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Original Article

Demonstration of Cellular Aging and Senescence in Serially Passaged Long-Term Cultures of Human Trabecular Osteoblasts

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Abstract. The proliferative capacity and cellular and biochemical characteristics of human trabecular bone osteoblasts were analysed throughout their replicative lifespan in vitro. Like several other cell types, human osteoblasts demonstrated a typical Hayflick phenomenon of cellular aging comprising a period of rapid proliferation until cumulative population doubling level (CPDL) 22 to 24, followed by a phase of slow growth and the final cessation of cell division at CPDL 32 to 34. Comparing young cells (less than 20% lifespan completed) and old cells (more than 90% lifespan completed) revealed a progressive increase in population doubling (PD) time, a decrease in attachment frequency, a decrease in the number of S-phase positive cells, a decrease in the rates of DNA, RNA and protein synthesis, an increase in the protein content per cell and an increased proportion of senescence-specific β-galactosidase positive cells. While osteoblastic production of collagen type I decreased progressively during aging, alkaline phosphatase activity dropped rapidly after the first few passages and then remained constant during the rest of the proliferative lifespan. Significant morphological changes from thin and spindle-shaped early passage young cells to large, flattened and irregularly shaped late passage old cells full of intracellular debris were observed. In comparison, osteoblasts established from an osteoporotic bone sample showed a maximum CPDL of less than 5, had a longer PD time and exhibited abnormal senescent morphology. Thus, we have demonstrated for the first time that human osteoblasts, like several other diploid cell types, have a limited proliferative capacity in vitro and undergo aging and senescence as measured by various cellular and biochemical markers. In addition, preliminary studies show that cells from osteoporotic bone have a severely reduced proliferative capacity. This model of bone cell aging facilitates study of the molecular mechanisms of osteoblast senescence as well as factors related to osteoblast dysfunction in patients with osteoporosis.

Keywords: Aging; Lifespan; Osteoblasts; Osteoporosis; Senescence

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Introduction

A decrease in the formation of new bone during bone remodelling is a crucial factor in the pathogenesis of bone loss observed during aging and in involutional osteoporosis [1,2]. Since osteoblasts are the primary effectors of bone formation in vivo, age-related changes in osteoblast functions may play an important role in this phenomenon. However, it is often not possible to study in vivo the molecular mechanisms which are responsible for alterations in osteoblastic functions during aging. Therefore, it is necessary to develop in vitro culture systems with normal diploid osteoblasts in order to investigate various cellular, biochemical and molecular aspects of aging of normal osteoblasts. Such systems can be of immense use not only for understanding the molecular basis of age-related alterations in cell function, but also for determining the responsiveness

of cells to various drug and hormone treatments with respect to the prevention and cure of age-related bone pathology.

During recent years, studies performed on a wide variety of cell types have established that normal diploid cells in culture can divide only a limited number of times, during which they undergo a plethora of changes culminating in the cessation of cell division. Since this phenomenon of cellular aging in vitro was first described by Hayflick [3,4] it is now generally known as the Hayflick phenomenon, and the limited division potential of normal cells is called the Hayflick limit [5]. There are several lines of evidence in support of the view that the limited division potential of cells and other changes occurring during their limited lifespan are a genuine expression of the process of aging. This evidence includes the inverse relationship between the age of the donor and maximum division potential of cells in culture; the direct relationship between a species' maximum achievable lifespan and the proliferative capacity of its cells in culture; and the decreased division potential of cells isolated from patients with premature aging syndromes, such as Werner's syndrome and progeria [5,6].

The Hayflick system of cellular aging in vitro has proved to be extremely useful for analysing various physiological, biochemical and molecular aspects of senescence and aging of a wide variety of cell types including fibroblasts, chondrocytes, smooth muscle cells, epithelial cells, endothelial cells, keratinocytes, melanocytes, glial cells and lymphocytes [5,6]. However, no systematic study has been published on *long-term serial passaging* of human osteoblasts which both describes the age-related changes in their morphology, growth characteristics and macromolecular synthesis, and determines their maximum division potential in vitro. Previous studies on the effects of aging on the cell proliferation and biochemical characteristics of osteoblasts have compared early passage primary cultures initiated from donors of different ages, but without following these cultures throughout their proliferative lifespan in vitro [7–11]. Therefore, we have characterized various age-related changes in human osteoblasts cultured from trabecular bone explants and serially subcultivated throughout their limited proliferative lifespan in vitro. Furthermore, we have compared the proliferative potential of osteoblasts obtained from normal bone with those observed in osteoblasts obtained from an osteoporotic bone sample. Some preliminary observations on the aging of human trabecular osteoblasts in culture have been published by us as a part of the proceedings of a conference [12].

Materials and Methods

Cell Culture

Human trabecular osteoblast cultures were established as described previously [13]. Briefly, trabecular bone samples were obtained from the femur heads or iliac crest of 7 normal individuals (males aged 2, 15 and 57 years; females aged 10, 68, 71 and 72 years). These patients were being treated either with hip replacement for osteoarthritis or with bone grafting for corrective surgery. No concurrent diseases which could affect bone metabolism were present in these donors. In order to establish cell cultures, residual fibrous tissue was dissected from bone samples that were washed extensively in phosphate-buffered saline (PBS), minced and digested with crude collagenase type IV (1 g/l in minimal essential medium (MEM) for 2 h at 37 °C. These fragments were cultured in phenol-red-free MEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, designated as the complete medium. Cells were allowed to grow out from bone explants and to form a confluent monolayer within 4 weeks. Osteoporotic bone samples were obtained from a 73-year-old woman suffering a low-energy traumatic hip fracture. There were no other medical conditions causing secondary osteoporosis in this patient. Trabecular bone samples from this donor were treated for initiating cell cultures as mentioned above.

In Vitro Subculturing

Normal and osteoporotic osteoblasts were grown in the complete medium described above. The cells were kept at 37 °C in 5% CO_2 in an incubator maintaining 95% humidity. The medium was changed twice a week and the cells were passaged (subdivided) by trypsinization at a split ratio of 1:2 after the cultures formed a confluent monolayer. The number of cells in a confluent cell layer was determined in a sample of cell suspension after trypsinization, using a Coulter counter (Coulter Electronics, UK). The number of population doublings (PDs) was calculated by the formula $\log N/\log 2$, where N is the number of cells in a confluent cell layer divided by the number of cells initially seeded in the culture flask.

Growth Characteristics

Changes in growth patterns during serial passaging were assessed by the following methods:

Short-Term Growth and Attachment Frequency. Every second or third passage, one-step growth characteristics of normal osteoblasts were studied to detect changes in their growth rates during aging. Osteoblasts were seeded in several 24-well plates at a density of approximately 5×10^3 cells per square centimetre. After 2, 4 and 6 h the attachment frequency was determined by detaching the cells in three wells and counting the number of cells using a Coulter counter. For growth analysis, cells in two wells were counted as above every day for the first 3 days and every second day for the next 14–16 days until the culture became confluent.

Lifespan Measurements. The maximum lifespan of both normal and osteoporotic osteoblasts was determined as the cumulative population doubling level (CPDL) achieved after serial passaging in vitro. Five parallel cell lines of normal osteoblasts and one line of osteoporotic osteoblasts were maintained throughout their lifespan in vitro. At confluence, the cells were split at 1:2 ratio and the number of cells counted using a Coulter counter to calculate CPDL and PD time. The cultures were considered to have reached the end of their proliferative lifespan when they failed to become confluent in 5 weeks in spite of twice-weekly changes of the culture medium.

Determination of the Number of S-phase Cells using Autoradiography. The proportion of cells synthesizing DNA (S-phase cells) was determined by [^{3}H]thymidine autoradiography. Cells were seeded in chamber slides (growth area 1 cm^2) in complete medium. In order to synchronize the cells, the medium was changed after 24 h to low serum medium (0.2% FCS). This induces cell quiescence and more than 99% cells become arrested in G_0 phase of the cell cycle in 3 days (as determined by both autoradiography and bromodeoxyuridine labelling). After 3 days of serum deprivation, 1 μCi/ml [^{3}H]thymidine (specific activity 87 Ci/mmol) was added together with complete medium. The osteoblasts were labelled with radioactive thymidine for different intervals (4, 24, 48 and 72 h). After labelling, cells were washed and fixed using ice-cold methanol. The slides were then processed for autoradiography using a standard procedure.

Morphological Changes

To assess for changes in cellular morphology during serial passaging, live osteoblasts were examined daily under a phase-contrast inverted microscope. Furthermore, at regular intervals, both normal and osteoporotic osteoblasts were fixed in –20 °C methanol for 30 min and stained with Giemsa stain for 60 min.

Staining for Actin Filaments. Osteoblasts were cultured in chamber slides using the same medium as described above. After 2–4 days the medium was aspirated and cells were washed, fixed and stained for actin filaments using fluorescein isothiocyanate (FITC)-conjugated phalloidin and were examined under UV light, as described previously for human fibroblasts [14].

Staining for Senescence-Associated β-Galactosidase Activity. Recently, it has been demonstrated that senescent cells exhibit increased β-galactosidase activity, which can be a useful biomarker of cellular aging [15]. This procedure was adopted for osteoblast cultures. In brief, the cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in Hank's buffer. The cells were washed in saline buffer and stained overnight in a staining solution containing 1 mg/ml X-gal, 40 mM citric/sodium phosphate buffer (pH 6), 5 mM potassium ferrocyanide, 150 mM sodium chloride and 2 mM magnesium chloride. This procedure leads to the blue staining specific to the senescent cells.

Macromolecular Characteristics

Protein Content. The protein content of cells was measured by the Bradford method as previously described [16]. Bovine serum albumin was used as a standard.

Synthesis of DNA, RNA and Protein. DNA, RNA and protein synthesis was measured by the incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{35}S]methionine, respectively. The cells were seeded in 24-well plates at a density of 10^4cells/cm^2. After 24 h, cells were incubated with medium containing either 4 μCi/ml [^{3}H]thymidine (specific activity 87 Ci/mmol), 4 μCi/ml [^{3}H]uridine (specific activity 44 Ci/mmol) or 5 μCi/ml [^{35}S]methionine (specific activity 1000 Ci/mmol) for 2 h or 72 h. Subsequently, the cells were washed, lysed in 0.1 N NaOH and precipitated at 4 °C by ice-cold 10% trichloroacetic acid (TCA). Gelman filters (Gelman Sciences, USA) were used to collect the TCA precipitates. The filters were dissolved in liquid scintillation fluid and counted in a β-counter.

Osteoblast Phenotype Markers

Changes in alkaline phosphatase (AP) and collagen type I were monitored during serial subculturing. Cells were trypsinized at each passage and were plated in 96-well plates (8×10^3 cells/cm^2). Cells were cultured in the absence or presence of 1,25-dihydroxyvitamin D_3 (calcitriol, 10^{-9}M) for 48 h. AP was measured in the cell layer by using *p*-nitrophenol (PNP) phosphate dissolved in diethanolamine buffer containing 2 mmol/l $MgCl_2$ (pH 9.8) as substrate. Incubation of substrate and cells was carried out at 37 °C for 30 min. Absorbance of PNP was determined by a plate reader at 405 nm.

Production of type I collagen was determined in the conditioned medium using a radioimmunoassay kit that measures the C-terminal propeptide of type I collagen (PICP; Farmos Diagnostica, Finland), as described previously [13,17].

Results

Cell Characteristics and Lifespan In Vitro

Osteoblast cultures established from 7 normals and 1 osteoporotic individual exhibited a limited proliferative capacity in vitro. The range of CPDL attained by various normal strains was between 10 and 33. Since one of these cell strains, designated OB/K-72 (culture initiated

from a 72-year-old healthy woman) was the best growing and longest lived in vitro, this cell strain was selected for the detailed studies reported here. A representative longevity curve (Fig. 1) from OB/K-72 line serially passage in culture shows that the normal osteoblasts could be grown continuously for 451 days, serially passage 34 times at a 1:2 split ratio and reached a maximum CPDL of 32.9. In comparison, osteoporotic osteoblasts obtained from a single patient had a severely limited proliferative capacity in vitro, which was much shorter than that of normal osteoblasts (Fig. 1). The osteoporotic osteoblasts were grown continuously for 201 days and were passaged 5 times at a split ratio of 1:2 reaching a maximum CPDL of 4.22. Characterization of the primary cultures of these cells showed a normal diploid karyotype and the complete osteoblastic phenotype, including the production of AP and osteocalcin, large quantities of collagen type I and minute quantities of collagen type III, and the ability to form a mineralized matrix in vitro [13,17,18].

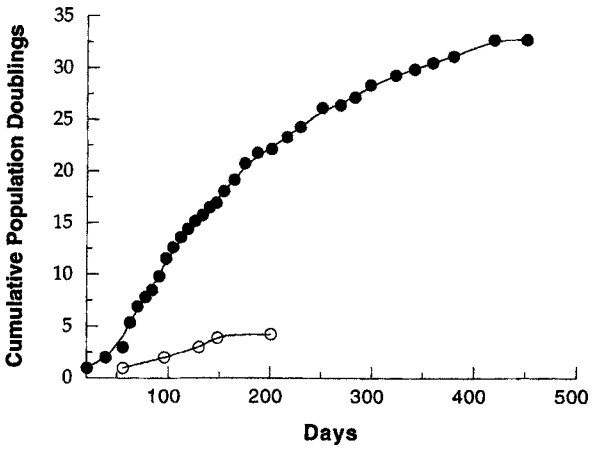


Fig. 1. Longevity curves of normal (*filled circles*) and osteoporotic (*open circles*) human trabecular osteoblasts serially passaged in vitro.

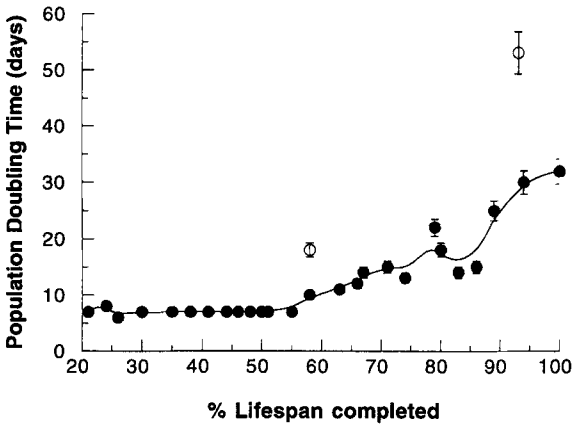


Fig. 2. Population doubling (PD) time as a function of percentage lifespan completed for normal (*filled circles*) and osteoporotic (*open circles*) human osteoblasts. Results represent the mean ± SEM of three independent measurements.

Longevity curves for normal and osteoporotic osteoblasts shown in Fig. 1 represent a typical Hayflick phenomenon. In normal osteoblasts, a period of rapid and regular growth and cell proliferation until about CPDL 22 was followed by slowing down of growth finally reaching the end of proliferative potential. Exponentially growing osteoblasts (less than 50% lifespan completed) were considered young, cells with between 50% and 80% of their lifespan completed were considered to be middle-aged and the slowly growing cells (more than 80% lifespan completed) were considered as old or senescent. Although cells could survive metabolically for quite some time after the last passage, there was no further increase in cell number and the cultures became progressively heterogeneous followed by detachment.

At each split the input and output of cells were determined and cell yield and PD time was calculated. Young exponentially growing osteoblasts had a relatively constant PD time of about 6–8 days (Fig. 2). A progressive increase in PD time was observed from passage 17 onwards (55% lifespan completed) until the end of the cells' lifespan. Senescent cultures which had completed more than 90% of the lifespan took approximately 25 days to double their number. During the short lifespan of osteoporotic osteoblasts the PD time increased from 18 days at passage 2 to 53 days at passage 4 (Fig. 2).

Figure 3 shows the cell yield per T25 culture flask (growth area 25 cm^2) as a function of percentage lifespan completed. For young cultures, the cell yield was around 8×10^5 cells per culture flask, decreasing to 5×10^5 cells per flask for middle-aged cells and finally to 1.2×10^5 per flask for very old cells. This decrease in cell yield is an indication of increased cell size of old osteoblasts compared with young osteoblasts. Additional flow cytometric studies confirmed these findings that the size of osteoblasts increased progressively during cellular aging in vitro (data not shown). Osteoporotic osteoblasts had a relatively low yield ranging from 1.0

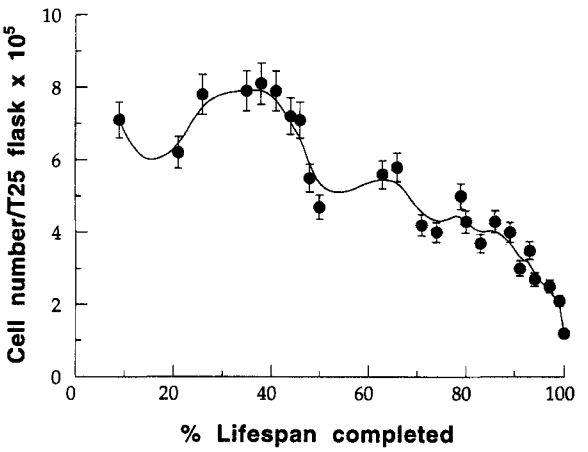


Fig. 3. Cell yield per T25 culture flask as a function of percentage lifespan completed for normal human osteoblasts. Results represent the mean ± SEM of three independent measurements.

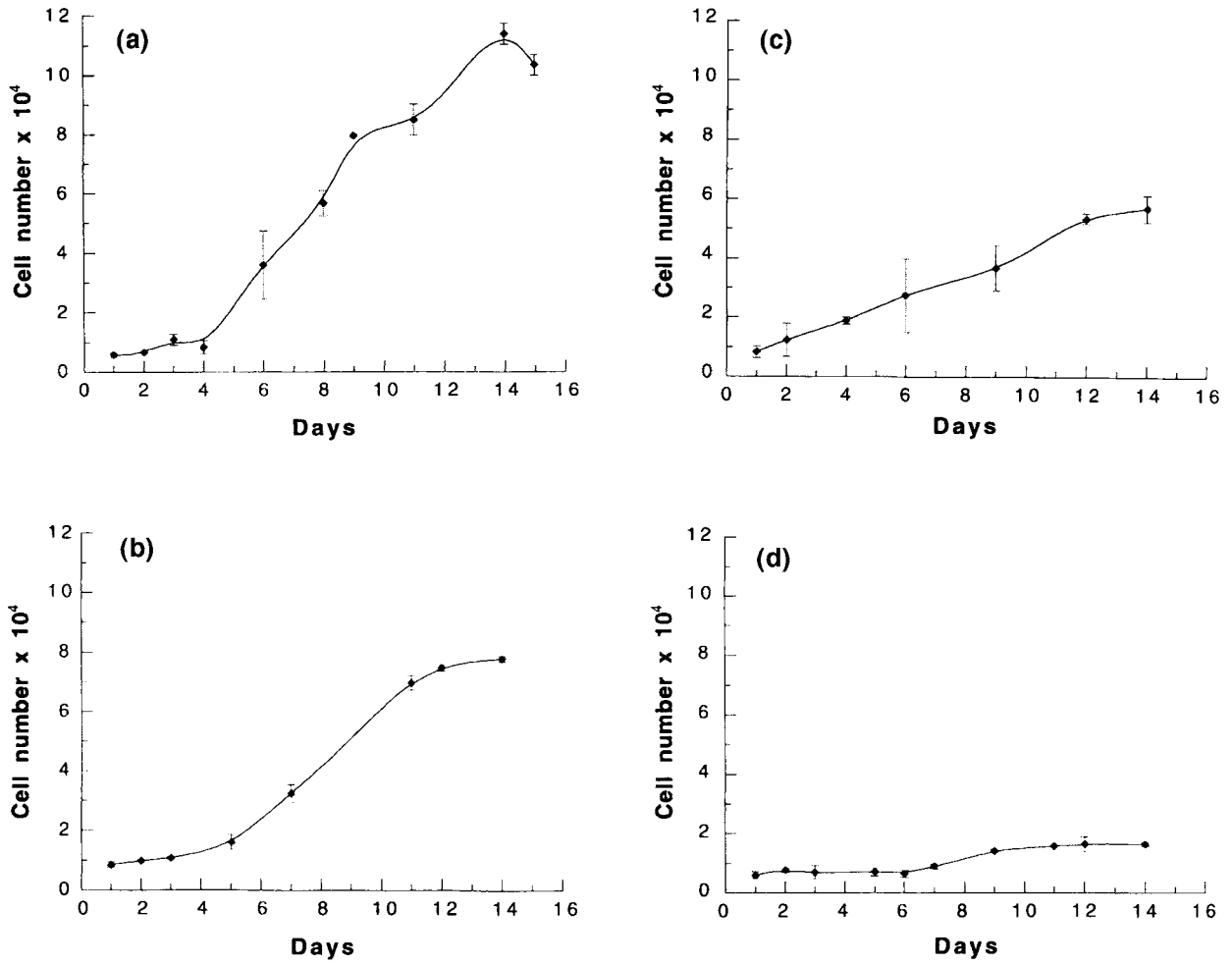


Fig. 4. One-step growth curves of normal human osteoblasts with **a** 18% **b** 61% **c** 80% and **d** 93% of their lifespan completed in vitro. Results represent the mean ± SEM of three independent measurements.

$\times 10^5$ to 1.7 $\times 10^5$ cells per flask during their short lifespan in vitro. Furthermore, we also found a decline in attachment frequency with increasing age in culture. Whereas young osteoblasts (both normal and osteoporotic) exhibited a high attachment frequency of 80–90%, late passage senescent cultures had an attachment frequency of only 45–55%.

Characteristics of short-term growth of normal osteoblasts were also examined at various points during the entire lifespan (Fig. 4). A clear age-related decline in the growth rate of osteoblasts was seen. In early passages (Fig. 4a, b) there was a short lag period followed by an exponential phase during which the cell number increased rapidly before slowing down and reaching a plateau. In late passages (Fig. 4c, d), the increase in cell number after the lag period was significantly retarded and the number of cells at confluence was dramatically reduced.

The slowing down of growth during aging was further confirmed by determining the proportion of cells undergoing the cell cycle, using [^{3}H]thymidine labelling and autoradiography. The number of S-phase cells was counted in at least 500 young osteoblasts (40% lifespan completed) and 400 old osteoblasts (80% lifespan completed). In young osteoblast cultures almost all cells (90–95%) entered S-phase during 72 h of labelling, whereas in old osteoblast cultures less than 15% of cells entered S-phase during 72 h. Similarly, the proportion of osteoblasts showing senescence-specific β-galactosidase staining also increased progressively from less than 5% in early passage cultures (total number of cells counted was 1600) to more than 95% (total number of cells counted was 1200) in late passage cultures (not illustrated).

Morphological Characteristics

Throughout the lifespan of normal and osteoporotic osteoblasts, cell cultures were examined daily under an inverted microscope and at various passage levels both as live cultures and after Giemsa staining. Normal

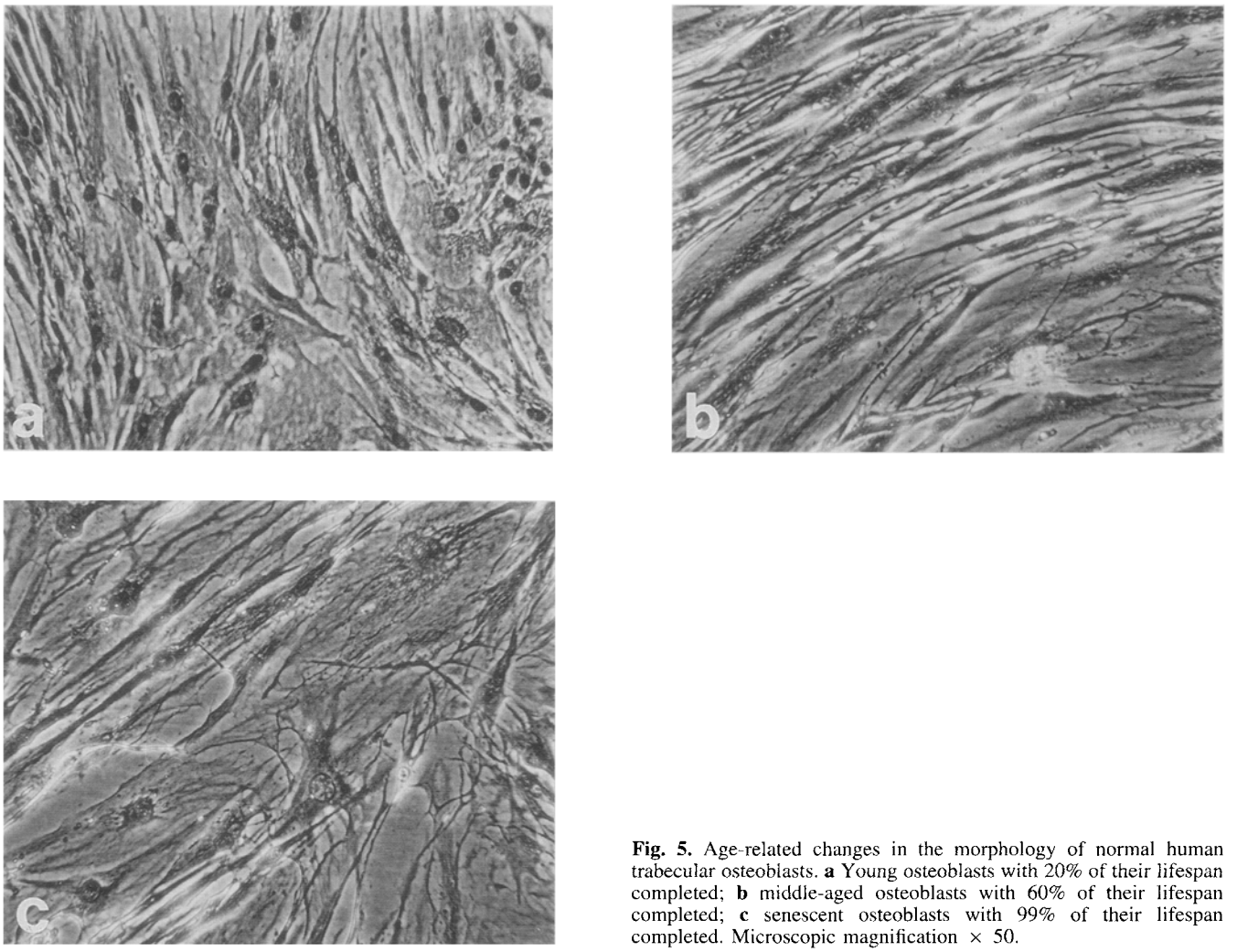


Fig. 5. Age-related changes in the morphology of normal human trabecular osteoblasts. **a** Young osteoblasts with 20% of their lifespan completed; **b** middle-aged osteoblasts with 60% of their lifespan completed; **c** senescent osteoblasts with 99% of their lifespan completed. Microscopic magnification × 50.

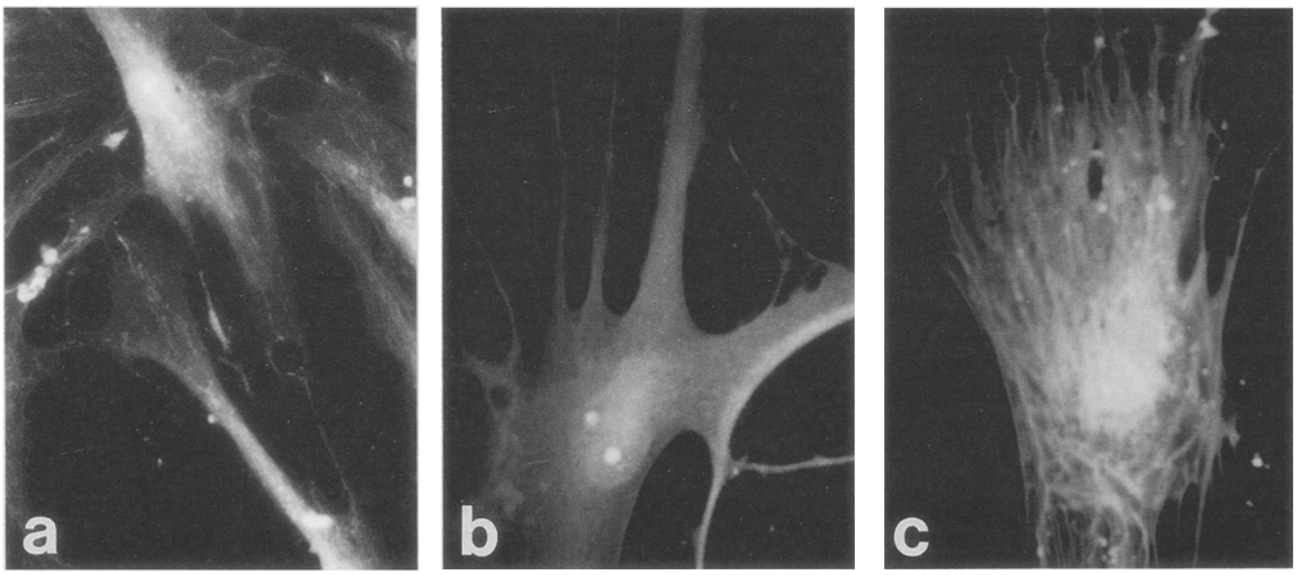


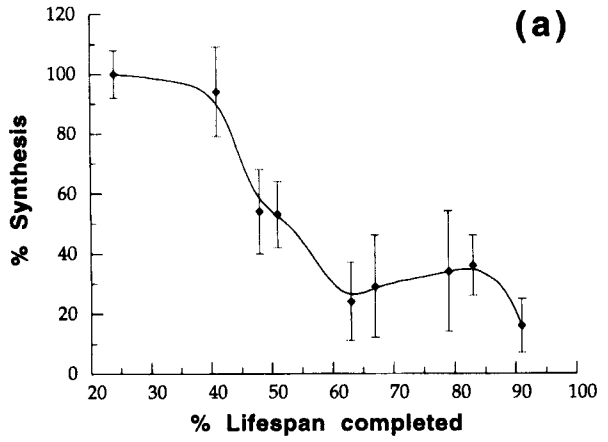
Fig. 6. Actin filaments of normal and osteoporotic osteoblasts visualized by fluorescent staining with FITC-conjugated phalloidin. **a** Young normal osteoblasts with 20% of their lifespan completed; **b** senescent normal osteoblast with 99% of their lifespan completed; **c** osteoporotic osteoblasts with less than 50% of their lifespan completed. Microscopic magnification × 200.

osteoblasts showed extensive morphological changes during cellular aging (Fig. 5). Young cells (less than 20% lifespan completed) were thin and spindle-shaped and they formed parallel arrays or 'finger-print-like' patterns in a confluent monolayer (Fig. 5a). Middle-aged cells (50–60% lifespan completed) became larger and heterogeneous and started to accumulate debris in their cytoplasm (Fig. 5b). Old cells (more than 90% lifespan completed) were large, flattened, irregularly shaped and often had more than one nucleus (Fig. 5c). In comparison, the osteoporotic cells, even at low passage levels, were large, flat and had heterogeneous senescent morphology with numerous cytoplasmic extensions.

Actin filaments of normal and osteoporotic osteoblasts of various passages were stained with FITC-conjugated phalloidin and examined under UV light. Similar to the observations in fibroblasts [14], the actin filaments were more abundant in old osteoblasts as compared with young osteoblasts (Fig. 6a, b). The filaments formed a diffuse pattern throughout the entire cell in both young and old cells. In comparison, the osteoporotic cells had a 'broken-bone-like' pattern (Fig. 6c) which is due to the disruption of lamellipodia formation by actin filaments.

Macromolecular Characeristics

DNA synthesis in osteoblasts was assessed by the incorporation of [^{3}H]thymidine into nuclei. Figure 7 shows the extent of DNA synthesis as a function of lifespan completed. For the purpose of comparison, DNA synthesis by young cells (24% lifespan completed) is considered as 100%. Both 2 h pulse labelling (Fig. 7a) and 72 h continuous labelling (Fig. 7b) exhibited a substantial decline in the level of DNA synthesis during aging. Old osteoblasts synthesized only 40% of the amount of DNA during pulse labelling and only 15% of the amount of DNA during continuous labelling as



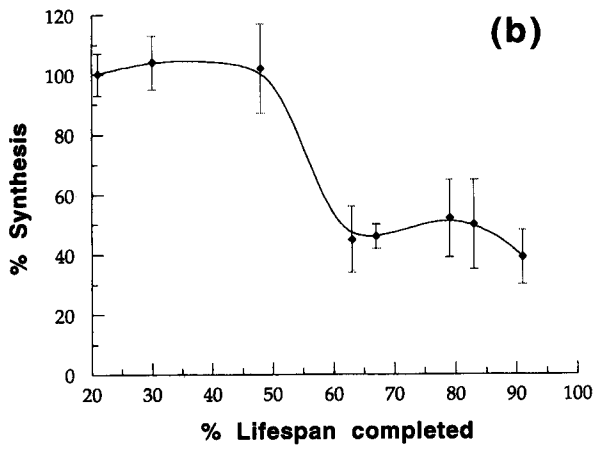
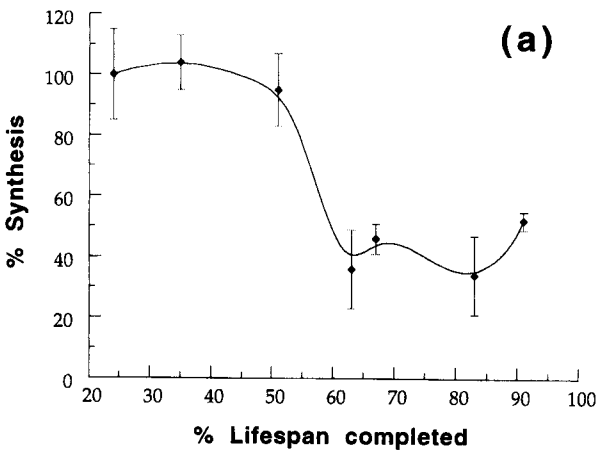


Fig. 7. DNA synthesis measured by [^{3}H]thymidine incorporation in TCA-insoluble material during the lifespan of normal osteoblasts. **a** Pulse labelling for 2 h; 100% synthesis corresponds to 7.6 × 10^4 c.p.m./10^6 cells. **b** Continuous labelling for 72 h; 100% synthesis corresponds to 9 × 10^5 c.p.m ./10^6 cells. Results represent the mean ± SEM of six independent measurements.



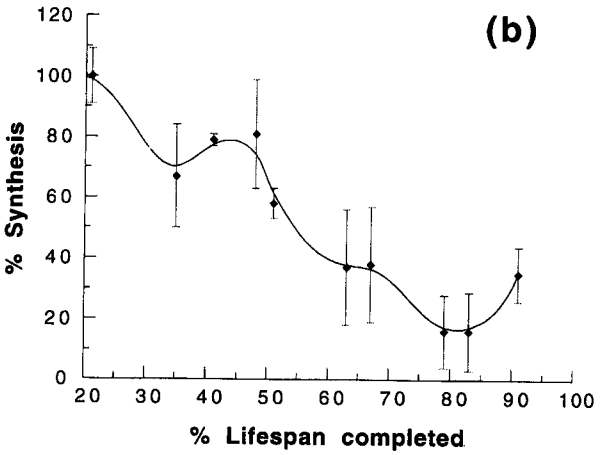


Fig. 8. Protein synthesis measured by [^{35}S]methionine incorporation in TCA-insoluble material during the lifespan of normal osteoblasts. **a** Pulse labelling for 2 h; 100% synthesis corresponds to 1.4 × 10^5 c.p.m./10^6 cells. **b** Continuous labelling for 72 h; 100% synthesis corresponds to 2.3 × 10^6 c.p.m./10^6 cells. Results represent the mean ± SEM of six independent measurements.

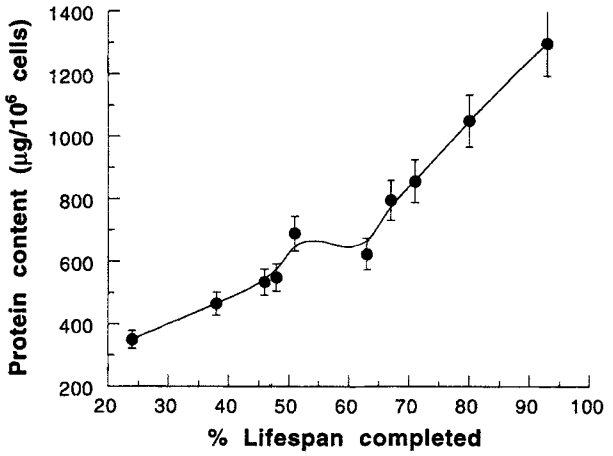


Fig. 9. Protein content ($\mu g/10^6$ cells) of normal osteoblasts as a function of percentage lifespan completed. Results represent the mean ± SEM of six independent measurements.

compared with young cells. This indicates that both the rate of DNA synthesis and the overall activity of DNA synthesis decreased with the cellular aging of human osteoblasts. Similarly, a 25% decline in the rate of RNA synthesis, as measured by [^{3}H]uridine incorporation, was observed in senescent osteoblasts as compared with young cells (data not shown).

Protein synthesis during aging also declined significantly (Fig. 8). The rate of protein synthesis, as determined by 2 h pulse labelling of cells with [^{35}S]methionine, declined by 60% in middle-aged cells as compared with young cells, with a slight or no further decline during the remaining lifespan (Fig. 8a). However, metabolic labelling of cells for 72 h showed that the overall protein synthetic activity of osteoblasts declined progressively during aging and reached the level of less than 20% in old cells as compared with young cells (Fig. 8b). Although protein synthesis declined during aging, protein content per cell showed a progressive increase during cellular aging (Fig. 9). In young cells the protein content was 350 $\mu g/10^6$ cells, which increased to 700 $\mu g/10^6$ cells in middle-aged cells and finally became 1250 $\mu g/10^6$ cells in old cells.

Markers of Osteoblast Phenotype

Levels of AP decreased rapidly during the first few passages, after which there was a low constant production of AP for the rest of the lifespan (Fig. 10a). Treatment of osteoblasts with 1,25-dihydroxyvitamin D_3 (calcitriol) increased the production of AP from 27 ± 3 to 49 ± 5 nmol PNP/min per 10^4 cells in young cultures. However, this stimulatory effect of calcitriol on AP production disappeared after about 30% of the lifespan was completed (Fig. 10a). A similar relationship between the levels of AP and percentage lifespan completed was observed in cells treated with calcitriol.

Osteoblastic production of PICP was estimated in the conditioned medium. A gradual decline in PICP

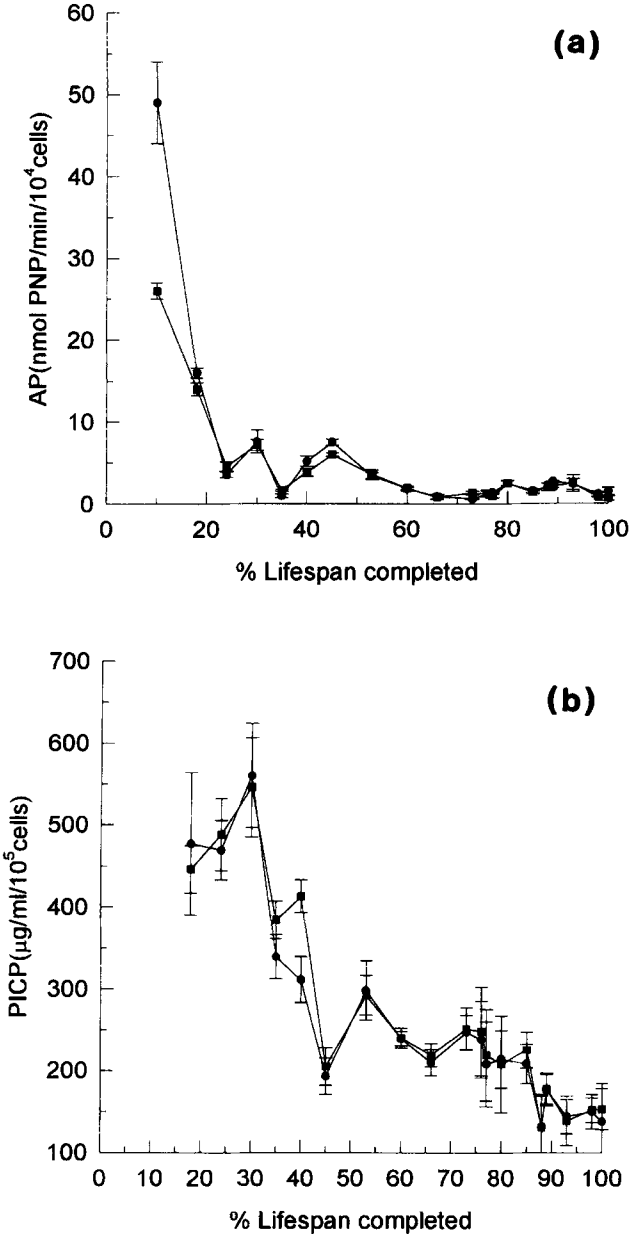


Fig. 10. Age-related changes in the production of alkaline phosphatase (AP) (**a**) and collagen type I (**b**) by human trabecular osteoblasts in vitro in the presence (*circles*) or absence (*squares*) of 10^9 M 1,25-dihydroxyvitamin D_3. AP was measured in the cell layer using *p*-nitrophenol phosphate as a substrate and collagen type I was estimated by measuring procollagen type I C-terminal propeptide (PICP) in the conditioned medium using a radioimmunoassay. Results represent the mean ± SEM of four independent measurements.

production with increasing age in culture was observed (Fig. 10b) and PICP production of senescent cells was 80% lower (150 ± 10 $\mu g/ml$ per 10^5 cells) than its production by young cells (510 ± 20 $\mu g/ml$ per 10^5 cells). Calcitriol did not cause any significant change in PICP production in young or old cells and a similar decline in PICP production was observed in calcitriol-treated cultures (Fig. 10b).

Discussion

In this study we have shown that normal human trabecular osteoblasts have limited proliferative capacity and a finite lifespan in culture. During a replicative lifespan of 30 to 35 population doublings, osteoblast cultures established from 7 healthy adult donors undergo a range of age-related cellular and biochemical changes which include the slowing down of growth and cell division, an increase in the number of non-cycling senescent cells, dramatic changes in morphology and cytoskeletal organization, and a decline in the synthesis of DNA, RNA and proteins. All these changes are considered to be the well-established characteristics of cellular aging in vitro [6,19,20]. While other cellular systems such as fibroblasts, endothelial cells, keratinocytes and lymphocytes have been utilized extensively to study the molecular mechanisms of cellular senescence and age-related pathology [20,21], our studies show that normal osteoblasts also undergo a similar process of cellular aging in culture.

Osteoblasts undergoing aging in vitro exhibit dramatic morphological and cytoskeletal changes which are comparable to those reported for fibroblasts [6,20,21]. However, the increased organization and bundling of actin filaments into rod-like structures known to occur in fibroblasts was not observed in senescent osteoblasts. Interestingly, osteoporotic osteoblasts had much higher levels of actin filaments which were spread throughout its multiple extensions of the cytoplasm. This alteration in actin organization may be a feature of impaired mobility [14].

We have also examined the β-galactosidase staining pattern of young and old osteoblast cultures as a marker of cellular aging. Recently, it has been demonstrated that senescent fibroblasts in vitro and in vivo exhibit increased β-galactosidase activity, which is considered a biomarker of aged cells [15]. We have observed a significant increase in the number of β-galactosidase positive cells during in vitro aging of osteoblasts. Although the functional role of increased β-galactosidase activity in old cells is not known, it may be related to increased lysosomal activities and altered cytosolic pH during aging [22].

An age-related decline in both the rate of synthesis and the total synthesis of DNA, RNA and proteins was observed in aging osteoblasts. These changes are similar to the ones reported for aging fibroblasts, keratinocytes and other cell types [23]. Although protein synthesis declined during aging, protein content per cell showed a three-fold increase. This increase in protein content during cellular aging is due to a decrease in the rate of protein degradation, which facilitates the accumulation of altered and abnormal proteins and possibly leads to cellular dysfunction and growth arrest [23,24].

Few previous studies have examined the effects of aging on osteoblast function in vivo and in vitro. Histomorphometric studies have shown that there is a decreased rate of bone formation with age as observed in bone biopsies of humans [25,26] and rats [27]. Although measurements of levels of AP [28] and type I collagen in human sera [29] showed an increase with age, this may reflect increased rate of bone turnover rather than the activity of individual osteoblasts, which may actually be decreased during aging [25–27]. In vitro studies have compared cellular characteristics of early passage cultures of osteoblasts obtained from young and old donors [8–11,30]. For example, it has been reported that the rate of synthesis of extracellular matrix proteins such as proteoglycans and collagen by cultured trabecular human osteoblasts was inversely related to donor age [10]. Similarly a donor-age-dependent decline in the expression of osteoblastic markers (AP, collagen type I and osteocalcin) during short-term growth of primary cultures of human osteoblasts has been reported [7,11].

Our studies on long-term serial passaging of human osteoblasts show that both total protein synthesis and osteoblast-specific type I collagen synthesis decrease with age in culture. Taken together, these studies indicate that the decline in the functional abilities of osteobalsts during cellular aging in vitro are comparable to the age-related changes observed in vivo. Furthermore, these studies also show that alterations in various structural, physiological, biochemical and molecular characteristics of osteoblasts during serial passaging in vitro are representative of cellular aging and senescence and not a process of terminal differentiation or dysdifferentiation [6,20,31].

One of the hallmarks of in vitro aging is decreased cellular responsiveness to hormones and growth factors, which underlies the failure of homeostasis during aging [20,21,31]. Calcitriol, among others, is a principal regulatory hormone of osteoblast functions and can exert multiple effects on osteoblast biology in vitro [32]. Similar to our previous findings [13,17], calcitriol induced a significant increase in AP production in young cultures but did not have any effects on the levels of PICP in the conditioned medium. We have found that the increase in AP production by calcitriol decreased rapidly during serial passaging. It is possible that these changes are due to down-regulation of calcitriol receptor expression or to changes in post-receptor pathways. It is also possible that under our culture conditions the rate of AP production was low due to the high rate of cellular proliferation [33] and that therefore small changes in AP production were not detectable.

In addition to our studies on normal osteoblast cultures, we have observed interesting differences between normal and osteoporotic osteoblasts. Although in this study only a single osteoporotic patient was studied and our results may be considered as preliminary, osteoblast cultures established from osteoporotic bone samples had a dramatically reduced capacity to divide and showed several features of accelerated senescence in vitro. These differences between the growth potential of normal and osteoporotic cells in vitro may be due to the presence of fewer osteoblasts in osteoporotic bone samples, so that these cells may already have undergone many more doublings during the establishment of primary cultures. On the other hand, the

observed accelerated senescence of osteoblasts may be a pathogenetic factor for the defective osteoblast functions observed in osteoporotic patients. Histomorphometric studies of iliac crest bone biopsies showed significantly reduced mean wall thickness in osteoporotic patients [34,35]. Also, cultured endosteal osteoblasts from patients with low-turnover osteoporosis exhibited decreased proliferative potential in short-term cultures [36]. The molecular mechanisms for osteoblast senescence and for the pathogenesis of osteoblast dysfunction in osteoporosis remain to be established by studying a larger number of osteoporotic patients. Furthermore, it also remains to be demonstrated whether there is a negative relationship between the age of the bone-biopsy donor and the maximum proliferative capacity during long-term cultivation in vitro, as observed for fibroblasts [6,20]. Finally, in spite of a limited number of bone samples analysed in this study, our results demonstrate that osteoblasts, like several other diploid cell types, undergo cellular aging and senescence during their limited proliferative lifespan in vitro, and that this model system can be extremely useful in order both to understand structural and functional alterations in the bone during aging and to develop new therapeutic strategies.

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