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Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells

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Abstract

Explant cultures of adult human trabecular bone fragments give rise to osteoblastic cells, that are known to express osteoblast-related genes and mineralize extracellular matrix. These osteoblastic cells have also been shown to undergo adipogenesis in vitro and chondrogenesis in vivo. Here we report the in vitro developmental potential of adult human osteoblastic cells (hOB) derived from explant cultures of collagenase-pretreated trabecular bone fragments. In addition to osteogenic and adipogenic differentiation, these cells are capable of chondrogenic differentiation in vitro in a manner similar to adult human bone marrow-derived mesenchymal progenitor cells. High-density pellet cultures of hOB maintained in chemically defined serum-free medium, supplemented with transforming growth factor-β1, were composed of morphologically distinct, chondrocyte-like cells expressing mRNA transcripts of collagen types II, IX and X, and aggrecan. The cells within the high-density pellet cultures were surrounded by a sulfated proteoglycan-rich extracellular matrix that immunostained for collagen type II and proteoglycan link protein. Osteogenic differentiation of hOB was verified by an increased number of alkaline phosphatase-positive cells, that expressed osteoblast-related transcripts such as alkaline phosphatase, collagen type I, osteopontin and osteocalcin, and formed mineralized matrix in monolayer cultures treated with ascorbate, β-glycerophosphate, and bone morphogenetic protein-2. Adipogenic differentiation of hOB was determined by the appearance of intracellular lipid droplets, and expression of adipocyte-specific genes, such as lipoprotein lipase and peroxisome proliferator-activated receptor γ2, in monolayer cultures treated with dexamethasone, indomethacin, insulin and 3-isobutyl-1-methylxanthine. Taken together, these results show that cells derived from collagenase-treated adult human trabecular bone fragments have the potential to differentiate into multiple mesenchymal lineages in vitro, indicating their developmental plasticity and suggesting their mesenchymal progenitor nature.

Keywords: Trabecular bone; Osteoblasts; Mesenchymal progenitors; Chondrogenesis; Osteogenesis; Adipogenesis

Introduction

Cultures of collagenase-treated adult human trabecular bone fragments are considered to be a reliable source of adult human osteoblastic cells (hOB) [20,38, 39,52,53] that can form in vitro a mineralized extracellular matrix, increase intracellular cAMP in response to parathyroid hormone, and express several osteoblast-related transcripts such as alkaline phosphatase (ALP), collagen type I (Col I), osteopontin (OP), osteonectin (ON), and osteocalcin (OC), which can be further elevated in response to 1α,25-dihydroxyvitamin D_3 [18,39,41,51–54]. During preparation of hOB explant cultures, collagenase pretreatment of trabecular bone fragments has been shown to effectively remove soft tissue components associated with bone surfaces, such as the periosteum and bone marrow, that may contain variable fractions of heterogeneous cells depending on the nature of the starting material (gender, donor age and site, amount of red versus yellow marrow, etc.)

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[39,52,53]. When these pretreated bone fragments are cultured as explants in low-calcium growth medium, cells that are surrounded by mineralized matrix and protected from collagenase treatment are subsequently able to migrate from the bone fragments and begin to proliferate [39,52,53]. While the origin of the hOB is still unclear, they have been proposed to represent osteocytes that have become liberated from their confinement and have once again become mitotic [52]. Some vasculature-associated cells such as pericytes, which have been reported to be able to differentiate into osteoblastic cells, may also contribute to the original cell population emerging from the bone fragments [9,49,52,55]. Yet other observations suggest that a proportion of the cells present on the surface of the bone fragments may be closely related to clonogenic, multipotential precursors present in the bone marrow, based on the detection of a STRO-1 antigen expressing subpopulation of cells in primary cultures of hOB [20]. Irrespective of the precise cellular origin of these hOB, they have served as a highly useful system to study osteoblast biology, including matrix biosynthesis, cell differentiation and maturation, response to various growth factors and hormones, and cell–matrix and cell–biomaterial interactions [18,39,41, 51–54,57–60].

The emerging discipline of tissue engineering and a need for a source of adult cells, which under controlled conditions can develop into distinct phenotypes, provided new impetus for evaluating the developmental potential of various adult cell types. Given that the cells derived from trabecular bone have been shown to form new bone and cartilage in vivo [19] and undergo adipogenesis in vitro [43], we have tested the hypothesis herein that mature trabecular bone harbors cells that display mesenchymal progenitor characteristics. This was prompted by a number of considerations. A key issue in the biological significance of mesenchymal progenitor cells has been their potential role as the contributing cells in tissue repair and wound healing. In this respect, it is noteworthy that bone tissues, by virtue of their weight-bearing role, constantly undergo precisely controlled remodeling. Fracture repair in bone generally involves an endochondral sequence, progressing from mesenchymal tissue to bone via a cartilage intermediate [17,70]. Also, ectopic bone induction, such as that resulting from implantation of demineralized bone matrix and/or its active ingredients, the bone morphogenetic proteins, could proceed in the absence of active marrow activity [48,68]. Finally, our previous studies have shown that the embryonic calvaria, an intramembranous bone that forms without a cartilage intermediate, contains chondroprogenitor cells that are able to differentiate into chondrocytes under certain conditions in vivo and in vitro [24–26,63].

In this study we have investigated the in vitro potential of adult trabecular bone-derived hOB to differentiate into chondrogenic as well as osteogenic and adipogenic lineages. For the sake of comparison, we have employed culture methods similar to those previously described for adult human bone marrow-derived mesenchymal progenitor cells [47].

Materials and methods

Preparation of collagenase-treated trabecular bone explant cultures

All chemicals were purchased from Sigma Chemicals (St. Louis, MO, US) unless otherwise stated. Trabecular bone fragments were obtained from the femoral head of patients (two females aged 42 and 58 years, and two males aged 47 and 54 years) undergoing total hip arthroplasty, with approval by the Institutional Review Board. None of the patients had a history of osteoporosis or avascular necrosis. Explant cultures were prepared based on a protocol first described by Robey and Termine [53] and modified by Sinha et al. [59]. Trabecular bone fragments were harvested using a bone curet, transferred to glass vials containing DMEM/F-12K (Speciality Media, Phillipsburg, NJ, US) supplemented with antibiotics (50 I.U. penicillin/ml, 50 μg streptomycin/ml, Cellgro, Herndon, VA, US), minced extensively with surgical scissors and washed repeatedly with DMEM/F-12K. Bone fragments were next transferred to a spinner flask containing DMEM/F-12K supplemented with 2 mM L-glutamine, 50 μg/ml ascorbate, 256 U/ml collagenase type XI and antibiotics, and incubated at 37 °C for 3–4 h in a humidified 95% air–5% CO_2 atmosphere until the cellular material on the bone surface disappeared, as assessed by light microscopy. Following extensive rinsing with 0.9% sodium chloride (Baxter, Deerfield, IL, US), bone fragments were then plated in tissue culture flasks containing calcium-free DMEM/F12-K supplemented with 10% fetal bovine serum (FBS, Premium Select, Atlanta Biologicals, Atlanta, GA, US), 2 mM L-glutamine, 50 μg/ml ascorbate and antibiotics. Explant cultures were maintained at 37 °C in a humidified 95% air–5% CO_2 atmosphere with the medium changed every 3–4 days. When the cells growing out the explants reached 70–80% confluence (after approximately 3–4 weeks), they were detached from the bottom of tissue culture flasks with 0.25% trypsin containing 1 mM EDTA (Gibco BRL, Life Technologies, Grand Island, NY, US), counted in a hemocytometer and plated as high-density pellet cultures or monolayers.

Chondrogenic differentiation of high-density pellet cultures

For chondrogenic differentiation, cells were plated as high-density pellet cultures in a chemically defined, serum-free DMEM (BioWhittaker, Walkersville, MD, US) as described previously [28,37,47,69]. Aliquots of 2×10^5 cells in 0.5 ml medium were pelleted by centrifugation at 500*g* for 5 min in 15 ml conical polypropylene tubes and the resulting cell pellets were supplemented with 10 ng/ml transforming growth factor-β1 (TGF-β1; R&D, Minneapolis, MN, US) to stimulate chondrogenic differentiation of the cells. Control cultures were maintained in a chemically defined, serum-free medium without TGF-β1. High-density pellet cultures were maintained for 3 weeks at 37 °C in a humidified 95% air–5% CO_2 atmosphere. The medium was changed every 3–4 days with TGF-β1 added fresh to the appropriate culture.

Osteogenic and adipogenic differentiation of monolayer cultures

For osteogenic and adipogenic differentiation, cells at the density of 1.5×10^5 cells/ml DMEM/F-12K (osteogenic differentiation) or DMEM (adipogenic differentiation), supplemented with 10% FBS and antibiotics, were plated in two-well chamber slides (Nalge Nunc, Naperville, IL, US) and grown to confluence. Osteogenic differentiation of confluent monolayer cultures was then induced with 50 μg/ml ascorbate, 10 mM β-glycerophosphate and 30 ng/ml human recombinant bone morphogenetic protein-2 (BMP-2; kindly provided by Genetics Institute, Cambridge, MA, US) [31], whereas adipogenic differentiation was induced with 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μg/ml insulin and 100 μM indomethacin

Table 1
PCR primer sets for amplification of lineage-specific genes and the length of amplified products

Gene	Primer sequence: sense/antisense	Product size (bp)	Reference
Osteogenic markers			
Col IA2	5′-GGACACAATGGATTGCAAGG-3′ 5′-TAACCACTGCTCCACTCTGG-3′	461	[36]
ALP	5′-TGGAGCTTCAGAAGCTCAACACCA-3′ 5′-ATCTCGTTGTCTGAGTACCAGTCC-3′	453	[47]
OP	5′-ACGCCGACCAAGGAAAACTC-3′ 5′-GTCCATAAACCACACTATCACCTCG-3′	483	Gene Bank Access No. BC 007016
OC	5′-ATGAGAGCCCTCACACTCCTC-3′ 5′-GCCGTAGAAGCGCCGATAGGC-3′	297	[36]
Adipogenic markers			
LPL	5′-GAGATTTCTCTGTATGGCACC-3′ 5′-CTGCAAATGAGACACTTTCTC-3′	276	[50]
PPARγ2	5′-GCTGTTATGGGTGAAACTCTG-3′ 5′-ATAAGGTGGAGATGCAGGCTC-3′	352	[47]
Chondrogenic markers			
Col II	5′-TTTCCCAGGTCAAGATGGTC-3′ 5′-CTTCAGCACCTGTCTCACCA-3′	377	[47]
Col IX	5′-GGGAAAATGAAGACCTGCTGG-3′ 5′-CGAAAAGGCTGCTGTTTGGAGAC-3′	516	Gene Bank Access No. NM 001851
Col X	5′-GCCCAAGAGGTGCCCCTGGAATAC-3′ 5′-CCTGAGAAAGAGGAGTGGACATAC-3′	703	[28]
AGN	5′-TGAGGAGGGCTGGAACAAGTACC-3′ 5′-GGAGGTGGTAATTGCAGGGAACA-3′	350	Gene Bank Access No. NM 001135
Internal control			
GAPDH	5′-GGGCTGCTTTTAACTCTGGT-3′ 5′-TGGCAGGTTTTTCTAGACGG-3′	702	[36]

[47]. Control cultures were grown without osteogenic or adipogenic supplements. Osteogenic and adipogenic stimulation was carried out for 4 and 2 weeks, respectively, with the media changed every 3–4 days and supplements added fresh to each culture.

Histological, histochemical and immunohistochemical analysis

Chondrogenic high-density pellet cultures were rinsed with phosphate buffered saline (PBS), fixed in 2% paraformaldehyde, dehydrated in ethanol, infiltrated with isoamyl alcohol and embedded in paraffin. Sections of 8 μm thickness were obtained through the center of each pellet and mounted on microscope slides. The sections were then stained with haematoxylin-eosin, Alcian blue or picro-Sirius red as described previously [13,14,21]. For collagen type II (Col II) or link protein (LP) detection, sections were pre-digested for 15 min at 37 °C with 300 U/ml hyaluronidase or 1.5 U/ml chondroitinase ABC, respectively. Sections were then incubated with the monoclonal antibodies, II-II6B3 (15 μg/ml PBS) specific to Col II or 8-A-4 (6 μg/ml PBS) specific to LP (Developmental Studies Hybridoma Bank, Iowa City, IA, US), for 1 h at 37 °C or overnight at 4 °C, respectively. Immunostaining was detected colorimetrically using Histostain-SP Kit for DAB (Zymed Laboratories Inc., San Francisco, CA, US). Osteogenic monolayer cultures were stained histochemically for ALP (Sigma Cat. No. 86-C) according to the manufacturer's protocol and for matrix mineralization using Alizarin red S as described previously [8]. Adipogenic monolayer cultures were stained histochemically for intracellular lipid droplets with Oil red O as described previously [47].

RNA isolation and RT-PCR analysis of gene expression

Total cellular RNA was extracted with Trizol reagent (Gibco BRL, Life Technologies, Grand Island, NY, US). For efficient RNA extraction from high-density pellet cultures, they were first briefly homogenized in Trizol reagent. The isolated RNA samples were converted to cDNA using random hexamers and Superscript II RNase H-Reverse Transcriptase (SuperScript First-Strand Synthesis System, Gibco BRL, Life Technologies, Grand Island, NY, US), and then amplified by PCR using AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT, US) and gene-specific primer sets listed in Table 1. Expression of the following genes was examined: collagen type I (*Col IA2*), alkaline phosphatase (*ALP*), osteopontin (*OP*), osteocalcin (*OC*), lipoprotein lipase (*LPL*), peroxisome proliferator-activated receptor γ2 (*PPARγ2*), collagen type II (*Col II*), collagen type IX (*Col IX*), collagen type X (*Col X*), and aggrecan (*AGN*). Amplifications were performed for 34 (*OC*) or 32 (all other genes) cycles consisting of 1 min denaturation at 95 °C, 1 min annealing at 60 °C (*OC*), 57 °C (*Col II, IX, X, AGN*), or 51 °C (all other genes) and 1 min extension at 72 °C, with the initial denaturation at 95 °C for 1 min and final incubation at 72 °C for 10 min. In all RT-PCR assays, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was analyzed to monitor RNA loading. RT-PCR products were analyzed by electrophoresis in 2% MetaPhor agarose gel (FMC Corp., Rockland, ME, US) containing ethidium bromide.

Results

Morphological observation of collagenase-treated trabecular bone explant cultures

After 3–4 h of collagenase treatment, the surface of the bone fragments appeared devoid of cellular material and soft tissue components as observed by light microscopy (Fig. 1A). When these bone fragments were plated in low calcium DMEM/F-12K medium, cells appeared migrating from the explants after approximately 10–14 days (Fig. 1B). With continued incubation in low calcium DMEM/F-12K medium, the cells pro-

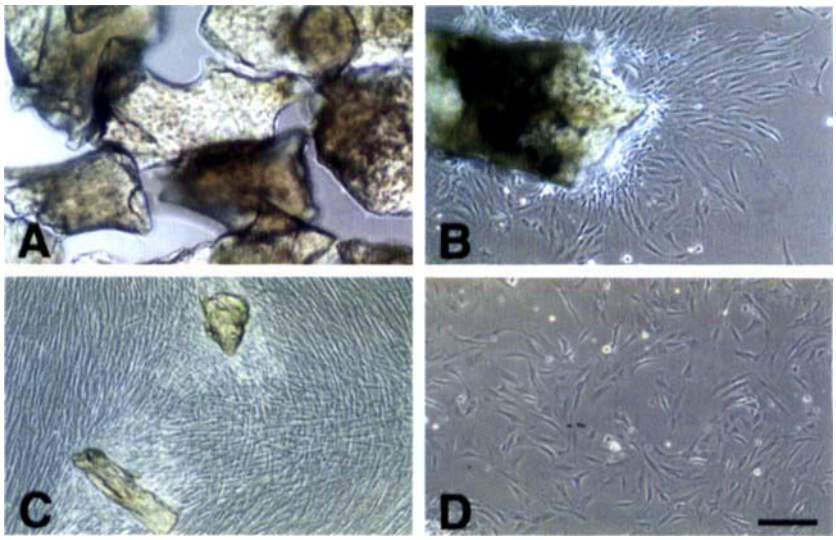

Fig. 1. Phase contrast photomicrographs of typical adult human trabecular bone explant cultures. (A) Appearance of adult human trabecular bone fragments after collagenase treatment. (B) hOB cells migrating from the bone fragments after approximately 10–14 days of explant culture. (C) Confluent monolayer of hOB cells after approximately 3–4 weeks of explant culture. (D) Appearance of hOB cells at the first passage. Bar = 200 μm.

liferated and formed a confluent monolayer after approximately 21–28 days (Fig. 1C). The cells appeared as a homogeneous fibroblastic cell population with mitotic figures (Fig. 1D). No differences in growth characteristics or cell morphology were noted among the different patient samples.

Histological and immunohistochemical examination of chondrogenic cultures

All high-density pellet cultures, formed by centrifugation, detached spontaneously from the bottom of polypropylene conical tubes within 24 h and were further cultured in suspension in a chemically defined, serum-free medium with or without TGF-β1. Over the 3 week culture period, pellet cultures treated with TGF-β1 increased in size, while omission of TGF-β1 prevented any size increase of the pellets (compare size in Fig. 2). Haematoxylin–eosin stained sections of 3 week TGF-β1-treated pellets showed morphologically distinct, chondrocyte-like cells embedded in abundant extracellular matrix (Fig. 2B and C). Alcian blue staining of these sections revealed the presence of a sulfated, proteoglycan-rich extracellular matrix (Fig. 2E and F), while picro-Sirius red staining showed prominent birefringent fibers present in the matrix and surrounding the cells (Fig. 2H and I). Cells within untreated pellets did not display chondrocyte-like morphology (Fig. 2A) or elaborate a proteoglycan-rich extracellular matrix (Fig. 2D), and no significant birefringent fibers in the matrix were detected (Fig. 2G). Also, only sections of TGF-β1-treated pellets immunostained for Col II (Fig. 2K and L) and LP (Fig. 2N and O) in the extracellular matrix, while neither Col II nor LP were detected in sections of untreated pellets (Fig. 2J and M). The cells from all tested donors responded similarly during chondrogenic high-density pellet cultures.

Histological and histochemical examination of osteogenic and adipogenic cultures

Confluent monolayer cultures treated for 10 days with the osteogenic supplements, ascorbate, β-glycerophosphate and BMP-2, showed a marked increase of ALP-positive cells (Fig. 3B) as compared to control cultures grown without osteogenic supplements (Fig. 3A). In cultures maintained for longer times, cells treated with osteogenic supplements began to produce mineralized matrix as observed by phase contrast microscopy and further confirmed by Alizarin red S staining (4 week treated cultures, Fig. 3D), while control cultures did not mineralize (Fig. 3C). Confluent monolayer cultures treated with the adipogenic supplements dexamethasone, IBMX, insulin and indomethacin showed the first adipocytic cells containing intracellular lipid droplets as early as treatment day 3, as observed by phase contrast microscopy and further confirmed by Oil red O staining (2 week treated cultures, Fig. 3F). Control cultures grown without adipogenic supplements showed no formation of adipocytic cells containing intracellular lipid droplets (Fig. 3E). The cells from all tested donors responded similarly in osteogenic and adipogenic culture conditions.

Expression of lineage-specific genes in chondrogenic, osteogenic and adipogenic cultures

Pre-confluent monolayer cultures of cells migrating from trabecular bone fragments served as a control

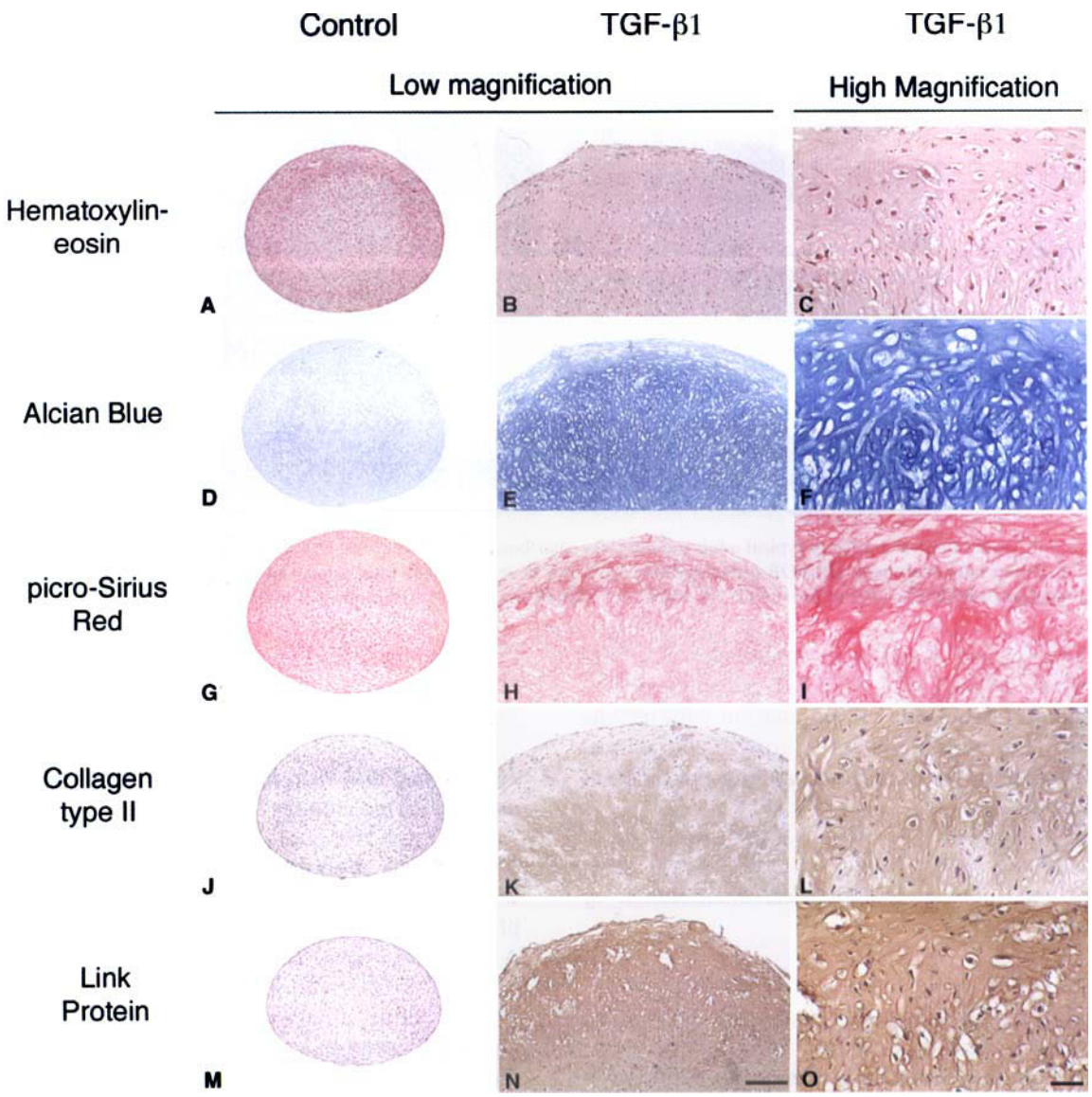

Fig. 2. Histological and immunohistochemical analyses of chondrogenic hOB cell pellet cultures. Left and central panel: Sections of cell pellets cultured without and with TGF-β1, respectively. Bar = 200 μm. Right panel: High magnification of sections of TGF-β1-treated cell pellets. Bar = 50 μm. From top to bottom: Sections were stained for haematoxylin/eosin (A–C), Alcian blue (D–F), picro-Sirius red (G–I), Col II (J–L) and link protein (M–O). Compared to untreated pellet cultures, TGF-β1-treated pellets increased substantially in size (compare size in left and central panel). The extracellular matrix of TGF-β1-treated pellets was rich in sulfated proteoglycans (D–F), birefringent fibers (G–I), specific cartilaginous matrix component such as Col II (J–L), and link protein (M–O).

population for gene expression analysis. These primary cells cultured without differentiation-stimulating agents showed the expression of *Col I* mRNA, but not other osteoblast-related genes such as *ALP*, *OP*, and *OC*. Expression of the adipocyte-specific genes, *LPL* and *PPARγ2*, or the chondrocyte-associated genes, *Col II*, *Col IX*, *Col X*, and *AGN*, was also not detected (Fig. 4, control). In contrast, cells cultured as monolayers and treated for 3 weeks with osteogenic supplements expressed *ALP*, *Col I*, *OP*, and *OC* genes, indicating osteogenic differentiation. Interestingly, these cells also expressed the *AGN* gene, but did not express other chondrocyte-associated or adipocyte-specific genes (Fig. 4, osteogenic). On the other hand, cells cultured as monolayers and treated for 2 weeks with adipogenic supplements expressed *LPL* and *PPARγ2* genes, indicative of adipogenic differentiation. These cells also expressed osteoblast-related genes *ALP*, *Col I*, and *OP*, but not *OC* or chondrocyte-associated genes (Fig. 4, adipogenic). Finally, cells grown as chondrogenic high-density pellet cultures for 3 weeks in chemically defined, serum-free medium supplemented with TGF-β1 expressed the chondrocyte-associated genes *Col II*, *IX*, *X* and *AGN*. These cells also showed expression of *Col I*

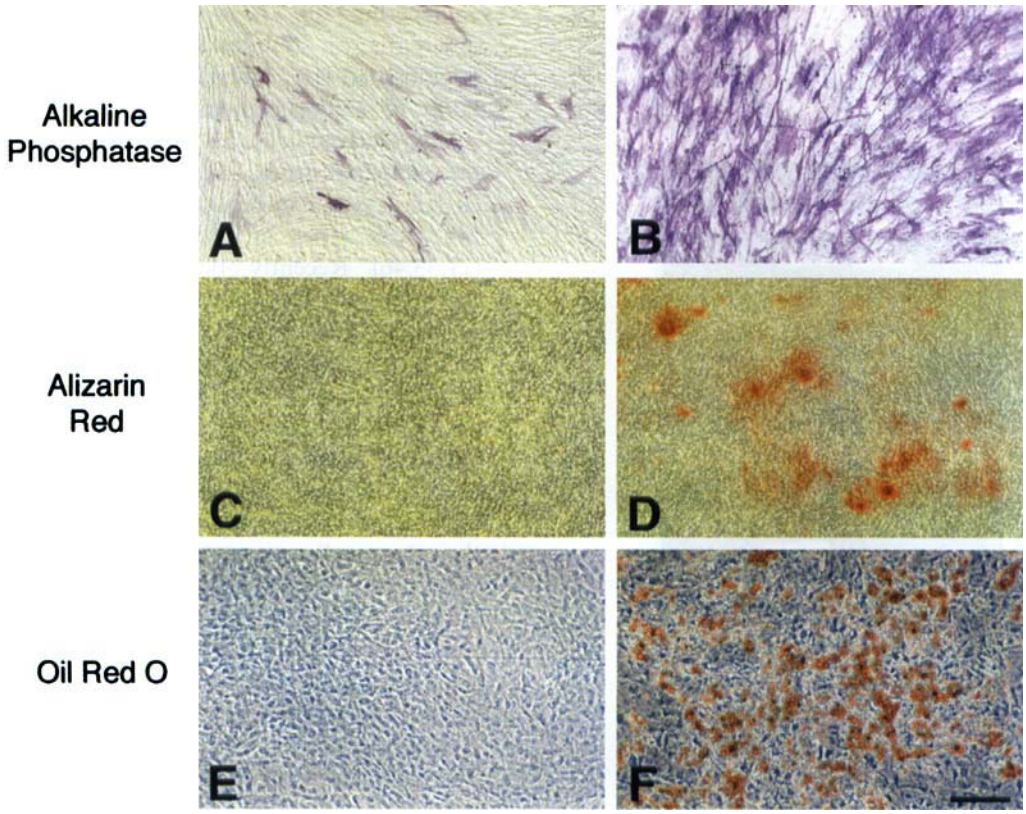

Fig. 3. Histological and histochemical analysis of osteogenic and adipogenic hOB cell monolayer cultures. hOB cells were cultured without and with differentiation-stimulating supplements (left and right panel, respectively). From top to bottom: Alkaline phosphatase (A, B), Alizarin red S (C, D), and Oil red O (E, F) staining. Cell cultures treated with osteogenic supplements showed an increased number of ALP-positive cells (B), and produced a mineralized extracellular matrix (D). Treatment of hOB cells with adipogenic supplements resulted in the formation of adipocytic cells containing intracellular lipid droplets (F). In untreated cell cultures, there was an absence of such phenotypes (A, C, E). Bar = 200 μm.

and *OP* genes, but not *ALP* or *OC*, or the adipocyte-specific genes, *LPL* and *PPARγ2* (Fig. 4, chondrogenic). The gene expression pattern in control and differentiation-stimulating conditions was identical for all tested donor cell populations.

Discussion

In this study, we have investigated the developmental potential of cells derived from adult human femoral trabecular bone, namely their ability to differentiate in vitro into cell types representative of chondrogenic, osteogenic and adipogenic lineages. Our results showed that cells derived from collagenase-treated trabecular bone fragments differentiated in vitro into these three examined mesenchymal lineages when cultured in defined conditions similar to those previously described for adult human bone marrow-derived mesenchymal stem cells (hMSC) [47].

A number of cell culture models are currently in use for the study of adult human primary osteoblasts, including osteoblast precursor cells originating from bone marrow [12,27,50], cells of the osteoblast lineage derived from explants of adult human trabecular bone [5] and collagenase-pretreated trabecular bone fragments [53]. The last method has been claimed to yield a more homogeneous osteoblastic cell population based on the observation that collagenase digestion of trabecular bone fragments efficiently removes connective tissue components so that cells are eventually derived only from those within the osteoid matrix [52]. When these collagenase-pretreated trabecular bone fragments were further plated in a low calcium medium to facilitate matrix dissolution, we observed that cell proliferation was first evident in close proximity to the surfaces of the explants and only after approximately 2 weeks of culture, consistent with the observation of others [39,52,53].

Interestingly, the predominant cell type in our explant cultures had an elongated, fibroblast-like morphology and, either in initial or post-confluent cultures, did not spontaneously acquire a more polygonal morphology, considered by some investigators to reflect a more "mature" osteoblast-like phenotype [60]. RT-PCR analysis of pre-confluent hOB cells that had just migrated from the bone fragments showed that these cells expressed only *Col I*, but not other osteoblast-related genes. Moreover, in further differentiation assays carried

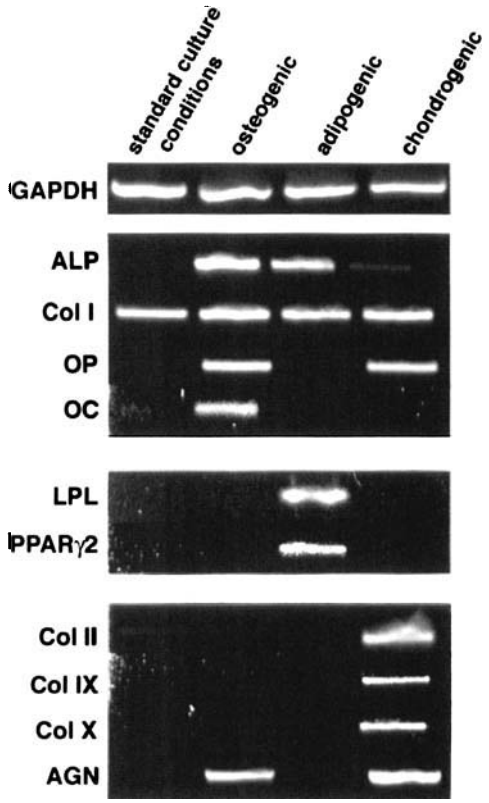

Fig. 4. RT-PCR analysis of the mRNA expression of lineage-specific genes in osteogenic, adipogenic and chondrogenic cultures. Pre-confluent monolayer cultures of hOB cells migrating from trabecular bone fragments served as a control population for gene expression analysis. Under osteogenic conditions, the hOB cells expressed osteoblast-related genes (*ALP*, *Col I*, *OP*, *OC*), and *AGN*. The cells treated with adipogenic supplements expressed adipocyte-specific genes (*LPL* and *PPARγ2*), and also *ALP* and *Col I*. Chondrogenic cell pellet cultures treated with TGF-β1 expressed cartilage-specific genes (*Col II*, *IX*, *X*, *AGN*), and osteoblast-related *Col I* and *OP*. Untreated cells (control) expressed only *Col I*, consistent with a fibroblast-like phenotype. The expression of *GAPDH* was analyzed as a control for the RNA loading.

out on first passage confluent monolayers of hOB cells, control cultures stained weakly for ALP, the most widely used biochemical marker of osteoblasts. These results suggest that the cells that migrated from collagenase-pretreated trabecular bone fragments, when cultured in standard culture conditions, displayed an undifferentiated and/or dedifferentiated cell phenotype.

To assess the in vitro developmental potential of hOB, we used a similar approach to that previously described for adult hMSC [47]. The multilineage differentiation potential of adult hMSC has been well established [30,34,42,47,67]. These cells, when cultured as high-density pellet cultures in a serum-free chemically defined medium containing dexamethasone, ascorbate, sodium pyruvate, proline and TGF-β1, have been shown to develop a chondrocyte-like phenotype [28,37,69]. This observation has opened the possibility of using these cells for the reconstruction of cartilage defects in tissue engineering [70]. The in vitro osteogenic and adipogenic differentiation abilities of hMSC have also been well documented [10,27,40,47]. However, there is a growing body of evidence suggesting that not only marrow stroma-derived cells, but also more defined cell types of mesenchymal origin, such as adipocytes, myoblasts and chondrocytes, can differentiate or transdifferentiate into other cell types in addition to their default lineage [11,33,46]. Recently, periosteally derived cells, which attain an osteoblast-like phenotype in culture, have been shown to differentiate into chondrocytes when further cultured in suspension in agarose gels [4]. Interestingly, Gundle et al. [19] have shown that osteoblastic cells derived from trabecular bone fragments, when implanted subcutaneously in diffusion chambers and continuously treated with osteo-inductive agents, give rise to mineralized tissue containing cartilage. It has also been shown that these cells can attain adipocytic as well as osteoblastic phenotypes in culture, and these results were verified by clonal population analysis [43]. However, these latter studies utilized an explant culture method that did not include collagenase pretreatment of trabecular bone fragments, and likely involved cells of heterogeneous origins. This prompted us to investigate the in vitro differentiation potential of a more homogeneous population of hOB derived from collagenase-treated adult human trabecular bone fragments.

In our study, we have shown that chondrogenic differentiation of hOB can be achieved in high-density pellet cultures in a serum-free, chemically defined medium containing TGF-β1. The size of TGF-β1 treated pellets increased over the 3 week culture period and, as previously shown for adult hMSC [28,37,69], this effect appeared almost entirely due to the deposition of extracellular matrix rather than to continued cell division, as evidenced by histochemical and immunohistochemical analysis. Furthermore, RT-PCR analysis revealed the expression of *Col II*, *IX*, *X* and *AGN* transcripts, characteristic of the chondrocyte phenotype. It is noteworthy that the expression of *Col X* was upregulated in the TGF-β1 treated hOB pellet cultures. The significance of *Col X* transcription at the early phase of chondrogenic differentiation is unclear, since Col X is generally considered a component of mature hypertrophic cartilage [11,23,64]. This may indicate that at this stage of culture, the hOB cells were in a transitional state, expressing transcripts characteristic of both osteoblastic and chondrocytic lineages [29]. It is noteworthy that Yoo et al. [69] also detected by immunostaining, as early as culture day 5, Col X associated with the cell surface of hMSC maintained under similar chondrogenic conditions.

In monolayer culture, osteoblastic differentiation involves a programmed developmental sequence, which is characterized by an early proliferative stage, followed by extracellular matrix development and maturation, and

matrix mineralization. During this process, ALP expression and activity progressively increase, then decrease when mineralization progresses. The cells also upregulate expression of several osteoblast-related genes such as *Col I*, *OP*, and *OC* [2,61]. In our study, hOB cultured in the presence of osteogenic supplements, ascorbate, β-glycerophosphate and BMP-2 showed an increased number of ALP-positive cells, expressed *ALP*, *Col I*, *OP* and *OC* transcripts and formed a mineralized matrix, all characteristic of the osteoblastic phenotype. Although many cell culture models employ dexamethasone as an osteo-inductive agent ([66], for review see [3,20]), the usage of BMP-2 seems to be more appropriate, since the osteo-inductive effect of BMP-2, in contrast to glucocorticoids, can be achieved both in vitro and in vivo [16]. The osteo-inductive effect of BMP-2 on human osteoblasts and human bone marrow stromal cell cultures has been reported [31]. Notably, in our osteogenic cultures, the hOB cells also expressed AGN, a proteoglycan core protein expressed predominantly in cartilaginous tissues [22]. The role of AGN in osteoblastic differentiation has not been investigated, although its expression has been found at low levels in ROS17/2.8 osteosarcoma cells and in intramembranous bone of the chick embryo [35,65]. Perhaps AGN functions as other small proteoglycans, such as decorin, in the mineralization process by binding to and regulating the fibril length of collagen [56]. That expression of decorin is selectively stimulated by BMP-2 in human osteoblasts and human bone marrow stromal cell cultures suggests, although indirectly, a similar mechanism for BMP-2 action on *AGN* gene expression in our culture system [31].

Furthermore, our results also showed that treatment of hOB monolayer cultures for 2 weeks with the adipogenic supplements, dexamethasone, IBMX, insulin and indomethacin, resulted in their conversion to adipocytes, as evidenced by the appearance of cells containing intracellular lipid droplets and gene expression of *LPL* and *PPARγ2*. These results are consistent with the known characteristics of the adipogenic differentiation pathway, that is not only accompanied by changes in cellular morphology and the formation of cytoplasmic lipid droplets, but also by transcriptional activation of many genes [1,32,43,47,62]. Interestingly, the hOB cultures treated with adipogenic supplements also showed *ALP* gene expression. However, adipocytes have been shown to express *ALP* [6,7,15,44]. Alternatively, only approximately 30–40% of the hOB cells in our adipogenic cultures become adipocytes, as evidenced by Oil red O staining of cytoplasmic lipid droplets and therefore the Oil red O negative cells could account for the detection of *ALP* by RT-PCR.

In our follow-up studies, immortalized primary hOB cells derived from collagenase-pretreated adult human trabecular bone fragments were used to derive two representative clonal subpopulations, staining weakly and strongly for ALP. Analysis of their differentiation potential showed that both clonal cell lines shared osteoblastic characteristics, but differed in their osteochondral potential, although they were equally capable of adipogenic differentiation [45]. This finding indicates cellular heterogeneity in the hOB population and suggests that it consists of cells that can differentiate into more than one mesenchymal lineage, but their developmental potentials differ. Nevertheless, it supports our hypothesis that the cells derived from collagenase-pretreated adult human trabecular bone fragments display mesenchymal progenitor characteristics. Our finding that the cells derived from human trabecular bone fragments, traditionally considered as osteoblastic cells, are able to develop into three distinct mesenchymal cell phenotypes under controlled in vitro culture conditions raise interesting questions on the developmental plasticity of cells normally residing within the mineralized matrix of mature bone. Unequivocal assignment of the cellular origin of hOB and direct comparison of the phenotypic characteristics of adult hOB and of adult hMSC are clearly needed to assess more accurately the differences between hOB and hMSC.

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