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Osteoblast recruitment from stem cells does not decrease by age at late adulthood

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Abstract

This study was aimed to characterize the ability of human bone marrow mesenchymal stem cells (MSC) to differentiate into osteoblasts in vitro. Twenty-three women and 20 men at late adulthood (52–92 years of age) were selected for the study. MSCs were isolated and cultured in vitro and alkaline phosphatase (ALP) activity, secretion of amino-terminal propeptide of type I procollagen (PINP), type III procollagen (PIIINP) and osteocalcin were analyzed. Matrix mineralization was analyzed by the von Kossa staining and by calcium quantification. We found that the ALP and PINP levels compared with control increased to 2.8- and 2.9-fold, respectively, when cells were cultured for three weeks. ALP activity, PINP and calcium deposition in response to dexamethasone treatment increased by age in women and was unchanged in men. Overall our data suggests that the osteogenic potential of MSCs does not decrease by age in either women or men at late adulthood.

Keywords: Mesenchymal stem cells; Bone marrow; Differentiation; Osteoblasts; Aging; PINP; ALP; Matrix mineralization

Age-related bone loss and osteoporosis are suggested to be caused by increased bone resorption and decreased bone formation [1]. Although bone loss at late adulthood is evident, the mechanism behind this phenomenon especially in the perspective of bone formation is not fully understood. An age-related atrophy of mesenchymal stem cells (MSC) has been proposed to cause a decrease in the number of osteoprogenitor cells and decreased bone formation capacity would then be due to lack of mature osteoblasts [2,3].

MSCs are multipotent cells, which can differentiate into various mesenchymal cell types, such as adipocytes, myoblasts, chondrocytes, tendon cells, and osteoblasts [4,5]. It has been demonstrated that MSCs isolated from bone marrow can be induced in vitro to differentiate into cells, which possess the characteristics of mature osteoblasts by glucocorticoids, β-glycerophosphate, ascorbate, and 10% fetal bovine serum [4]. During the differentiation process, osteoblast phenotype markers appear in the following order: after the proliferation of osteoblast precursors, mature osteoblasts produce a large amount of type I collagen [6], which is followed by alkaline phosphatase (ALP) expression which reaches a maximum during the latter phase of matrix maturation before the matrix mineralization, which is controlled by bone matrix protein osteocalcin [7,8].

In vitro MSCs form colonies in which cells have fibroblastic morphology, hence the literature uses term colony forming unit-fibroblasts (CFU-Fs) [9]. Some previous studies have shown that the number of ALP-positive CFU-Fs is maintained in patients younger than 50 years when compared to patients older than 50 years [10,11]. However, other studies have shown that the number of ALP-positive CFU-Fs decreases during the first two decades of life but is maintained after that

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[12–14]. Controversially, Martinez et al. [15] found that ALP activity increased by age after 50 years of age. However, in the Martinez's study mature osteoblasts were used instead of osteoblast precursors or stem cells.

The aim of this study was to try to answer the question whether the osteogenic potential of human MSCs indeed decreases at late adulthood. We used the human MSC differentiation model, in which the bone formation is initiated by proliferation of osteoblast precursors/stem cells and is followed by osteoblast maturation and finally ends in calcification of the extracellular matrix. We found, that the osteogenic capacity does not show any decline by age. In contrast, there was a slight age-dependent increase in a variety of osteogenic markers, including PINP in women. To our knowledge, there are no previous data available on the influence of aging at late adulthood on the osteogenic potential of human MSCs.

Materials and methods

Human MSC isolation and culture. Human bone marrow was harvested from 43 patients, who were operated for hip fracture or osteoarthrosis were randomly selected between 2000 and 2003 for the study. Twenty three of the patients were women (from 52 to 92 years of age) and 20 were men (from 54 to 86 years of age). The Ethical Committee of The Northern Ostrobothnia Hospital District approved the study.

Bone marrow was obtained from the femoral collum and trochanteric region, during the operation. Approximately 5-ml bone marrow was placed into a 50-ml Falcon tube (Greiner, Germany) with 10 ml medium: phenol red free α-modified essential medium with L-glutamine (α-MEM; Gibco, Paisley, UK) buffered with 20 mM Hepes (Gibco) and containing 10% heat-inactivated fetal bovine serum (FBS; Bioclear, Netherlands), and 10 ml/L of antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin; Gibco) and transported to the laboratory for further processing within 5 min.

The bone marrow samples were plated into 25 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) and cultured in 5 ml above described medium with 100 nM dexamethasone (Dex) (Sigma, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After the cell-attachment period of two days, culture medium was removed, and cultures were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS; Gibco). Fresh medium was added and half of the medium was replaced two times per week until near-confluence.

For analysis by different biochemical assays human MSCs were washed with PBS and adherent cells detached using trypsin–EDTA solution (Gibco). Cells were counted and plated into 24-well plates at a density of 5000 cells/well. During the final culture half of the medium with ingredients was changed twice a week.

Number of ALP-positive CFU-Fs. When bone marrow was transported to laboratory, Ficoll–Paque Research Grade (Pharmacia Biotech, Uppsala, Sweden) was used according to the manufacturer's instructions to isolate mononuclear cells. Cells were seeded at a density 50,000 cells/cm^2 and cultured above described medium with 100 nM Dex. After 1 week in culture, cells were stained for alkaline phosphatase. Colonies consisting more than 5 cells were defined as CFU-Fs and a CFU-F was considered ALP positive when at least one cell positively stained for ALP. The average of the number of ALP-positive CFU-Fs was computed.

ALP staining. Human MSCs in 24-well plates in four replicate wells were cultured with 100 nM Dex or without (control). At day 21, staining was performed by a standard procedure according to the manufacturer's instructions (Sigma Diagnostics). The human MSCs were washed three times with PBS, fixed in 3% paraformaldehyde (PFA) for 10 min. Cells were then incubated with Naphthol AS-BI phosphate containing 0.1 M sodium nitrite and Fast red violet for 15 min. Hematoxylin was used as a counterstain. The number of CFU-Fs and cells staining positive for ALP was viewed with a light microscope.

Specific ALP activity. Human MSCs in 24-well plates in four replicate wells were cultured without (control) or with 100 nM Dex. At days 7, 14, and 21, cultured cells were assayed as described below. The assay buffer containing 0.1% Triton X-100, pH 7.6, was added to each well, and the plates were frozen. After thawing, enzyme activity was determined using 0.1 mM 4-*p*-nitrophenylphosphate (Sigma) as substrate and absorbance was read at 405 nm in a plate reader (Victor 2, Wallac Oy, Turku, Finland). Each sample was measured in duplicates. The protein contents of the wells were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). The enzyme activities were expressed as units/mg protein (specific ALP activity).

Determination of procollagen propeptides (PINP and PIIINP). Human MSCs in 24-well plates were cultured with 100 nM Dex or without (control). At days 7, 14, and 21, cultured cells were assayed as described below. The concentration of amino-terminal propeptide of type I procollagen (PINP) was measured using commercially available RIA (Orion Diagnostica, Espoo, Finland), which had only a minimal 1.2% cross-reaction with monomeric Col1 domain, which is a degradation product of intact PINP. The assay was done in sequential saturation and we used duplicate 0.05 ml aliquots of 1:2 or 1:5 diluted lysates of cells (see below). The intra- and interassay coefficients of variation of PINP in serum samples were 4.6–10.3% and 3.1–10.8%, respectively. The concentration of amino-terminal propeptide of type III procollagen (PIIINP) (also in sequential saturation) was measured in duplicate 0.2 ml aliquots of 1:5 diluted lysates of cells with a commercially available RIA (Orion Diagnostica, Espoo, Finland). The intra- and interassay coefficients of variation of PIIINP in serum samples were 4.4–6.1% and 4.1–18.0%, respectively.

Fluorescence staining of anti-PINP. Human MSCs in 24-well plates in four replicate wells were cultured with 100 nM Dex or without (control). At day 21, cultured cells were stained for PINP as described below. The PFA-fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were stained by using polyclonal PINP antibody for 30 min, rinsed thoroughly in PBS, and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibodies (DAKO, Glostrup, Denmark) for 30 min. The samples were viewed with a fluorescence microscope.

Measurement of osteocalcin. Human MSCs were cultured in 24-well plates in four replicate wells with Dex, βGB, and AA and the concentration of osteocalcin in the culture medium was measured on days 0, 14, 21, 28, and 35. Osteocalcin was determined in duplicates with an in-house immunoassay for total osteocalcin (T-OC) [16] which was modified from two-site assays described by Hellman et al. [17]. Assay T-OC detects both human and bovine osteocalcin and the within-assay and between-assay CVs are less than 10%.

Western blot. For Western analysis, human MSCs were seeded at 4×10^3 cells/cm^2 into 60 mm^2 Petri dishes, with 100 nM Dex. The cells were cultured in duplicate dishes 14 and 21 days. Cultures were rinsed with PBS and then extracted with RIPA buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and Complete EDTA-free protease inhibitor tablets Boehringer Mannheim, Mannheim, Germany) for 30 min on ice. The total protein concentration of the lysate was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Samples with equal amounts of total protein were loaded onto SDS–polyacrylamide gel and proteins were electrophoretically transferred to Immobilon-P filter (Millipore Corporation, Bedford, Mass). The filter was immunolabeled with rabbit anti-PINP and anti-PIIINP antibodies and peroxidase-linked anti-rabbit IgG antibody (Amersham Life Sciences, Little Chalfont, UK) was used as

secondary antibody. Proteins were detected with ECL (Amersham Life Sciences).

Calcium quantification and von Kossa staining. Human MSCs were plated into 24-well plates. Cells were cultured in four replicate wells in (1) control only, (2) 100 nM Dex, (3) 10 mM sodium β-glycerophosphate (βGB) (Sigma), and (4) 100 nM Dex, 10 mM βGP, and 0.05 mM ascorbic acid-2-phosphate (AA) (Sigma). For calcium quantification the cultures were characterized at days 21, 28, and 35. The cells were washed three times with Ca^{2+} and Mg^{2+}-free PBS and incubated overnight at room temperature in 0.6 M HCl. Calcium determination was based on the reaction of calcium with *o*-cresolphthalein-complexone according to the manufacturer's instruction (Roche Diagnostics Corporation, IN, USA). The colorimetric reaction was read at 570 nm in a plate reader. For von Kossa staining, cells were cultured for 14, 28, and 42 days and the cultures were washed three times with Ca^{2+} and Mg^{2+}-free PBS, fixed in PFA for 10 min. Bone nodules were detected with von Kossa [18] staining for calcium. The calcification was viewed with the light microscope and in a stripping table.

Statistical analysis. The statistics were evaluated by using Student's *t* test and linear regression analysis. Values are expressed as means ± SD or ±SE. In the figures *, **, and *** represent the Student's *t* test *p* values of $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Results

Human MSCs form colonies and differentiation initiate under optimal conditions in vitro

After 7 days of culture, human MSCs started to form distinct colonies. We were able to demonstrate a positive correlation between the number of ALP-positive CFU-Fs and the ALP activity of the cell lysate ($r = 0.781$, $n = 6$). To examine in detail the effect of different culture conditions in the differentiation process, human MSCs were treated with Dex for 21 days after seeding for 1–3 days. The attachment period of two days was found to induce highest ALP activity at 3 weeks of culture when the ALP activity reached it's maximum (Fig. 1A). All measurements of specific ALP activity showed response for Dex treatment at all tested passages (1.–4.) (Fig. 1B), however, there was some variation in Dex-responses between different subcultures (data not shown). To determine the optimal cell number for ALP expression, the human MSCs were plated for 2500, 5000 or 10,000 cells/2 cm^2 well. The highest specific ALP activity was observed when 5000 human MSCs/well, were added (Fig. 1C).

Human MSC-derived osteoblasts show high specific ALP activity and PINP secretion at 3 weeks

The cells were characterized for ALP activity, PINP and PIIINP secretion. During the first days of culture the specific ALP activity decreased rapidly (data not shown), until a slow increase was observed after 7 days at Dex-treated cells. The maximal specific ALP activity was achieved by day 21, just before the initial matrix mineralization. Dex induced an average of 1.8-, 1.9-, and 2.8-fold increases, in the specific ALP activity compared with control at 7, 14, and 21 days, respectively (Fig. 2A). Secretion of PINP was highest after 14 days with Dex and control, and was maintained in the Dex-treated cells. PINP levels were 2.9-fold higher in the Dex-treated group compared to control after 21 days. PIIINP levels remained low during the observation period (Fig. 2B). Western blotting showed expression of PINP at 14 and 21 days, and expression of PIIINP only at day 14 (Fig. 2C). To confirm these observations, we also stained control and Dex-treated human MSCs for the ALP (Fig. 2D) and PINP (Fig. 2E). Dex, once again increased the number and proportional area of ALP- and PINP-positive cells.

Human MSCs form mineralized bone after 3 weeks in vitro

To test whether human MSCs could form mineralized bone nodules in vitro, we grew these cells under four different culture conditions: (1) control, (2) Dex, βGP, and AA, (3) Dex, and (4) βGP. As shown in Fig. 3A, MSCs did not form mineralized bone nodules

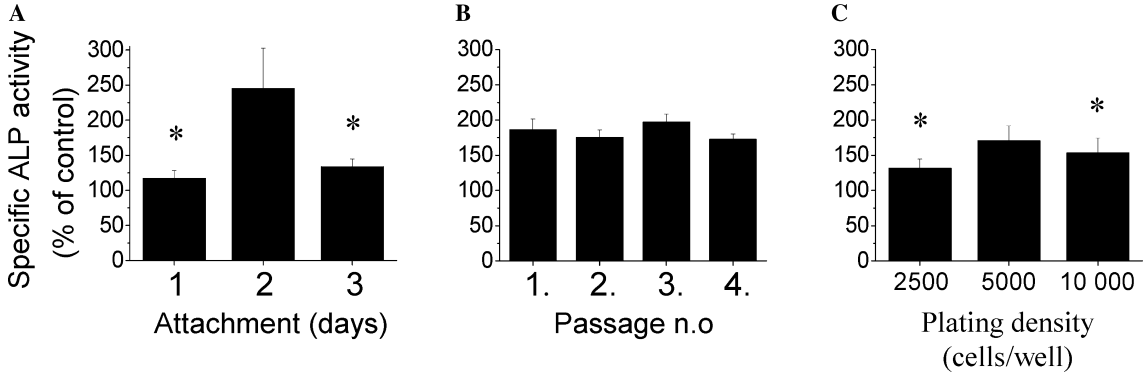

Fig. 1. The effects of (A) cell-attachment period, (B) passage number, and (C) plating density on human MSC cultures. Cells were treated without (control) and with 100 nM Dex and specific ALP activity was assayed at day 21. The columns show the means ± SE (*$p < 0.05$ Student's *t* test) compared with the highest value.

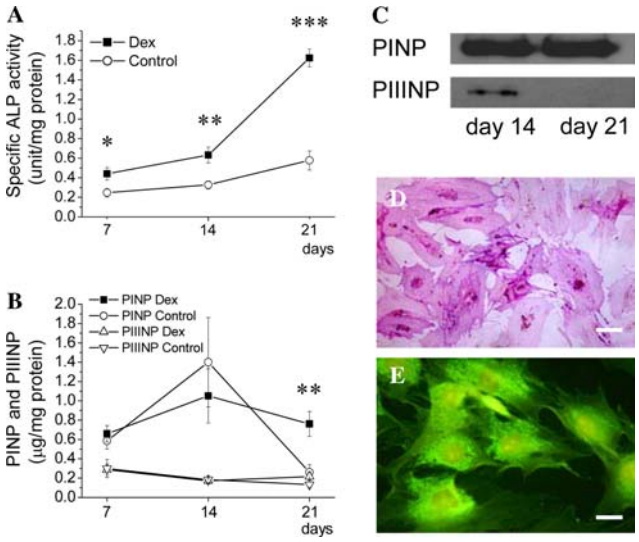

Fig. 2. The dynamics of osteoblastic markers in human MSC culture. Cells were treated without (control) and with 100 nM Dex. (A) Specific ALP activity and (B) secretion of PINP and PIIINP were analyzed. The results are expressed as means ± SE. The p values from Student's t test between control and Dex are shown as asterisks ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). Human MSCs were treated with 100 nM Dex and (C) Western blot analysis for expression of PINP and PIIINP was assayed. For (D) ALP staining and (E) Anti-PINP fluorescent staining cells were grown in the presence of 100 nM Dex for 21 days. Bar, 0.02 mm.

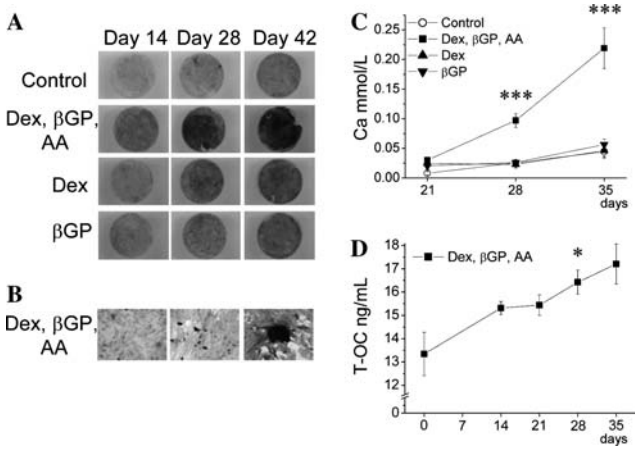

Fig. 3. Human MSCs form colonies and mineralize extracellular matrix in vitro. Cells were grown in (1) control, (2) Dex, βGP, AA, (3) Dex, and (4) βGP. (A) At days 14, 28, and 42 the cells were washed, fixed and stained for calcium phosphate by the von Kossa method. (B) Mineralized nodules were formed when cells were treated with Dex, βGP and AA, magnification 40×. (C) The calcium deposition was determined at days 21, 28, and 35. The results are expressed as means ± SE. The p values from Student's t test between control and Dex, βGP, AA are shown as asterisks ($***p < 0.001$). (D) Human MSCs were grown in Dex, βGP and AA. At days 0, 14, 21, 28, and 35 osteocalcin was analyzed from culture medium. The results are expressed as means ± SE. The p value from Student's t test between 0 and 28 days are shown as asterisk ($*p < 0.05$).

when only one supplement was present. However when Dex, βGP, and AA were all added, mineralized bone appeared as demonstrated by positive staining for von Kossa. The number and size of bone nodules was studied up to 42 days at culture (Figs. 3A and B).

Bone formation, as evaluated by calcium levels, increased readily after 21 days at Dex, βGP, and AA-treated cultures. Calcium levels were 3.8- and 4.9-fold higher compared with control at 28 and 35 days, respectively. In cells treated with only one supplement, mineralization was initially weak and remained weak throughout the culture period (Fig. 3C). Osteocalcin concentration in the medium increased during the culture period of 5 weeks and the increase was statistically significant on day 28 (1.2-fold, $p = 0.03^*$) when compared to the beginning of the culture (Fig. 3D).

MSC mediated in vitro bone formation is not declined by age

After careful preparation of the bone formation assay for repeatability as described above, the effect of age on PINP concentration, specific ALP activity and calcium concentration were examined. Human MSCs were grown in the presence of Dex for 21 days. The relationship between age and PINP concentration was determined. Linear regression analysis did not show a significant change ($r = 0.590$, $p = 0.124$) in male cells, but an evident increase in cells from women ($r = 0.712$, $p = 0.00199$) (Figs. 4A and B). Similar results were ob-

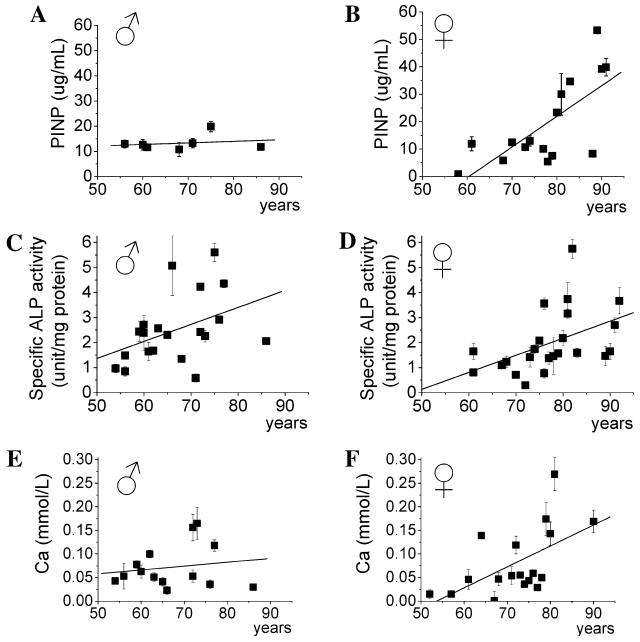

Fig. 4. The effect of age on the collagen synthesis, ALP activity and extracellular matrix mineralization by differentiated osteoblasts. The human MSCs were cultured for 21 days in the presence of Dex, (A,B) PINP concentration and (C,D) specific ALP activity were assayed. (E,F) The cells were cultured for 35 days in the presence of Dex, βGP, and AA and calcium concentration was assayed. Each point represents means ± SD. Female and male groups are indicated in figures by ♀ and ♂ symbols, respectively.

tained at the age-specific ALP activity relationship. Once again, linear regression analysis did not indicate significant change by age for males ($r = 0.420$, $p = 0.0654$). Female cells, again, showed a significant increase by age ($r = 0.467$, $p = 0.0248$), although this increment was not as clear as for PINP (Figs. 4C and D). The relationship between age and calcium concentration on day 35 in the presence of Dex, βGP, and AA was determined. Again, there was no significant change in male cells ($r = 0.162$, $p = 0.580$), but a significant increase by age in the calcium concentration ($r = 0.573$, $p = 0.0129$) in women (Figs. 4E and F).

Discussion

Osteoporosis is one of the serious diseases impairing the aging population. Explanations for this condition have been sought for already more than six decades. Albright et al. [19] related the postmenopausal osteoporosis to estrogen deficiency. In women bone remodellation increases by 90% at the menopause as assessed by biochemical markers [20], whereas age-related bone loss in men is not so aggressive. Estrogen might be the most important factor of age-related osteoporosis, at least in females, but other explanations have also been studied. Bone loss in osteoporosis is thought to be partly caused by decreased bone formation [1] whilst estrogen effects most clearly bone resorption. Decreased bone formation can result from both impaired osteoblast recruitment and differentiation from stem cells in the bone marrow or decreased activity of mature osteoblasts. This study was focused to clarify the role of osteoblast recruitment from bone marrow mesenchymal stem cells in elderly patients.

It is shown in this study that there is an interesting positive correlation between osteoblast markers (PINP concentration, specific ALP activity and calcium concentration) and the patient's age in osteoblast formation assay when MSC from postmenopausal women were used. We found also that MSCs from older male patients sustained their osteoblast differentiation and bone formation capacity (Fig. 4).

The MSC isolation and culture was developed for reproducibility. It was first demonstrated that the human bone marrow-derived MSCs were able to proliferate and subsequently differentiate into mature osteoblasts, which were capable of CFU and bone formation [9]. The first osteoblastic markers (Fig. 2), ALP and PINP appeared, when cells were grown for three weeks. PINP/PIIINP ratio was used to confirm absence of fibroblastic phenotype in the culture [21]. ALP and fluorescence staining confirmed that up to 98% of all cells obtained the osteoblastic phenotype. These cells were also able to mineralize the collagen matrix and finally, at the latest stage, synthesize osteocalcin, which are considered to be hallmarks of terminal osteoblast differentiation [22]. It is notable that in the model used here there is very few osteoblasts present in the initial culture and that marginal specific ALP activity at first days of culture decreases rapidly when undifferentiated ALP-negative stem cells proliferate (see Results).

In previous literature there are no reports on the effects of age on PINP expression, ALP activity, and extracellular matrix mineralization at late adulthood by human MSCs. Hence, data presented here are reflected to studies employing different methodologies. Studies by Justesen et al. [10] and Stenderup et al. [11] clearly show that the number of ALP-positive CFU-Fs is maintained during aging when patients younger than 50 years were compared to patients older than 50 years. On the other hand, an age-related decline was demonstrated by D'Ippolito et al. [12], Majors et al. [13], and Nishida et al. [14] up to the age of 30 years with little or no change thereafter. Controversially, Martinez et al. [15] found that osteoblastic ALP activity increased by age after fifth decade of life. Martinez study's finding is now supported by the data presented here. In this study ALP activity and other biochemical markers were used as determinants of osteoblast differentiation rather number and size of the ALP-positive CFU-Fs. However, we do not believe that this explains the discrepancy between this study and previous data since we were able to demonstrate positive correlation between the number of ALP-positive CFU-Fs and the ALP activity of the lysate.

The main finding of this study is that after menopause the osteogenic potential of human MSCs does not decrease. Hence, the pathogenesis of the age-related osteoporosis is neither due to impaired human MSC differentiation capacity nor MSC number and explanations should be looked elsewhere, for example nutrition which might influence osteoblasts activity in vivo. It is well-known fact among orthopedic surgeons that osteoporotic bone has as good healing capacity as normal bone, albeit the fractures might be somewhat different. This study supports and explains this clinical finding. The question that emerges now is what are the factors that maintain and even stimulate osteoblastogenesis from MSCs of elderly patients? Cooper et al. [23] have shown that activity of 11β-hydroxysteroid dehydrogenase type 1, which appears in human osteoblasts and converts hormonally inactive cortisone into cortisol [24], increases by age in primary osteoblast cultures. It is notable that this and all previous studies [4,25] characterize the Dex-induced differentiation of cells into osteoblasts. Second, as shown by Batge et al. [26] there is an age-dependent increase of transforming growth factor-β receptors in human osteoblast-like cells in vitro. This might explain via paracrine mechanism the observed increment in osteoblast recruitment [27,28]. We conclude that further studies are now required to clarify which of

the mechanisms suggested above explains our finding, which demonstrates the unique capacity of stem cells to enable tissue regeneration even at late course of life.

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