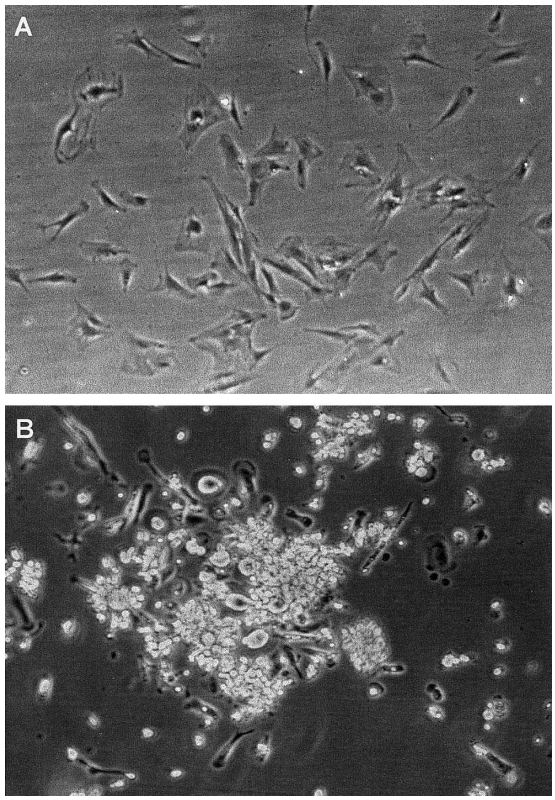

FIG. 1. Morphology of cultured bone marrow cells. Bone marrow mononuclear cells were immunopurified using the monoclonal antibody STRO-1. (A) STRO-1$^+$ cells. (B) STRO-1$^-$ cells. Original magnification ×100.

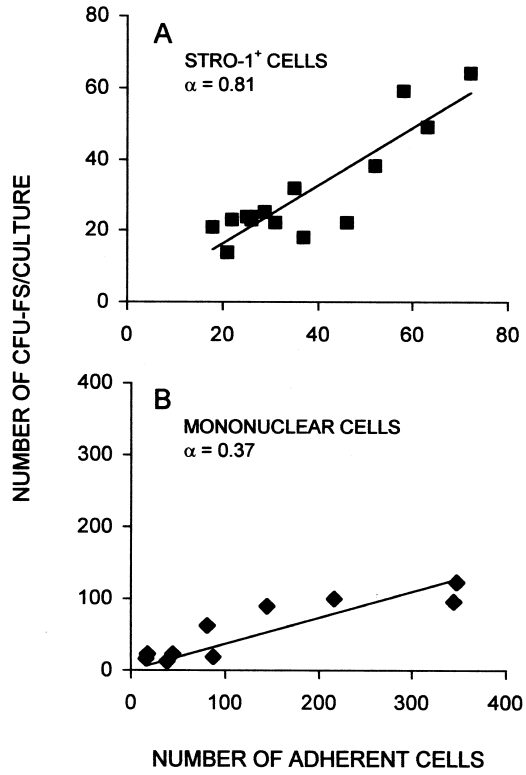

FIG. 2. Number of CFU-Fs counted after 13 days of in vitro growth plotted versus number of adherent cells with fibroblastic morphology counted after 5 days of in vitro growth. Bone marrow was aspirated from 6 young donors: 3 women and 3 men. Mononuclear cells from all 6 donors were isolated. Four of the donors (2 men and 2 women) were subjected to immunopurification using STRO-1 antibody. (A) STRO-1$^+$ cells. (B) Total bone marrow mononuclear cells.

was assayed using AR-S staining. Because of the limited number of cells obtained from each donor, these experiments were performed only in a subgroup of the participants. Because we were not able to determine the number of cells in these highly confluent cultures, mineralization capacity was determined by comparing cultures treated with L-ascorbic acid 2-phosphate with control cultures. These increments were then compared between young donors (n = 7), old donors (n = 6), and patients with osteoporosis (n = 3). We found no difference in L-ascorbic acid 2-phosphate–induced mineralization among young donors (1.5 ± 0.2-fold increase compared with control [no treatment]), old donors (1.9 ± 0.3), and patients with osteoporosis (1.2 ± 0.1). The absolute values for the incorporation of calcium into matrix were 205 ± 102 mmol of Ca^{2+}, 97 ± 33 mmol of Ca^{2+}, and 195 ± 98 mmol of Ca^{2+} for young donors, old donors, and patients with osteoporosis, respectively.

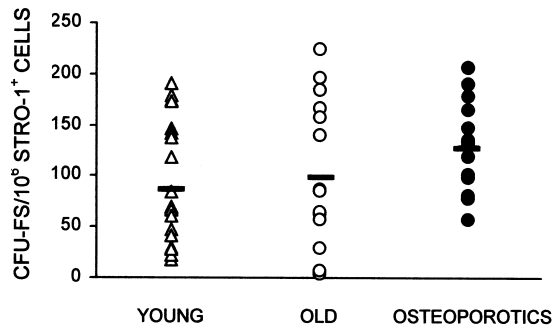

FIG. 3. Effect of donor age and osteoporosis on the number of CFU-Fs. Bone marrow was aspirated from iliac crest of 23 young donors (10 women and 13 men), 15 old donors, and 13 patients with osteoporosis. STRO-1$^+$ cells were seeded in four chamber slides (1×10^5 cells/cm^2). After 13 days, cultures were fixed, stained, and the number of CFU-Fs was determined.

Correlation with bone mass measurements

We found no significant correlation between BMD measurements at the spine or femur neck and the total number of CFU-Fs, CFU-F sizes, or ALP$^+$ CFU-Fs in old donors or in patients with osteoporosis (data not shown).

DISCUSSION

Several histomorphometric studies performed on iliac bone biopsy specimens have shown an age-related decrease

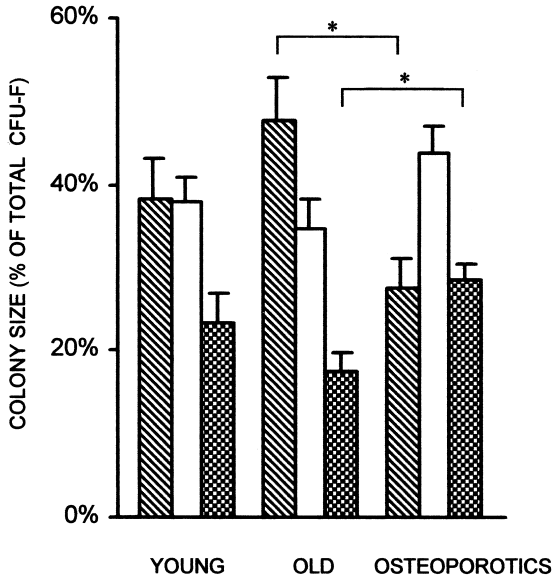

FIG. 4. Effect of donor age and osteoporosis on the size distribution of CFU-Fs. Bone marrow was aspirated from iliac crest of 23 young donors, 15 old donors, and 13 patients with osteoporosis. STRO-1^+ cells were seeded in four slide flasks (1×10^5 cells/cm^2). Colony sizes were divided into three groups: ▧, 1 mm^2 $< x <$ 5 mm^2; □, 5 mm^2 $< x <$ 10 mm^2, ▪, $>$ 10 mm^2. The percentage of CFU-Fs in each size category was determined for each donor. Results presented as mean ± SEM ($*p < 0.05$).

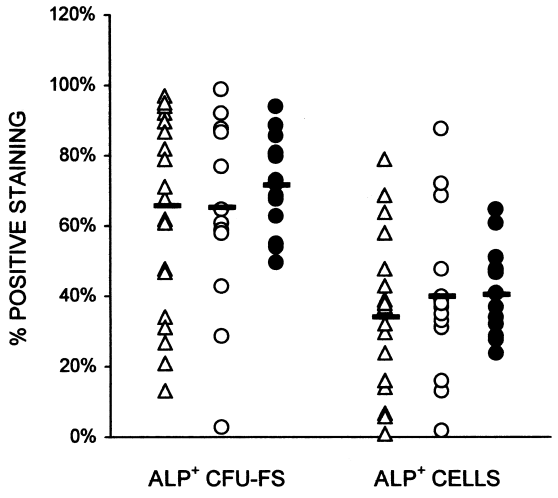

FIG. 5. Effect of donor age and osteoporosis on the cellular expression of ALP. Bone marrow was aspirated from iliac crest of 23 young donors, (△) 15 old donors (○), and 13 patients with osteoporosis (●).

in the mean wall thickness, which is an estimate of in vivo osteoblastic bone-forming capacity.[1,2] These changes were even more pronounced in some studies in patients with osteoporosis.[3,4] Theoretically, decreased bone formation can result from either impaired OB recruitment and differentiation from stem cells in the bone marrow or decreased activities of mature OBs. Our data suggest that during aging and in patients with osteoporosis, the number of osteoprogenitor cells as well as their proliferative potential and osteogenic commitment are maintained.

TABLE 3. EFFECT OF AGE ON MEASURED PARAMETERS

	Young	*Old + OP*	p *Value*
Number (n)	23	28	
Age (years)	22–44	58–83	
STRO-1^+ cells (%)	27 ± 7	24 ± 1	NS
CFU-F number/culture	87 ± 12	113 ± 12	NS
CFU-F size (mm^2)	7.2 ± 0.5	6.9 ± 0.3	NS
CFU-F size distribution			
1 mm^2 $< x <$ 5 mm^2 (%)	38 ± 5	38 ± 4	NS
5 mm^2 $< x <$ 10 mm^2 (%)	38 ± 3	39 ± 3	NS
$>$10 mm^2 (%)	24 ± 4	23 ± 2	NS
ALP positive CFU-Fs (%)	66 ± 6	68 ± 4	NS
ALP positive cells (%)	34 ± 5	40 ± 3	NS

Low-density mononuclear cells were enriched in MSCs using STRO-1 antibody. STRO-1^+ cells were seeded (1×10^5 cells/cm^2) into chamber slides. After 13 days cells were stained for ALP.

OP, patients with osteoporosis.

To examine for the presence of stem cell and osteoprogenitor cell atrophy during aging, several investigators have used assays for quantifying their number in bone marrow based solely on their ability to adhere to plastic surfaces. However, these cultures are heterogeneous and contaminated with hematopoietic nonmesenchymal cells.[11] It is plausible that contamination of the MSC cultures with nonmesenchymal cells may influence their growth kinetics and may increase growth variability among donors.[32,33] It also is possible that some of the adherent, nonmesenchymal cells may be classified falsely as CFU-Fs or spuriously increase CFU-F size, which may affect the results obtained. To overcome these limitations, we used STRO-1 antibody for positively selecting MSCs. Our findings show a better correlation between the number of the initial STRO-1^+ cells and the number of CFU-Fs obtained than those found in the case of unfractionated mononuclear marrow cells, which supports the usefulness of STRO-1 antibody selection when assaying the number of CFU-Fs.

In addition to the total number of CFU-Fs, the colony size distribution is an important parameter denoting the proliferative potential among the heterogeneous population of CFU-Fs. We have used methods reported by previous investigators in the biogerontology field who have used colony size distribution to detect age-related changes in the proliferative capacity of cells undergoing in vitro senescence.[34] Although previous investigators measured the sizes of a few colonies per culture[24,25] or estimated the sizes of the colonies from measurements of the diameters,[35,36] we have performed systematic measurements of sizes of all colonies obtained from each donor. We did not measure the total number of cells per colony in all cultures because it was not practically possible. Instead, we estimated the cell density per colony and found a good corre-

lation between the colony size and the estimated cell density per colony, suggesting that CFU-F sizes reflect true cell growth.

Expression of the ALP enzyme has been used generally in stromal cell cultures as a marker of osteogenic commitment[37] and recently it was suggested that it is a marker of the osteoadipoprogenitor cell population.[38] We found that between 60% and 70% of CFU-Fs in STRO-1^+ cell cultures were ALP^+. Based on extensive study of the cellular phenotype characteristics of stromal cell cultures, Stewart et al.[28] suggested that STRO-1^+/ALP^- represents a less differentiated osteoblastic phenotype and that with progression in osteogenic differentiation, the cells become STRO-1^+/ALP^+ and then STRO-1^-/ALP^+, indicating that our cultures represent earlier stages of OB differentiation. We have defined an ALP^+ colony by the presence of a least one ALP^+ cell in accordance with the definition used by previous investigators.[24,25] To overcome the potential lack of specificity associated with this low-threshold definition and because of variations in staining intensity in different colonies, we determined the total number of ALP^+ cells per culture.

Previous human studies that examined the relationship between aging and the CFU-F population showed variable results. The discrepancies can be attributed to differences in study subjects, sampling site of bone marrow, and methods of MSC isolation. Several studies have used bone marrow obtained during surgical procedures for treatment of osteoarthritis or other operations,[21,23–25] or as postmortem samples.[22] These donors may not be a good representative sample of the normal aging population. Also, bone marrow was obtained from different sites (spine[22] and iliac crest[21,23]), which may influence the composition of bone marrow and the occurrence of MSCs. Furthermore, the techniques used for establishing the MSC cultures were different. Some authors used bone marrow aspirates[21,23] and others used "flushing" techniques to remove bone marrow cells from the attached bone fragments.[22,24,25] Also, several studies defined the osteoprogenitor cells as cells that form adherent colonies of CFU-Fs,[21–25] but a recent study identified osteoprogenitor cells among the nonadherent cell population based on their ability to express osteocalcin, osteonectin, and bone-ALP.[39] Although it is not clear whether all these variables overestimate or underestimate the age-related changes in the MSC population in bone marrow, it shows the difficulties involved in comparing different studies.

We did not find significant age-related changes in the number of CFU-Fs or in ALP^+ CFU-Fs. In previous studies that reported the presence of an age-related decline in the number of CFU-Fs,[21–23] the major decline took place up to the age of 30 years with little or no change thereafter. This is in accordance with our findings because the age of our normal population sample was between 22 and 83 years. The reported decline in the number of CFU-Fs thus may indicate a change of the skeletal dynamics from a "modeling" mode characteristic of skeletal growth to a "remodeling" mode characteristic of maintenance of the adult skeleton. This also may explain the large age-related decrease in the number of CFU-Fs observed in some rodent studies[15–18] as they continue to grow throughout most of their lifespan.

The function of the OB is to form a mineralized matrix. Studying the effect of donor age on matrix formation ability may reveal abnormalities that cannot be detected using surrogate markers of OB functions, for example, ALP production. Unfortunately, there are only few available quantitative assays for measuring bone matrix formation by the OBs in vitro and in vivo. The available assays require a large number of cells and a long culture period, which makes it difficult to process a large number of samples necessary for clinical studies. Under our culture conditions, in vitro mineralization was observed in the presence of ascorbic acid[9,11] and was not dependent on dexamethasone as was reported in other in vitro OB cell culture models.[23] We measured bone matrix formation ability using a semiquantitative staining method with AR-S. We found no major differences between cultures of young donors, old donors, and patients with osteoporosis in matrix-forming ability. These findings are consistent with the presence of normally functioning OBs in old donors and in patients with osteoporosis. Previous studies using mature OBs obtained from trabecular bone explants from donors at different ages did not reveal a decrease in matrix formation in vitro as visualized by von Kossa staining.[40] In contrast, in studies using rodent OBs an age-related decrease in rate of bone formation was established.[16,41,42] Because we measured in vitro mineralization capacity in a small number of donors, our results should be considered preliminary and further investigations are needed to explore the effect of aging on this important aspect of the biological functions of OBs.

We found that cells obtained from patients with osteoporosis exhibited growth and functional characteristics similar to age-matched controls. Interestingly, the percentage of large colonies was higher in cultures established from patients with osteoporosis, suggesting a good proliferative potential of the CFU-Fs. This may be related to the increased rate of bone remodeling and turnover observed in osteoporotic patients that requires continuous recruitment of osteoprogenitor cells.[43] Similar to our findings, Oreffo et al.[25] reported maintenance of the number of CFU-Fs in the osteoporotic population. In contrast, these authors reported a decrease in number of ALP^+ CFU-Fs in patients with osteoporosis but the control subjects examined in this study were not age-matched and suffered from severe osteoarthritis and therefore may not be representative of the normal aging population. Only few experimental studies have previously examined the biological characteristics of osteoporotic OBs. We have previously reported that marrow stromal OBs obtained from patients with osteoporosis exhibited similar proliferative and differentiation responses to stimulation by growth hormone and calcitriol compared with cells from age-matched controls.[44] Also, Marie et al. showed that mature OBs obtained from trabecular bone from patients with osteoporosis exhibited reduced growth potential in vitro only in the subgroup with histomorphometric signs of decreased bone formation.[45] Thus, OBs obtained from patients with osteoporosis exhibit normal biological characteristics except in a subgroup with a severe form of the disease.

Based on previous studies in humans[22,23] and in rodents[15–18] we assumed a minimum relevant difference of 50% in the maximal decrease in the number of CFU-Fs between young donors and old donors. With the number of donors used (27 young individuals and 15 old individuals), the risk of type 2 error (the chance of not detecting a real difference) can be estimated as 20% in this study. In addition, because BMD measurements were performed in both elderly patients and in patients with osteoporosis, it is not likely that the lack of differences in CFU-F number and biology between normal elderly donors and patients with osteoporosis was caused by inclusion of patients with osteoporosis in the control group.

Our studies do not support the hypothesis that the observed decrease in bone formation is the result of decreased number of osteoprogenitor cells in bone marrow. It is possible that during bone remodeling, the number of recruited osteoprogenitor cells is dependent on the composition of bone microenvironment including the availability of growth factors and hormones necessary for OB commitment and not on the actual number of osteogenic stem cells. Some of these mechanisms have been addressed in experimental studies. Pfeilschifter et al.[46] showed that mature OBs obtained from donors with different ages exhibited decreased responsiveness to some growth factors. Also, Kveiborg et al.,[47] using a long-term culture model for in vitro OB aging, showed that senescent OBs expressed lower constitutive levels of insulin-like growth factor 1 (IGF-1) and IGF-binding protein 3 messenger RNA (mRNA), known to exert anabolic effects on the OBs. Finally, the maintenance of osteoprogenitor cell population in the bone marrow during aging and in patients with osteoporosis suggests that therapeutic interventions should aim at increasing the functional activity of these cells to avoid the age-related and osteoporosis-related bone loss.

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REFERENCES

1. Brockstedt H, Kassem M, Eriksen EF, Mosekilde L, Melsen F 1993 Age- and sex-related changes in iliac cortical bone mass and remodeling. Bone **14:**681–691.
2. Kragstrup J, Melsen F, Mosekilde L 1983 Thickness of bone formed at remodeling sites in normal human iliac trabecular bone: Variations with age and sex. Metab Bone Dis Relat Res **5:**17–21.
3. Eriksen EF, Hodgson SF, Eastell R, Cedel SL, O'Fallon WM, Riggs BL 1990 Cancellous bone remodeling in type I (postmenopausal) osteoporosis: Quantitative assessment of rates of formation, resorption, and bone loss at tissue and cellular levels. J Bone Miner Res **5:**311–319.
4. Cohen-Solal ME, Shih MS, Lundy MW, Parfitt AM 1991 A new method for measuring cancellous bone erosion depth: Application to the cellular mechanisms of bone loss in postmenopausal osteoporosis. J Bone Miner Res **6:**1331–1338.
5. Parfitt AM 1990 Bone forming cells in clinical conditions. In: Hall BK (ed.) The Osteoblast and Osteocyte. The Telford Press, London, UK, pp. 351–426.
6. Friedenstein AJ 1976 Precursor cells of mechanocytes. Int Rev Cytol **47:**327–359.
7. Friedenstein AJ, Chailakhyan RK, Gerasimov UV 1987 Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet **20:**263–272.
8. Owen M 1988 Marrow stromal stem cells. J Cell Sci Suppl **10:**63–76.
9. Kassem M, Risteli L, Mosekilde L, Melsen F, Eriksen EF 1991 Formation of osteoblast-like cells from human mononuclear bone marrow cultures. APMIS **99:**269–274.
10. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR 1999 Multilineage potential of adult human mesenchymal stem cells. Science **284:**143–147.
11. Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL 1996 Isolation and characterization of osteoblast precursor cells from human bone marrow. J Bone Miner Res **11:**312–324.
12. Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV 1994 Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology **134:**277–286.
13. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MA 1980 Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood **56:**289–301.
14. Friedenstein AJ, Chailakhjan RK, Lalykina KS 1970 The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet **3:**393–403.
15. Egrise D, Martin D, Vienne A, Neve P, Schoutens A 1992 The number of fibroblastic colonies formed from bone marrow is decreased and the in vitro proliferation rate of trabecular bone cells increased in aged rats. Bone **13:**355–361.
16. Quarto R, Thomas D, Liang CT 1995 Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif Tissue Int **56:**123–129.
17. Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ 1996 Age-related changes in osteogenic stem cells in mice. J Bone Miner Res **11:**568–577.
18. Kahn A, Gibbons R, Perkins S, Gazit D 1995 Age-related bone loss. A hypothesis and initial assessment in mice. Clin Orthop **313:**69–75.
19. Xu CX, Hendry JH, Testa NG, Allen TD 1983 Stromal colonies from mouse marrow: Characterization of cell types, optimization of plating efficiency and its effect on radiosensitivity. J Cell Sci **61:**453–466.
20. Brockbank KG, Ploemacher RE, van PC 1983 An in vitro analysis of murine hemopoietic fibroblastoid progenitors and fibroblastoid cell function during aging. Mech Ageing Dev **22:**11–21.
21. Nishida S, Endo N, Yamagiwa H, Tanizawa T, Takahashi HE 1999 Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. J Bone Miner Metab **17:**171–177.
22. D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA 1999 Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J Bone Miner Res **14:**1115–1122.

23. Majors AK, Boehm CA, Nitto H, Midura RJ, Muschler GF 1997 Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. J Orthop Res **15:** 546–557.
24. Oreffo RO, Bennett A, Carr AJ, Triffitt JT 1998 Patients with primary osteoarthritis show no change with ageing in the number of osteogenic precursors. Scand J Rheumatol **27:**415–424.
25. Oreffo RO, Bord S, Triffitt JT 1998 Skeletal progenitor cells and ageing human populations. Clin Sci **94:**549–555.
26. Gronthos S, Graves SE, Ohta S, Simmons PJ 1994 The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood **84:**4164–4173.
27. Simmons PJ, Torok SB 1991 Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood **78:**55–62.
28. Stewart K, Walsh S, Screen J, Jefferiss CM, Chainey J, Jordan GR, Beresford JN 1999 Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. J Bone Miner Res **14:**1345–1356.
29. Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ 1995 Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106–01 BSP). J Biol Chem **270:**9420–9428.
30. Vestergaard P, Hermann AP, Orskov H, Mosekilde L 1999 Effect of sex hormone replacement on the insulin-like growth factor system and bone mineral: A cross-sectional and longitudinal study in 595 perimenopausal women participating in the Danish Osteoporosis Prevention Study. J Clin Endocrinol Metab **84:**2286–2290.
31. Stenderup K 1999 Effect of age and osteoporosis on growth potential of osteoprogenitor cells in human bone marrow. Candidate thesis, University of Aarhus, Aarhus, Denmark.
32. Kuznetsov SA, Friedenstein AJ, Robey PG 1997 Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol **97:**561–570.
33. Friedenstein AJ, Latzinik NV, Gorskaya YF, Luria EA, Moskvina IL 1992 Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner **18:** 199–213.
34. Smith JR, Whitney RG 1980 Intraclonal variation in proliferative potential of human diploid fibroblasts: Stochastic mechanism for cellular aging. Science **207:**82–84.
35. Gronthos S, Simmons PJ 1995 The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood **85:**929–940.
36. Owen ME, Cave J, Joyner CJ 1987 Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J Cell Sci **87:** 731–738.
37. Rodan GA, Noda M 1991 Gene expression in osteoblastic cells. Crit Rev Eukaryot Gene Expr **1:**85–98.
38. Bianco P, Gehron RP 2000 Marrow stromal stem cells. J Clin Invest **105:**1663–1668.
39. Long MW, Ashcraft EK, Normalle D, Mann KG 1999 Age-related phenotypic alterations in populations of purified human bone precursor cells. J Gerontol A Biol Sci Med Sci **54:**B54–B62.
40. Koshihara Y, Hirano M, Kawamura M, Oda H, Higaki S 1991 Mineralization ability of cultured human osteoblast-like periosteal cells does not decline with aging. J Gerontol **46:**B201–B206.
41. Inoue K, Ohgushi H, Yoshikawa T, Okumura M, Sempuku T, Tamai S, Dohi Y 1997 The effect of aging on bone formation in porous hydroxyapatite: Biochemical and histological analysis. J Bone Miner Res **12:**989–994.
42. Liang CT, Barnes J, Seedor JG, Quartuccio HA, Bolander M, Jeffrey JJ, Rodan GA 1992 Impaired bone activity in aged rats: Alterations at the cellular and molecular levels. Bone **13:**435–441.
43. Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL 1988 Bone formation rate in older normal women: Concurrent assessment with bone histomorphometry, calcium kinetics, and biochemical markers. J Clin Endocrinol Metab **67:**741–748.
44. Kassem M, Brixen K, Mosekilde L, Eriksen EF 1994 Human marrow stromal osteoblast-like cells do not show reduced responsiveness to in vitro stimulation with growth hormone in patients with postmenopausal osteoporosis. Calcif Tissue Int **54:**1–6.
45. Marie PJ, Sabbagh A, de VM, Lomri A 1989 Osteocalcin and deoxyribonucleic acid synthesis in vitro and histomorphometric indices of bone formation in postmenopausal osteoporosis. J Clin Endocrinol Metab **69:**272–279.
46. Pfeilschifter J, Diel I, Pilz U, Brunotte K, Naumann A, Ziegler R 1993 Mitogenic responsiveness of human bone cells in vitro to hormones and growth factors decreases with age. J Bone Miner Res **8:**707–717.
47. Kveiborg M, Flyvbjerg A, Rattan SI, Kassem MS 2000 Changes in the insulin-like growth factor-system may contribute to in vitro age-related impaired osteoblast functions. Exp Gerontol **35:**1061–1074.

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