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Coexpression of Osteogenic and Adipogenic Differentiation Markers in Selected Subpopulations of Primary Human Mesenchymal Progenitor Cells

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Abstract Knowledge of the basic mechanisms controlling osteogenesis and adipogenesis might provide new insights into the prevention of osteoporosis and age-related osteopenia. With the help of magnetic cell sorting and fluorescence activated cell sorting (FACS), osteoblastic subpopulations of mesenchymal progenitor cells were characterized. Alkaline phosphatase (AP) negative cells expressed low levels of osteoblastic and adipocytic markers. AP positive cells expressed adipocytic markers more strongly than the AP negative cell populations, thus suggesting that committed osteoblasts exhibit a greater adipogenic potential. AP negative cells differentiated to the mature osteoblastic phenotype, as demonstrated by increased AP-activity and osteocalcin secretion under standard osteogenic culture conditions. Surprisingly, this was accompanied by increased expression of adipocytic gene markers such as peroxisome proliferator-activated receptor-γ2, lipoprotein lipase and fatty acid binding protein. The induction of adipogenic markers was suppressed by transforming growth factor-β1 (TGF-β1) and promoted by bone morphogenetic protein 2 (BMP-2). Osteogenic culture conditions including BMP-2 induced both the formation of mineralized nodules and cytoplasmic lipid vacuoles. Upon immunogold electron microscopic analysis, osteoblastic and adipogenic marker proteins were detectable in the same cell. Our results suggest that osteogenic and adipogenic differentiation in human mesenchymal progenitor cells might not be exclusively reciprocal, but rather, a parallel event until late during osteoblast development. J. Cell. Biochem. 104: 1342–1355, 2008.

Key words: mesenchymal progenitor cells; TGF-beta; BMP-2; adipogenesis; osteoblastic differentiation

Bone is a highly organized structure formed through a complex process, which involves the proliferation and differentiation of progenitor cells into osteoblasts. Evidence exists that osteoblasts arise from multipotential mesenchymal stem cells residing in the bone marrow stroma, which are capable of differentiating into several cell lines that form bone, cartilage, adipose and other connective tissue [Owen, 1988; Beresford, 1989; Gimble et al., 1996; Nuttall and Gimble, 2000].

Various clinical studies show that the decrease in bone volume associated with osteoporosis and age-related osteopenia is accompanied by an increase in bone marrow adipose tissue [Meunier et al., 1971; Gimble et al., 1996; Nuttall and Gimble, 2000; Justesen et al., 2001].

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A number of in vitro studies support the hypothesis that a high degree of plasticity exists between adipocytic and osteoblastic pathways [Bennett et al., 1991; Oreffo et al., 1997; Thompson et al., 1998; Prichett et al., 2000; Justesen et al., 2001; Abdallah et al., 2004; Nuttall and Gimble, 2004], based on the lineage-specific marker expression. Mesenchymal progenitor cells cultured from explants of adult trabecular bone serve as an excellent system to investigate the biology of normal human bone cells. When cultured in standard osteogenic media these cells have been shown to express markers of the osteoblast phenotype, including the capacity to produce type I collagen, synthesize osteocalcin (Oc) and exhibit alkaline phosphatase (AP) activity [Beresford et al., 1984; Gundle and Beresford, 1995; Gronthos et al., 1997, 1999; Nutall et al., 1998; Siggelkow et al., 1998a,b, 1999; Martinez et al., 1999; Lecanda et al., 2000]. AP levels show characteristic changes during differentiation in culture. The developmental expression of genes reflecting growth, extracellular matrix maturation and mineralization was shown in primary cultures of rat calvarial osteoblasts [Owen et al., 1990]. The AP mRNA expression and enzyme activity increased during the matrix maturation phase, whereby Oc remained at low levels. However, when Oc expression increased, AP levels decreased, signifying the beginning of the mineralization phase [Owen et al., 1990]. Previously, we described this developmental sequence in mesenchymal progenitor cells derived from human healthy donors [Siggelkow et al., 1999, 2004]. The mesenchymal progenitor cell cultures were comprised of a heterogeneous population of cells, including osteogenic cells at different stages of differentiation [Noth et al., 2002; Sakaguchi et al., 2004]. This cellular heterogeneity complicates the interpretation of the effect of differentiation factors, indicating a need for a more homogeneous population. The use of monoclonal antibodies that bind to cell surface markers offers the opportunity to obtain a more homogeneous subpopulation of cells. Expression of AP, known to be an early marker of osteogenic differentiation, was employed as a cell surface marker for FACS analysis of mesenchymal progenitor cells [Siggelkow et al., 1998b, 1999] and positive and negative immunoselection from human bone marrow cell cultures [Rickard et al., 1994; Herbertson and Aubin, 1997; Gronthos et al., 1999]. In this study, we compared the expression of osteogenic and adipogenic markers in AP positive (AP^{pos}) and AP negative (AP^{neg}) subpopulations from human mesenchymal progenitor cells cultures directed towards the osteogenic phenotype. In addition, AP^{neg} cells were induced towards the osteogenic and adipogenic lineages applying osteogenic conditions including TGF-β1 and BMP-2. Surprisingly, AP^{pos} cells stained positive for oil-red and AP, indicating a coexpression of osteoblastic and adipocytic markers on the protein level. Finally, this was in line with the co-localization of adipogenic and osteogenic marker proteins in differentiated osteoblasts on the single cell level by immunogold electron microscopy.

MATERIALS AND METHODS

Materials

Cell culture disposables were purchased from Nunc (Roskilde, Denmark), all cell culture media and FCS from Biochrom (Berlin, Germany) and medium supplements (antibiotics, glutamine) from GIBCO-BRL (Eggenstein, Germany). All other reagents were purchased from Sigma Chemical Co. (Munich, Germany) unless otherwise stated. 1,25-$(OH)_2$-D_3 (Hoffman La-Roche, Basel, Switzerland), recombinant human BMP-2 and natural human TGF-β1 were purchased from Promocell (Heidelberg, Germany). The Oc detection kit was purchased from Metra Biosystems (Palo Alto, CA).

Primary Antibodies

The anti-human bone AP-mAb, a mouse anti-human monoclonal IgG (Metra Biosystems), was characterized previously [Siggelkow et al., 1998b; Heinemann et al., 2000]. The lipoprotein lipase (LPL)-specific mAb 5D2 was kindly provided by John D. Brunzell from the University of Washington. The specificity and reactivity of the 5D2-mAb against human LPL has been shown previously [Peterson et al., 1992; Chang et al., 1998]. The PPARγ2-mAb (E8: sc-7273) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For detection of Oc we used a rabbit polyclonal antibody against bovine Oc (BTI, MA). For surface analysis we used CD45-PE and CD34-PE from Immunotech (Prague), CD90-FITC from Becton Dickinson (Heidelberg), CD44-FITC from Bender Med. Systems (Vienna) and CD105-FITC from Biolegend, San Diego.

Mesenchymal Progenitor Cell Cultures

Bone specimens were isolated from the iliac crest, proximal or distal femur of 15 patients undergoing surgery, 14 male, 1 female, ranging in age from 16 to 83 years, mean age 57.27 ± 22.1, median 65 years. Bone specimen from the iliac crest were taken from donors during fracture repair due to trauma without any signs of bone disease, including osteoporosis or autoimmune disorders. Specimen from the proximal or distal femur were taken form donors operated on for arthroplasty due to osteoarthritis, bone specimen were taken from the most distal point away from the arthritic joint. The study was approved by the local ethical committee of the Medical Faculty of the University of Goettingen and informed consent was obtained from all patients. Mesenchymal progenitor cells cultures were obtained as described previously [Siggelkow et al., 1998a,b, 1999]. The cell populations established by this method have been characterized as a mesenchymal stem cell-like population capable of differentiation into osteoblasts, chondrocytes, and adipocytes [Noth et al., 2002; Tuli et al., 2003]. Cells isolated by our method are also able to differentiate into osteoblasts, adipocytes, and chondrocytes (unpublished results).

Cells were maintained at 37°C in a humidified 95% air–5% CO_2 atmosphere in DMEM/10% FCS with glutamine (58.5 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml), hereafter named standard conditions. The medium was changed twice a week. After 4 weeks, cells were washed with phosphate buffered saline (PBS) and detached with the help of 0.05% trypsin-EDTA for 5–10 min at 37°C. Cells were plated in 75-cm^2 flasks at a density of 4×10^3 cells/cm^2 and are defined as first passage (P1) cells. Cells were sorted by MACS and by FACS according to their AP expression as described below.

Magnetic Cell Sorting

Magnetic cell sorting (MACS) of P1 cells was performed with the MACS System (Vario-MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany) under sterile conditions following the manufacturer's recommendations. Prior to being sorted by MACS, the cells were resuspended in PBS/1% BSA and a small aliquot (10^5 cells) was used for FACS analysis (unsorted fraction).

Cells were harvested as described above, cell density was adjusted to $\sim 10^6$ cells/ml and cells were incubated with the anti-AP antibody. The incubations were performed in PBS at room temperature for 20 min and cells were washed in PBS after each step. Samples (3×10^6 cells) were incubated with the anti-AP antibody diluted 1:50 in PBS. Cells were resuspended in 180 μl PBS and incubated with 20 μl MACS Microbeads mAb (Miltenyi Biotec, Bergisch-Gladbach, Germany), a rat anti-mouse monoclonal IgG. Finally, cells were incubated with FITC-conjugated rabbit anti-rat antibody (DAKO, Denmark) diluted 1:50 in order to characterize cell populations by FACS directly before and after MACS sorting. Prior to being sorted by MACS, the cells were resuspended in PBS/1% BSA and a small aliquot (10^5 cells) was removed for FACS analysis (unsorted fraction). After washing with buffer (PBS/1% BSA/5 mM EDTA) and ice cold PBS/1% BSA, the cells were applied to a steel wool column and placed in a strong magnetic field. The AP^{neg} cells (depleted fraction) were collected as the eluate, while the AP^{pos} cells remained attached to the magnetized matrix.

FACS Analysis

For FACS analysis of surface markers the following monoclonal antibodies were used CD 45-PE, CD 14-FITC, CD 34-PE (hematopoetic lineage) and CD 44-FITC, CD 90-FITC and CD 105-FITC (mesenchymal stem cell markers) diluted 1:50. For FACS analysis, cells were incubated with FITC-conjugated rabbit anti-rat antibody (DAKO) diluted 1:50. Cells were analyzed as described [Siggelkow et al., 1998b] with FACScan 81533 (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). Appropiate controls were performed which consisted of unstained cells as autofluorescence control and cells incubated only with the FITC- or PE conjugated antibody to check for unspecific binding. Analysis was carried out with FACS Research Software Version 2.1.

Fluorescence Activated Cell Sorting

Cells were plated in 75-cm^2 flasks at a density of 4×10^3 cells/cm^2. Before being sorted, P1 cells were cultured under standard conditions for 4 weeks. Cells were trypsinized, stained with the primary mAb anti-AP antibody and incubated with FITC conjugated secondary antibody, as described for MACS. Cells were

resuspended to $\sim 10^6$ cells/ml and sorted using FACS Vantage SE (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). Analyses were performed with Cell Quest Pro Software Version 2000.

Cultivation and Differentiation of AP^{neg} Cells

AP^{neg} cells were plated at a density of $4 \times 10^3/cm^2$ cells in 24-well plates in standard medium and maintained for 2 weeks. At this time, cultures were stimulated for 2 or 7 days with a combination of osteogenic factors (OF), including 2 mM β-glycerolphosphate, 50 μg/ml L-ascorbic acid, 10^{-8} M dexamethasone, 4×10^{-8} M 1,25-$(OH)_2$-D_3 and either 1 ng/ml TGF-β1 or 50 ng/ml BMP-2. The incubation medium contained DMEM with 0.1% BSA and the control was performed applying the solvent (ethanol and citrate buffer <0.01%). Ascorbic acid was added every day and all other factors every 2 days. After 2 and 7 days, supernatants were collected for further analysis.

AP-Activity Assay

AP-activity was measured as described before [Siggelkow et al., 1998a, 1999]. AP^{neg} cells in 24-well plates were lyzed after differentiation treatment and assayed in duplicate for AP-activity, which was normalized to cell number and results were expressed as nmol/10^5 cells/min.

Oc Protein Secretion, Detection of Mineralization and Oil Red O Staining

Oc secretion was measured in duplicate (lowest detection level: 2 ng/ml) in supernatants from AP^{neg} cells in 24-well plates after differentiation treatment by applying an ELISA (Metra, Mountain View). Values were corrected for background levels present in the culture medium, normalized to cell numbers and the results expressed as ng/10^5 cells/day.

Mesenchymal progenitor cells (P1, unsorted) were plated in six-well plates at a density of 4×10^3 cells/cm^2 under standard conditions. At confluence, the medium was changed to OF incubation medium with either TGF-β1 or BMP-2. After 24 days, the degree of formation of mineralized nodules was determined by alizarin red staining [Bodine et al., 1996]. Replicate wells were stained for AP-activity and lipid vacuoles.

AP^{neg} cells in 24-well plates were fixed after differentiation treatments with ice-cold 90% ethanol/PBS for 20 min followed by double-staining for AP-activity and lipid vacuoles. After being rinsed in distilled water, cells were stained using a SIGMA AP kit (86-C) according to the manufacturer's instructions. A stock solution of oil Red O was prepared from 0.5% (w/v) oil Red O in 99% isopropanol. Cells were stained with this stock solution diluted to 0.3% (v/v) with distilled water for 15 min. After this time, the cells were rined with distilled water, photographed and stored in PBS at −20°C.

PCR Analysis

RT-PCR was performed using a previously described protocol [Siggelkow et al., 2003, 2004]. Semiquantification of RT-PCR products was performed by a competitive PCR approach using exogenous DNA competitors ("mimics") as an internal control [Köhler, 1995]. Amplifications were performed in a Primus PCR cycler (MWG-Biotech, Ebersberg, Germany) for the following cDNAs: LPL [Rickard et al., 1996], aP2 [Ahdjoudj et al., 2001], and PPARγ2 [Thomas et al., 1999]. The ribosomal gene L7 [Hemmerich et al., 1993] was amplified as house-keeping gene. Primer sequences (Table I) for these genes were synthesized by MWG-Biotech. All PCR reactions were carried out at a cycle number (25–35) ensuring a linear amplification profile with the following programs (L7: 2 min at 94°C, cycles of (1 min at 94°C, 1 min at 54°C, 2 min 72°C)); (aP2: 1 min at 94°C, cycles of (1 min at 94°C, 1 min at 55°C, 1 min at 72°C)); (LPL: 2 min at 94°C, cycles of

TABLE I. PCR Primer Sequences

	Primer sequence (5′–3′)		PCR product size (bp)	
Transcript name	Sense	Antisense	mRNA	Mimic
L7	AGATGTACAGAACTGAAATTC	ATTTACCAAGAGATCGAGCAA	378	548
LPL	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC	276	442
aP2	GTACCTGGAAACTTGTCTCC	GTTCAATGCGAACTTCAGTCC	418	597
PPARγ2	CAGTGGGGATGCTCATAA	CTTTTGGCATACTCTGTGAT	390	542

(30 s at 94°C, 2 min at 55°C, 2 min at 72°C)); (PPARγ2: 2 min at 95°C, cycles of (40 s at 94°C, 50 s at 55°C, 50 s at 72°C)) with a final 10 min incubation at 72°C.

Reaction products were analyzed by electrophoresis in 1.5% (w/v) agarose gels and visualized by ethidium bromide staining under UV light. In all experiments the expression of each gene was quantified as target to mimic ratio and normalized to the ribosomal house-keeping gene L7. Sequence analysis of the PCR products was performed (Seqlab, Germany).

For real-time PCR a Mastercycler Eppendorf Realplex^2 detection system with Mastercycler ep gradients software was used. Reactions were set up in 96-well plates using the following concentrations: 0.2 μM each sense and antisense primers for Oc, RUNX2, β-actin, osterix (OSX), aP2, PPARγ2, LPL (Table II). 4.5 μl Platinum Sybr Green qPCP superMix-UDP (Invitrogen) and 1 μl cDNA obtained from 250 to 400 ng RNA in 10 μl. A two-step amplification protocol was chosen, consisting of initial denaturation at 95°C for 2 min followed by 40 cycles with 15 s denaturation at 95°C, 15 s annealing at 60°C and 20 s extension at 68°C. Finally a dissociation protocol was performed to control specificity of amplification products: 15 s at 95°C, 15 s at 58°C, and 15 s at 95°C. Expression of the cDNA of interest was measured relative to the expression of house-keeping gene β-actin by the threshold-cycle (C_t) method [Livak and Schmittgen, 2001; Kubista et al., 2006].

Electron Microscopy

For the ultrastrucutral analysis, mesenchymal progenitor cells (P1) were cultured for 7 days with OF + BMP−2 (50 ng/ml) as described above. Cells were trypsinized, fixed and dehydrated at 4°C and washed in 0.2 HEPES after each step. Cells were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 0.2 M HEPES buffer (pH 7.4) for 15 min, treated with 10 mM ammonium chloride in 0.2 M HEPES for 45 min, dehydrated in a graded series of ethanol up to 70% and embedded in the acrylic resin LR-Gold (London Resin Company, Reading, England). For electron microscopy, ultrathin sections were cut with a Reichert ultramicrotome and collected on formvar coated nickel grids.

Gold Labeling of the Antibodies

Preparation of 8 and 16 nm gold particles and the labeling of the primary antibodies were performed following standards protocols [Miosge et al., 1999] and 16 nm gold particles were coupled to the AP- and Oc-antibodies, while 8 nm gold particles were coupled to the PPARγ2 and LPL antibodies.

Immunogold Histochemistry

Nickel grids were incubated for 15 min at room temperature with TBS. Thereafter, the grids were incubated with gold labeled anti-AP (1:50), Oc (1:80), PPARγ2 (1:50) or LPL (1:50) antibodies diluted with TBS for 16 h at 4°C. For double labeling, two of the antibodies coupled to gold particles of differing sizes (16 and 8 nm) were incubated in parallel [Miosge et al., 2003]. After having been rinsed with water and stained with uranyl acetate (15 min) and lead citrate (5 min), sections were examined with a Zeiss EM 109 electron microscope. To exclude unspecific binding of the colloidal gold probes to anionic binding sites, we incubated control sections with the pure gold solution under the same conditions as described above. All controls were negative.

Statistical Analysis

All experiments were reproduced at least twice using P1 cells from healthy donors. Statistical analysis of control and stimulated Oc levels was

TABLE II. Primer Sequences for Real Time PCR

Transcript name	Primer sequence (5′–3′)		PCR product size (bp)	Accession no.	T_m (°C)
	Sense	Antisense			
RUNX2	TTC CAG ACC AGC AGC ACT C	CAG CGT CAA CAC CAT CAT T	181	NM_004348	63
Osx	GCA GCT AGA AGG GAG TGG TG	GCA GGC AGG TGA ACT TCT TC	359	NM_152860	60
AP	TGC ACC ATG ATT TCA CCA	TTA GCC ACG TTG GTG TTG	161	NM_001826	56
OC	CAT GAG AGC CCT CAC A	AGA GCG ACA CCC TAG AC	310	NM_199173	57
aP2	GTA CCT GGA AAC TTG TCT CC	GTT CAA TGC GAA CTT CAG TCC	418	NM_001442	63
PPARγ2	TCT CTC CGT AAT GGA AGA CC	GCA TTA TGA GAC ATC CCC AC	300	NM_005037	55
LPL	AGA GCC AAA AGA AGC AG	GGC AGA GTG AAT GGG AT	182	NM_000237	59
Beta-actin	CTG GAA CGG TGA AGG TGA CG	AGT CCT CGG CCA CAT TGT GA	71	NM_001101	60

performed with the Mann–Whitney nonparametric test. $P < 0.05$ was considered significant (GraphPad Prism version 3.0).

RESULTS

Characterization of the Cell Population Isolated From Bone Specimen

In addition to the formerly published characterization of the cell population [Siggelkow et al., 1998a,b, 1999] we performed additional experiments to reveal the mesenchymal origin of the cells. When stimulated accordingly, cells differentiate to adipocytes, chondrocytes and osteoblasts (data not shown). Using surface markers for FACS analysis cells showed the following profile (mean ± SD of three donors) CD 45: 4.7 ± 2.9%, CD 34: 4.0 ± 2.6% (hematopoetic origin) CD44: 10.6 ± 4.8%, CD90: 93.7 ± 5.6%, CD105: 34.9 ± 20.2% (mesenchymal stem cell markers).

Isolation of Subpopulations of Mesenchymal Progenitor Cells

The number of spontanously AP positive cells varies in unstimulated cultures of mesenchymal progenitor cells between 2% and 60% [Marie et al., 1989; Siggelkow et al., 1998b]. In our hands, the MACS method did not reveal AP^{pos} fractions higher than 80%. The enrichment was dependent on the number of AP^{pos} cells in the starting population, with an increase in AP^{pos} cells only after ostegenic stimulation. Because our aim was the analysis of pure or at least nearly pure populations, we did not reculture the mixed populations but applied the FACS sort instead. However, the number of cells also sorted by this method was still too small to be recultured, therefore, cell fractions were analyzed directly after isolation. The purity of each AP subpopulation was analyzed by FACS (Fig. 1). Examples for the number of AP^{pos} and AP^{neg} cells in different fractions analyzed is shown in Table III.

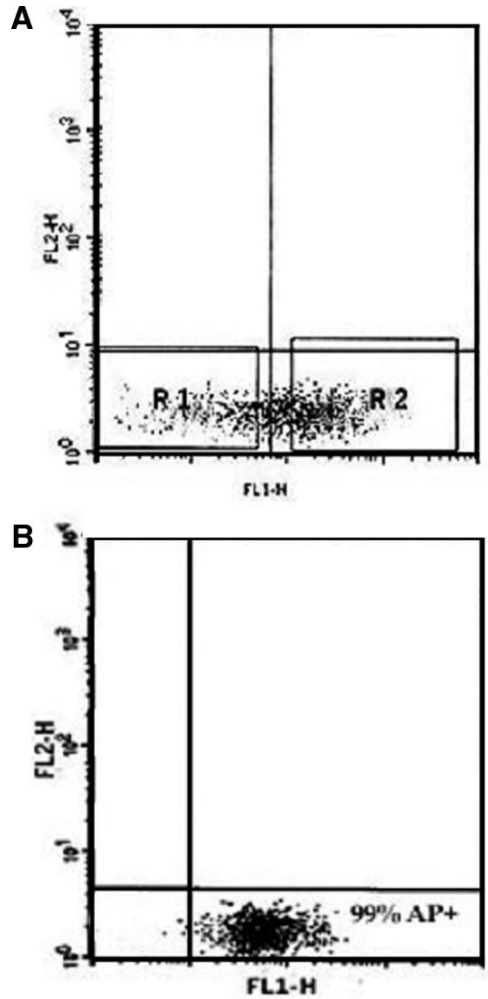

Fig. 1. Isolation of AP^{pos} subpopulations by FACSort (**A**) and control of their purity by FACS (**B**). Cells were stained with the primary mAb for AP and incubated with FITC conjugated antibody. A: intact cells of the starting population are depicted as a Dot-Plot. AP subpopulations (R1, AP-negative; R2, AP-positive) were identified within the FL1-H Channel (green fluorescence, *x*-axis) considering background fluorescence (unspecific binding of FITC conjugated antibody) B: FACS analysis after enrichment to 99% AP^{pos} cells.

TABLE III. Percent of AP^{pos} Cells in Different Populations of Mesenchymal Progenitor Cells Generated by MACS or FACS

In present	Unstimulated cultures		Osteogenic stimulation before analysis for 7 days			Osteogenic stimulation before analysis for 14 days		
Donor identifier	KA	GS	GH	KA	DJ	GH	KA	DJ
Starting population	5	40	25	20	14	82	72	85
AP^{pos} fraction	55	90	55	67	50	99	90	92
AP^{neg} fraction	1	0	X	4	X	X	0	7

Number of AP^{pos} cells in different cell populations. X: not enough cells for analysis.

Expression of Bone-Related Markers in the AP^{neg} Populations

We determined the expression of bone-related markers under osteogenic differentiation conditions in a population of AP^{neg} cells. MACS was used to obtain enough AP^{neg} cells for the differentiation studies. As determined by FACS analysis with the AP antibody, the purity check showed <1% AP^{pos} cells and therefore >99% AP^{neg} cells.

The AP^{neg} cells were recultured for a period of 2 weeks under standard conditions (DMEM/10% FCS). A recheck by FACS analysis for AP revealed that the sorted cells remained negative (<0.9% AP^{pos}) after 2 weeks.

AP-activity increased after 2 days and continued to increase up to 7 days in AP^{neg} cells under all differentiation conditions (OF, OF + TGF-β1, OF + BMP-2). The degree of AP staining (Fig. 2A) correlated with the levels of AP-activity assayed in cell lysates (Fig. 2B). The maximal and most rapid increase in AP-activity was found with TGF-β1 added to the OF cocktail (Fig. 2B). This increase was up to fourfold after 2 days and sixfold after 7 days, compared to the AP-activity measured in OF treated cells without TGF-β1. As seen in Figure 2, under control conditions when the incubation medium consisted of DMEM/0.1% BSA, no changes in AP levels were detectable by AP-activity staining (Fig. 2A) or assay (Fig. 2B).

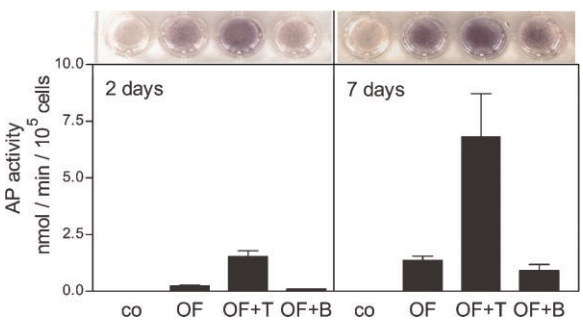

Fig. 2. Effect of osteogenic factors on the AP activity in AP^{neg} cells isolated by MACS. Cells were cultured for 2 weeks under standard conditions before addition of osteogenic factors (OF): β-glycerolphosphate (2 mM), asc (50 μg/ml), dex (10^{-8} M) $1,25(OH)_2D_3$ (4×10^{-8} M) and either OF + (T) TGF-β1 (1 ng/ml) or OF + (B) BMP-2 (50 ng/ml) for 2 and 7 days. Cultures were fixed and doublestained for AP-activity. Cell layers were also lyzed, assayed for AP-activity and results expressed as nmol/min/10^5 cells (lower panel). Values are expressed as the mean ± SEM from quadruplicate experiments. Co, controls. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Oc secretion increased significantly ($P < 0.05$) under all differentiation conditions after 2 and 7 days compared to control conditions, as shown in Figure 3. Maximal Oc secretion was found after 7 days by addition of either TGF-β1 (15-fold) or BMP-2 (17-fold), compared to control cells (Fig. 3).

Expression of Adipocytic Markers in AP^{neg} Populations Sorted by MACS

Development of an adipogenic phenotype was confirmed by semiquantitative RT-PCR analysis of the genes PPARγ2, LPL and aP2, known to be upregulated in the adipocyte differentiation process. While PPARγ2 was induced after 2 days and decreased after 7 days, LPL and aP2 increased after 2 and 7 days of incubation with OF. The adipogenic response was increased up to 27-fold after stimulation with BMP-2. TGF-β1 nearly completely prevented the induction of PPARγ2, LPL, and aP2. AP^{neg} cells exhibited detectable levels of PPARγ2, LPL, and aP2 under control conditions (Fig. 4).

Comparison of AP^{neg} and AP^{pos} Osteoblast-Like Cell Populations Isolated by FACS Sorting Under Standard Conditions

FACS sorting was employed to isolate highly enriched AP^{neg} and AP^{pos} cell populations for the comparison of osteoblastic and adipocytic marker expression. AP^{neg} and AP^{pos} cells were isolated by FACS sort from mesenchymal progenitor cell cultures from three donors

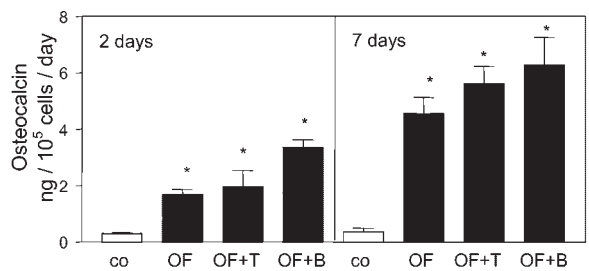

Fig. 3. Effect of osteogenic factors on Oc secretion in AP^{neg} cells isolated by MACS. Cells were cultured for 2 weeks under standard conditions before addition of osteogenic factors (OF) and either OF + 1 ng/ml TGF-β1 (T) or OF + 50 ng/ml BMP-2 (B) for 2 and 7 days. Oc protein levels were assayed by ELISA in supernatants of cultures which had been incubated for a total of 2 and 7 days and results expressed as ng/10^5 cells/day. Values are expressed as the mean ± SEM from quadruplicate experiments. Differences between the values from control and treated cultures were analyzed by Mann–Whitney test (*$P < 0.05$); co, controls.

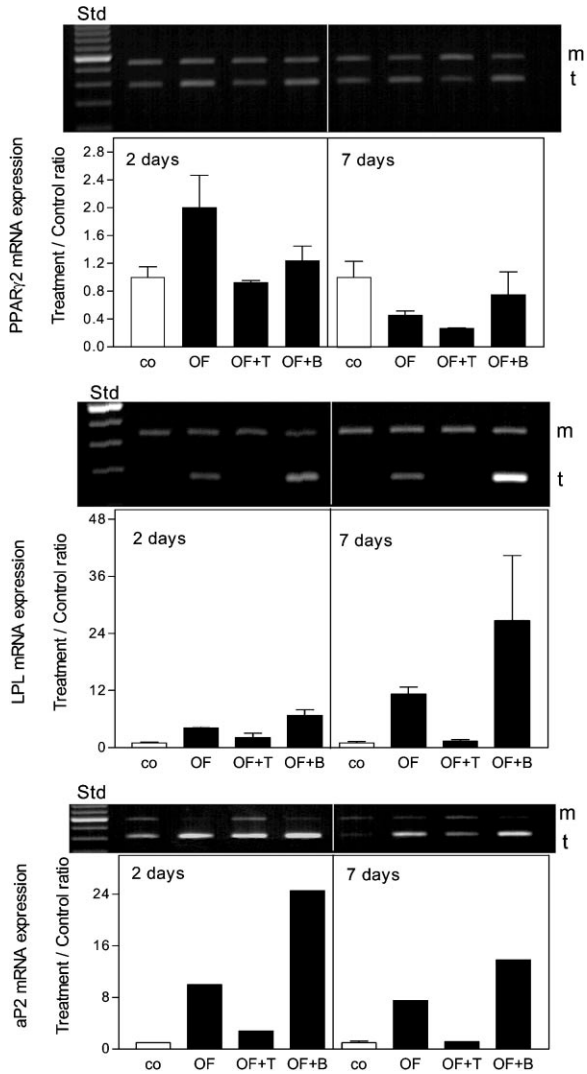

Fig. 4. Effect of osteogenic factors on adipocyte-related gene marker expression in AP^{neg} cells isolated by MACS. Gene expression of PPARγ2, LPL and aP2 was determined by semiquantitative RT-PCR. AP^{neg} cells isolated by MACS (secondary cultures) were cultured for 2 weeks under standard conditions before addition of osteogenic factors (OF) and either OF + 1 ng/ml TGF-β1 (T) or OF + 50 ng/ml BMP-2 (B) for 2 and 7 days. Values are the mean ± SEM of two independent wells. The mRNA levels were normalized to L7 levels and the induction is expressed relative to control (co) cells (arbitrarily set to 1). The size of both mimic (m) and target (t) is given in bp in Table I. Std: 100 bp DNA standard.

grown under standard conditions. Although the number of AP^{pos} cells in stimulated cultures was higher, we used unstimulated conditions to be able to compare the results. In contrast to the experiments described before, cells were not recultured but analyzed directly after sorting.

The AP^{neg} and AP^{pos} cells were characterized by mRNA expression of RUNX 2, Osx, AP, and Oc as osteoblastic and PPARγ, LPL, and aP2 as adipocyte-related markers (Fig. 5). Gene expression for alkaline phosphatase was high in AP^{pos} cells and low in AP^{neg} cells as expected after sorting for the AP protein. The very low AP gene expression in AP protein negative cells might be explained by differences between protein and RNA expression in these cells. In addition, cells vary in AP activity and the binding of the antibody might depend on a certain amount of protein. Therefore AP^{neg} cells comprise a population of cells very low in AP protein. RUNX2 and OSX gene expression was increased in AP^{pos} cells clearly showing the transition to the osteoblastic phenotype. Oc was expressed at a very low level indicating only a few differentiated cells (data not shown). However, the amount of the adipocytic transcription factor PPARγ2 was clearly higher in AP^{pos} cells confirming the coexpression of markers of osteogenesis and adipogenesis. LPL gene expression was detected in unstimulated cells at a very low level which was again higher in AP^{pos} versus AP^{neg} cells. The marker of late adipogenic differentiation aP2 is expressed in both phenotypes with no differences between AP^{pos} and AP^{neg} cells.

Light Microscopic Analysis and Ultrastructural Immunoelectron Microscopy Co-Localization of Osteogenic and Adipogenic Markers

Since we have shown an effect of TGF-β1 and BMP-2 on the differentiation of cell fractions characterized by AP protein expression, we were interested if the effect was reproducible in mixed cultures of mesenchymal progenitor cells. Development of a mature osteoblastic phenotype was confirmed by alizarin red staining of mineralized nodules (Fig. 6C,D). To validate the simultaneous expression of markers for both lineages under these conditions, we performed double staining for cytoplasmic lipid accumulation and AP activity. Cells treated for 24 days in addition to OF with either TGF-β1 or BMP-2 exhibited positive staining for AP and mineralized nodules (Fig. 6C,D) compared with the unstained control (Fig. 6A,B). However, a different pattern of cytoplasmic lipid accumulation was found under these two different conditions. Cells treated with BMP-2 showed oil Red O-stained lipid droplets in parallel to the osteogenic differentiation (Fig. 6D), whereas no lipid accumulation was detectable in TGF-β1 treated cultures (Fig. 6C). Therefore, we used osteogenic

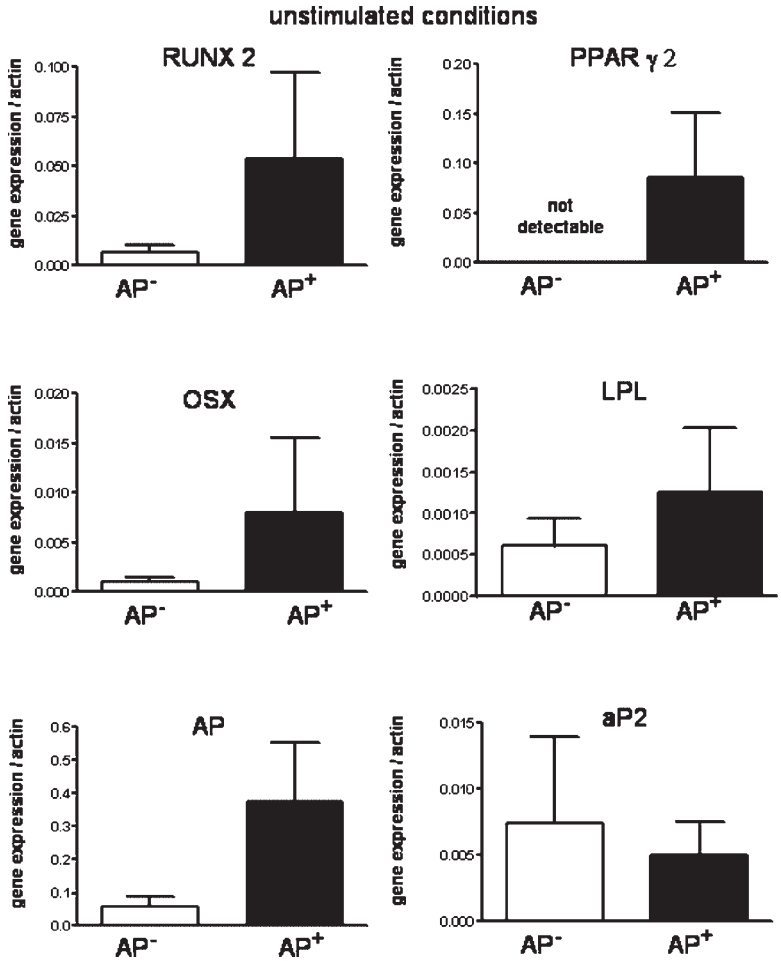

Fig. 5. Osteoblast- and adipocyte-related gene marker expression in AP^{neg} and AP^{pos} cell populations isolated by FACSort. Mesenchymal progenitor cells cultures from three donors were grown for 4 to 8 weeks under standard conditions. AP^{neg} and AP^{pos} cell populations were isolated with FACSort. Gene expression of AP, RUNX2, OSX and Oc as osteoblastic and PPARγ2, LPL and aP2 as adipocytic markers was assessed in both AP^{neg} and AP^{pos} cell populations by real-time PCR. The mRNA levels were normalized to beta-actin levels. Mean values ± SEM from three patients are depicted.

conditions including BMP-2 to investigate the coexpression of both adipocyte and osteoblast-related proteins on the same cell using immunogold double labeling at the ultrastructural level. We found a parallel expression of osteoblast specific proteins AP and Oc with adipogenic markers PPARγ2 and LPL (Fig. 6F, G, and H). PPARγ2 protein was localized in the nucleus and cytoplasm and LPL protein in the cytoplasm of the cell.

DISCUSSION

In the present study, we employed MACS and FACSort to isolate AP^{neg} and AP^{pos} populations of mesenchymal progenitor cells. With the MACS method pure AP^{neg} populations were generated whereas only enrichment to 70–80% of AP^{pos} cells was achieved. Using FACSort instead of MACS increased the number of AP^{pos} cells to 92–99% but at the cost of a very low cell yield. Hence, the AP^{pos} cells were not recultured but analyzed directly after isolation by FACSorting.

Here we demonstrate that the AP^{neg} osteoblasts constitutively express PPARγ2, a transcription factor that is involved in preadipocyte differentiation, as well as aP2 and LPL, both of which are known PPARγ2 target genes [MacDougald and Mandrup, 2002]. When AP^{neg} mesenchymal progenitor cells were treated with osteogenic stimulation in the presence or absence of BMP-2 we found an increase in PPARγ2 gene expression. This was accompanied by a remarkable induction of aP2 and

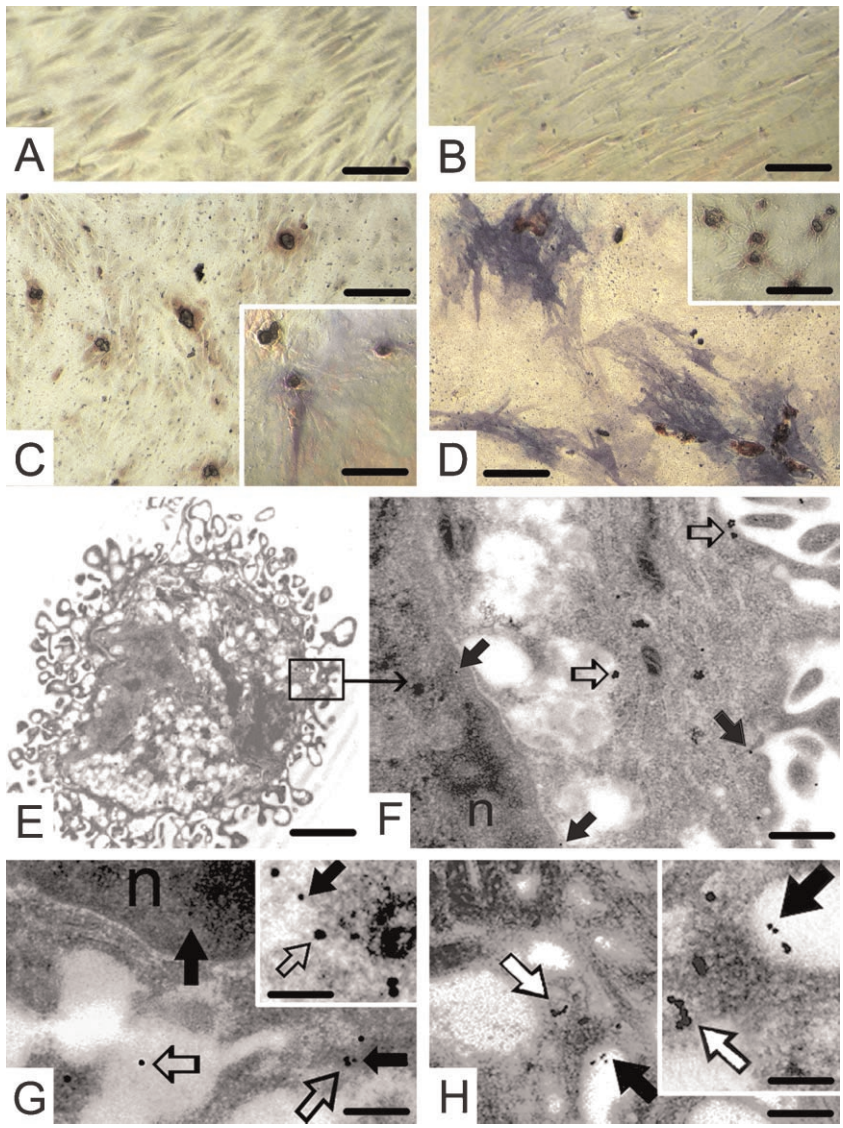

Fig. 6. Adipogenesis and osteogenesis in mesenchymal progenitor cells cultivated under different culture conditions: (**A**) negative alizarin red staining, and (**B**) negative AP-activity and oil Red O staining for cells kept in control medium. **C**: Alizarin red positive mineralized nodules in cells grown in OF + TGF-β1 medium, as well as positive AP-activity (inset). **D**: Oil Red O stained lipid vacuoles and AP-activity were detected only in cultures treated with OF + BMP-2, alizarin red positive mineralized nodules (inset). Bars = 27 μm. **E**: Ultrastructural analysis, a representative cell exhibits abundant cytoplasmic processes, bar = 0.35 μm. **F**: Immunogold histochemistry, double labeling of a cell kept in OF + BMP-2 medium, small gold particles represent PPARγ2 (black arrows) and large gold particles represent AP (open arrows). **G**: Double labeling for PPARγ2 (black arrows) and large gold particles represent osteocalcin (open arrows), the inset gives a higher magnification. **H**: Double labeling with small gold particles for LPL (black arrows) and large gold particles for osteocalcin (open arrows), higher magnification in inset. Bars = 0.25 μm and 0.12 μm in insets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

LPL gene expression. When using FACSort to compare the AP^{pos} and AP^{neg} cell population we can show that PPARγ2 and LPL expression was higher in AP^{pos} than in AP^{neg} cells The latter observation suggests that the gene expression of PPARγ2 is spontaneously high enough to activate the adipocyte differentiation program in committed osteoblasts expressing AP protein.

These data are surprising because PPARγ2 is an early transcriptional regulator of adipogenesis, which has been shown to be upregulated as mesenchymal progenitor cells undergo adipogenesis [Nutall et al., 1998]. Clinical observations and in vitro models document an inverse relationship between bone marrow adipocytes and osteoblasts [Bennett et al., 1991; Oreffo et al., 1997; Thompson et al.,

1998; Prichett et al., 2000; Justesen et al., 2001; Abdallah et al., 2004; Nuttall and Gimble, 2004]. However, previous data reported by Garcia et al. [2002] using murine neonatal calvaria-derived cells indicated that the expression level of several adipocyte markers changed in the time-course of osteoblast maturation in parallel with the expression of a set of osteoblast markers. The authors suggested that the differentiation of calvarial cells followed the osteogenic pathway, but that the commitment to this pathway might not imply the complete repression of the differentiation of mesenchymal cells along the adipocyte pathway. The possibility that the existence of two different cell types resulted in this parallel expression of osteoblastic and adipogenic markers was not excluded. That each single cell has the potential to differentiate into both pathways is supported by studies using differentiated osteoblasts, which adapt the adipogenic phenotype when cultured under adipogenic conditions [Nutall et al., 1998; Song and Tuan, 2004; Schilling et al., 2007]. This process termed redifferentiation was also shown for adipocytes adapting the osteoblastic phenotype when cultured under osteogenic conditions [Nutall et al., 1998; Schilling et al., 2007]. An increase in adipogenesis using osteoblastic stimulation as described in our study was not reported in any of the studies.

LPL and aP2 gene expression greatly increased when AP^{neg} mesemchymal progenitor cells were grown in the presence of BMP-2, but inhibited when TGF-β1 was added instead. This observation, together with the induction of the osteogenic markers Oc and AP, confirms that TGF-β1 promotes osteogenesis while blocking adipogenesis in human mesenchymal stem cells, as has been shown before [Nutall et al., 1998; Choy and Derynck, 2003]. The members of the TGF-β superfamily play a crucial role in bone development, tissue remodeling and healing. These cytokines are capable of inducing the differentiation of mesenchymal stem cells into osteogenic cells. The effects of TGF-β and BMP-2 cytokines on bone-derived osteoblasts mediated by p38 kinase-dependent and Smad protein-dependent mechanisms are age-dependent and are involved in balancing adipogenic and osteogenic differentiation [Noth et al., 2003; Moerman et al., 2004]. BMP-2, which also belongs to the TGF-β superfamily, is a potent stimulator of osteoblast differentiation in vitro and bone formation in vivo [Hogan, 1996].

In our study, we saw a differential effect of TGF-β1 and BMP-2 on osteoblast differentiation. While TGF-β1 further increased AP activity and Oc secretion in stimulated AP^{neg} cultures, BMP-2 stimulated Oc maximally, but without further increase in AP activity. This differential effect on AP and Oc suggested that BMP-2 is involved preferentially in late osteoblastic differentiation according to the differentiation model proposed by Owen in which AP expression decreases in parallel to an increase in Oc in the matrix mineralization phase [Owen et al., 1990]. In this regard, the parallel increase of adipogenic markers this late during osteogenic differentiation is surprising. To validate the gene expression data, we, therefore, also investigated the protein level by determining intracellular lipid accumulation and mineralization. The ability to accumulate lipid was induced only in the presence of BMP-2 and was not observed when TGF-β1 was added instead. The induction of mineralization, however, was clearly induced in the presence of either BMP-2 or TGF-β1.

However, the parallel adipocytic and osteoblastic differentiation could still be an effect of different populations of mesenchymal progenitor cells. To investigate the possibility of a parallel differentiation event we used immunogold histochemistry in single cells. In fact, we can show the coexpression of both osteoblastic (AP and Oc) and adipogenic (PPARγ and LPL) proteins in the same cell. Our results suggest, that a terminal, differentiated osteoblast induced by osteogenic conditions including BMP-2, can also express the full developmental program of adipocyte differentiation, including marker genes and proteins as well as cytoplasmic lipid accumulation.

Our data, indicating that BMP-2 induced osteogenic and adipogenic markers occur in parallel in our primary human mesenchymal progenitor cells, are supported by data obtained from mesenchymal cell lines or from mesenchymal cells of other species. Applying a murine mesenchymal pluripotent cell line, CH310T1/2, permanently transfected with either BMP-2 or BMP-4 cDNA in an eukaryotic expression vector, Ahrens et al. [1993] demonstrated that BMP-2 and BMP-4 mediate osteoblastic differentiation and adipogenesis in parallel. Recently, it has been shown that exogenous or

endogenous BMP-4 participates in adipocyte lineage determination in CH310T1/2 murine mesenchymal progenitor cells [Bowers et al., 2006; Bowers and Lane, 2007; Otto et al., 2007]. Chen et al. [1998] reported that BMP-2 can induce both osteoblast and adipocyte differentiation in a 2T3 mesenchymal cell line, cloned from a transgenic mouse containing the BMP-2 promoter driving the SV-40 T antigen. BMP-2 induced 2T3 cells to differentiate into mature osteoblasts or adipocytes, depending upon culture conditions. In a murine embryonic stem cell line the incubation with BMP-2 induced adipogenesis, as well as osteoblastic differentiation of hypertrophic chondrocytes [zur Nieden et al., 2005]. However, these data were generated applying cell lines, or murine cells and they have not been corroborated at the single cell level.

In summary, we have demonstrated that human mesenchymal progenitor cells display a parallel increase in both osteogenic and adipogenic markers under osteogenic conditions including BMP-2. This double differentiation potential was confirmed in subpopulations of AP^{neg} and AP^{pos} cells. Our results suggest that osteogenic and adipogenic differentiation in human mesenchymal progenitor cells are not exclusively reciprocal but possibly occur in parallel until late during osteoblast differentiation.

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