Journal of Cellular Biochemistry 74:357–371 (1999)

Inhibition of Osf2/Cbfa1 Expression and Terminal Osteoblast Differentiation by PPARγ2

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Abstract Cells of the bone marrow stroma can reversibly convert among different phenotypes. Based on this and on evidence for a reciprocal relationship between osteoblastogenesis and adipogenesis, we have isolated several murine bone marrow-derived clonal cell lines with phenotypic characteristics of osteoblasts or adipocytes, or both. Consistent with a state of plasticity, cell lines with a mixed phenotype synthesized osteoblast markers like type I collagen, alkaline phosphatase, osteocalcin, as well as the adipocyte marker lipoprotein lipase, under basal conditions. In the presence of ascorbic acid and β-glycerophosphate—agents that promote osteoblast differentiation—they formed a mineralized matrix. In the presence of isobutylmethylxanthine, hydrocortisone, and indomethacin—agents that promote adipocyte differentiation—they accumulated fat droplets, but failed to express adipsin and aP2, markers of terminally differentiated adipocytes. Furthermore, they were converted back to matrix mineralizing cells when the adipogenic stimuli were replaced with the osteoblastogenic ones. A prototypic cell line with mixed phenotype (UAMS-33) expressed Osf2/Cbfa1—a transcription factor required for osteoblast differentiation, but not PPARγ2—a transcription factor required for terminal adipocyte differentiation. Stable transfection with a PPARγ2 expression construct and activation with the thiazolidinedione BRL49653 stimulated aP2 and adipsin synthesis and fat accumulation, and simultaneously suppressed Osf2/Cbfa1, α1(I) procollagen, and osteocalcin synthesis. Moreover, it rendered the cells incapable of forming a mineralized matrix. These results strongly suggest that PPARγ2 negatively regulates stromal cell plasticity by suppressing Osf2/Cbfa1 and osteoblast-like biosynthetic activity, while promoting terminal differentiation to adipocytes. J. Cell. Biochem. 74:357–371, 1999.

Key words: peroxisome proliferator-activated receptor; osteoblasts; adipocytes; cell differentiation; transcription factors; thiazolidinedione

Even though the cells that comprise the bone marrow stroma are derived from a common stem cell, they can serve several diverse functions, including support of hematopoiesis and osteoclastogenesis, fat accumulation, and bone formation [Owen and Friedenstein, 1988]. This functional adaptation is apparently accomplished by the plasticity of some of the stem cell progeny as exemplified by the ability of stromal cells to convert between the osteoblast and adipocyte phenotype. Thus, a stromal cell type known as the Westen-Bainton cell exhibits PTH receptors and high alkaline phosphatase (AP) activity; gives rise to osteoblasts during fetal development and in hyperparathyroidism. On the other hand, when marrow hematopoietic activity is reduced using chemotherapeutic agents, these cells convert into adipocytes and can support myeloid cell production [Bianco et al., 1988, 1993; Weiss, 1988; Rouleau et al., 1988; Westen and Bainton, 1979]. Further, adipocytes isolated by limiting dilution from cultures of rabbit bone marrow can form bone in diffusion chamber implants [Bennett et al.,

Grant sponsor: National Institutes of Health; Grant number: RO3 AG15605-01; Grant number: PO1-AG13918; Grant number: 5K07 AG00718-03; Grant sponsor: Department of Veteran Affairs; Grant sponsor: Donald W. Reynolds Foundation.

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Received 1 December 1998; Accepted 7 January 1999

1991]. Conversely, the addition of fatty acids to cultures of osteoblastic cells causes them to differentiate into adipocyte-like cells [Diascro et al., 1998].

It is likely that interconversion of stromal cells among phenotypes, as well as commitment to a particular lineage with suppression of alternative phenotypes, is governed by specific transcription factors. Indeed, osteoblast-specific factor 2/core binding factor a1 (Osf2/Cbfa1) is required for commitment of mesenchymal progenitors to the osteoblast lineage. Thus, mice that are deficient in this factor lack osteoblasts and mineralized bone matrix [Komori et al., 1997]. Further, expression of Osf2/Cbfa1 in fibroblastic cells induces transcription of osteoblast specific genes [Ducy et al., 1997]. On the other hand, CCAAT/enhancer binding protein α (C/EBPα), C/EBPβ, and C/EBPδ, as well as peroxisome proliferator activated receptor γ1 (PPARγ1) and PPARγ2 orchestrate adipocyte differentiation [Spiegelman and Flier, 1996; Yeh et al., 1995; Wu et al., 1996; Shao and Lazar, 1997]. The introduction of C/EBPα in fibroblastic cells induces adipocyte differentiation [Freytag et al., 1994; Samuelsson et al., 1991] and transfection of fibroblastic cells with PPARγ2, and subsequent activation with an appropriate ligand causes the development of adipocytes [Tontonoz et al., 1994].

Identification of the factors that determine terminal differentiation of osteoblasts and adipocytes in the marrow is of considerable interest because of evidence that age-related bone loss may be due to increased adipogenesis at the expense of osteoblastogenesis. Indeed, using SAMP6 mice, a murine model of age-related osteopenia, we have previously established a tight association among reduced number of osteoblast progenitors, decreased bone formation, and decreased bone mass [Jilka et al., 1996]. This decrease in osteoblastogenesis is accompanied by increased adipogenesis and myelopoiesis, as well as decreased osteoclastogenesis, the latter attributable to a reduction in the stromal/osteoblastic cells that support osteoclast formation [Jilka et al., 1996; Kajkenova et al., 1997].

Here, we have cloned a murine bone marrow-derived cell line, designated UAMS-33, with characteristics of both pre-osteoblasts and pre-adipocytes, and used it to investigate the role of transcription factors in terminal differentiation. We present evidence that PPARγ2 induces the terminal differentiation of these cells to adipocytes and simultaneously suppresses Osf2/Cbfa1 expression and terminal differentiation to osteoblasts.

MATERIALS AND METHODS

Establishment of Murine Marrow Cell Cultures

Adult (4–6 months old) SAMR1 mice were obtained from our mouse colony established from breeders provided by Dr. Toshio Takeda, Kyoto University (Kyoto, Japan). Mice were maintained in accordance with the National Institutes of Health (NIH) guidelines on the care and use of laboratory animals. Cultures were established from the marrow cells of femurs and tibiae as previously described [Jilka et al., 1996] and maintained in α-MEM (ICN Biochemicals, Aurora, OH) supplemented with 15% heat-inactivated FBS (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of Murine Bone Marrow-Derived Cell Lines

Marrow cells obtained from SAMR1 mice were cultured for 6 days, harvested by trypsin-EDTA digestion, and exposed to uncoated magnetic beads Dynabeads M-450 (Dynal, Lake Success, NY) to remove phagocytic macrophages and endothelial cells [Fei et al., 1990]. At this point, the cell preparation contained stem cells capable of giving rise to colonies of fibroblastic cells (CFU-F), adipocytes (CFU-AD), and osteoblasts (CFU-OB), as determined using the assays described below. In addition, these cells were capable of providing support for myelopoiesis and osteoclastogenesis, using the assays described below.

Foci of transformed cells that developed during the subsequent 12–20 weeks of culture were isolated using cloning cylinders. The primary isolates were subjected to two rounds of limiting dilution subcloning by seeding the cells at 1 cell per well in a 96-well plate. The resultant cell lines were classified according to their ability to differentiate into fat-laden adipocytes and/or matrix mineralizing osteoblasts, as described below.

Stimulation and Histological Assessment of Osteoblast and Adipocyte Differentiation

Cells were seeded at a density of 5×10^3 cells/cm^2 in α-MEM medium supplemented with

15% fetal bovine serum (FBS) (basal medium). To stimulate adipogenesis, cells were maintained for 3 days in medium containing 0.5 mM isobutylmethylxanthine (IBMX), 0.5 μM hydrocortisone, and 60 μM indomethacin (Sigma Chemical Co., St. Louis, MO), referred to as IHI medium. Then the medium was changed to basal medium and cells were maintained in culture for 3 more days [Dorheim et al., 1993]. Alternatively, cells were maintained for 3 days in basal medium, and then for an additional 5 days in medium containing 0.5 μM dexamethasone (Sigma). Adipocyte cells were visualized with Oil Red O. For the stimulation of osteoblastogenesis, cells were maintained for 3 days in basal medium, and then changed to medium containing 0.2 mM ascorbic acid and 10 mM β-glycerophosphate, referred to as ASC + BGP medium, for 6 days. Mineralization was visualized by von Kossa staining.

Osteoclast Formation

Cells (5×10^3 per cm^2 in a 48-well plate) were cultured for 8 days with 4×10^4 nonadherent bone marrow cells obtained from adult SAMR1 mice, as a source of osteoclast progenitors. Cultures were maintained in α-MEM medium supplemented with 10% FBS in the presence or absence of 10 nM $1,25(OH)_2D_3$ with a change of one-half the medium after 4 days of culture. Osteoclasts were identified as previously described by staining for tartrate-resistant acid phosphatase (TRAPase), by detection of bound ^{125}I-calcitonin (data not shown), and by their ability to excavate bone from the surface of smooth cortical bone slices (data not shown) [Girasole et al., 1994].

Myelopoiesis

Cells (1×10^3 per cm^2 in 12 cm^2 flasks) were cultured with 0.6×10^6 nonadherent bone marrow cells obtained from adult SAMR1 mice, as a source of myeloid progenitors. As a positive control, 2×10^6 freshly isolated bone marrow cells were cultured for 1 week to obtain an adherent stromal cell population and than nonadherent cells were added. As a negative control, cultures of nonadherent marrow cells were cultured alone. Cultures were maintained in α-MEM medium supplemented with 12.5% FBS, 12.5% horse serum (Hyclone, Logan, UT), 2 μM 2-mercaptoethanol, and 10 mM hydrocortisone. Myelopoiesis was assessed by determining the number of nonadherent cells present in the culture supernatant at 7, 14, and 21 days later [Hauser et al., 1996]. At each time point, after collection of nonadherent cells, cultures were refed using one-half of conditioned and one-half of fresh medium. The morphology of collected cells was examined on cytospins stained by Wright-Giemsa dye. Ninety-eight percent of the nonadherent cells exhibited morphological characteristics of myeloblasts, myelocytes, or neutrophils as determined by their size, distribution, and intensity of staining, and the shape of nucleus.

Enzymatic and Biochemical Assays

Calcium in the cell monolayer was dissolved in 0.1% acetic acid and determined colorimetrically using Calcium Binding Reagent (Sigma). Cell number was estimated by protein determination of the cell layer using BCA Protein Assay Reagent (Pierce, Rockford, IL), or by the amount of lactate dehydrogenase in the cell layer as previously described [Jilka et al., 1996]. In the latter, cell number is expressed as the change in OD^{490} per minute. AP activity was determined on fixed cells in a 96-well plate using p-nitrophenol as substrate as previously described [Jilka et al., 1996]. Lipoprotein lipase (LPL) was released from cells by incubation at 37°C for 1 h in α-MEM containing 10 U/ml of heparin, and activity was determined using [^{3}H]-triolein (NEN Life Science Products, Boston, MA) [Nilsson-Ehle and Schotz, 1976]. PTH-induced cAMP was determined by incubating cells for 10 min at 37°C in the presence of 100 nM bPTH(1-34) (Bachem California, Torrance, CA). cAMP was extracted from the cell layer with acidic ethanol, dried, and assayed by immunoassay Biotrak (Amersham Life Sciences, Arlington Heights, IL). Osteocalcin was determined by radioimmunoassay (RIA) (Biomedical Technologies, Stoughton, MA). Type I collagen present in the extracellular matrix was determined by enzyme-linked immunosorbent assay (ELISA) after extracting the cell layer with 20 mM NaOH for 8 min, followed by rinsing with phosphate-buffered saline (PBS) containing 0.05% TWEEN-20 according to established procedures [O'Grady and Kumar, 1994]. Briefly, after incubation with 1% bovine serum albumin (BSA), collagen was detected using an indirect capture ELISA with goat anti-type I collagen and horseradish peroxidase-conjugated rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL). Bound enzyme-labeled

antibody was detected by incubation with ABTS substrate (Vector Laboratories, Burlingame, CA) and OD^{410} was then determined using a plate reader. A standard curve was generated by absorption of 2–200 ng/well of rat tail collagen (Sigma) and used to quantify the amount of collagen present in the extracellular matrix.

Fluorescence Activated Cell Sorting (FACS)

After trypsinization, cells were resuspended at $2–3 \times 10^6$ cells/ml in PBS and incubated for 20 min with 2 μg/ml Nile Red to label fat-containing cells [Greenspan et al., 1985; Gimble et al., 1994]. Cells were analyzed on a FACScan (Becton-Dickinson, San Hose, CA) multiparameter flow cytometer. Fluorescence was detected using a 575/26 bandpass filter. The median channel number (fluorescence intensity) was held constant for uninduced cell populations for all analyzed samples. Labeled cells were collected and analyzed in duplicate.

RNA Isolation and Northern Blot Analysis

Total RNA and poly$(A)^+$ RNA were prepared using RNAesy (Qiagen, Chatsworth, CA) and FastTrack (Invitrogen, San Diego, CA) isolation kits, respectively. RNA samples were electrophoresed on 1.2% agarose gels containing 1.9% formaldehyde and blotted onto a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Hercules, CA). Prehybridization and hybridization of Northern blots were performed as previously described [Lecka-Czernik et al., 1997]. The cDNA inserts were labeled to high specific activity using the DECAprime II Kit (Ambion, Austin, TX). The cDNAs used for this studies were as follows: 1.4-kb fragment of murine LPL [Kirchgessner et al., 1987]; 1.8-kb fragment of human C/EBPα [Johnson et al., 1987]; 270-bp fragment of 5′ sequence specific for murine Osf2/Cbfa1 [Ducy et al., 1997]; 1.2-kb fragment for rat osteocalcin [Lian et al., 1989]; 1.5-kb fragment of human α1(I) procollagen [Murano et al., 1991], 1.5-kb fragment of murine PPARγ, which recognized both isoforms [Gimble et al., 1996a]; 0.6-kb fragment of aP2 [Bernlohr et al., 1984]; and 0.8-kb fragment of adipsin [Min and Spiegelman, 1986]. Human and rat cDNAs were used where the homology of transcripts between these two species and mice was greater than 80%. Washed filters were exposed to X-ray film Biomax MS-1 (Eastman Kodak, Rochester, NY). Equivalence of loading and transfer of RNA was assessed by determination of 18S rRNA, or by determination of ChoB expression [Harpold et al., 1979].

Western Blot Analysis of PPARγ2 Protein

Protein extracts from total cells were harvested in 50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate; 0.1% SDS; 0.1% PMSF, and analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). PPARγ2 protein was detected using rabbit anti-PPARγ2 antibody from Affinity Bioreagents (Golden, CO), according to the protocol provided by manufacturers. Immunoreactive proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences).

Transfection with PPARγ2 Expression Vector

UAMS-33 cells were transfected with the full-length cDNA for PPARγ2 cloned into expression vector pEF-BOS [Mizushima and Nagata, 1990; Gimble et al., 1996a]. In this vector, the sequence of interest is under the control of the promoter for human translation elongation factor EF-1α. For positive selection of transfectants, cells were cotransfected with pL2 plasmid bearing the gene responsible for neomycin resistance [Chen and Okayama, 1987]. Transfection was carried out as previously described [Gorman et al., 1983]. Subconfluent cells were synchronized overnight by addition of 3 mM thymidine. Cells (3×10^6 cells/ml) were released from thymidine block for 5 h and electroporated with a 280-V pulse using GenePulser Electroporator (Bio-Rad Laboratories) with 20 μg of plasmid of interest and cotransfected with 10 μg of pL2. After electroporation, the cells were cultured for 18 hours in basal medium with 5 mM sodium butyrate to increase efficiency of transfection and then changed to basal medium and cultured for an addition 48 h. The medium was then supplemented with 0.5 mg/ml G418 (Gibco-BRL Life Technologies, Gaithersburg, MD) and cells allowed to grow for 2 weeks with a medium change at 4-day intervals. Colonies were isolated using cloning cylinders and replated in duplicates. Transfected cells expressing active PPARγ2 were detected by culturing in the presence of 5 μM of BRL49653 and assessing fat cell development using Oil Red O.

Statistics

Statistically significant differences between mean values were detected by analysis of vari-

ance (ANOVA), using SigmaStat (SPSS, Chicago, IL) after establishing the homogeneity of variances and normal distribution of the data.

RESULTS

Characterization of Murine Bone Marrow-Derived Cell Lines

Forty-five clonal cell lines were isolated by limiting dilution subcloning from foci of transformed cells that arose in long-term cultures of murine bone marrow cells. Their doubling time ranged from 20 to 35 h (not shown). When maintained under basal conditions, all of these cells exhibited a polygonal morphology (Fig. 1A,D,G). Of these, 15 formed a mineralized matrix when incubated with ASC + BGP, but they did not accumulate fat in response to adipogenic stimulation with IHI. Results from a representative cell line, OB-6, are shown in Figure 1A–C. Another 20 cell lines, exemplified by AD-2 cells (Fig. 1D–F), differentiated into adipocytic cells as evidenced by the formation of large lipid droplets stained with Oil Red O after exposure to IHI. These cell lines however could not form mineralized bone nodules when maintained under pro-osteoblastogenic conditions. The remaining 10 cell lines could differentiate into either osteoblastic or adipocytic cells when cultured in appropriate media. Figure 1G–I depicts this phenomenon in a prototypic cell line from this group, UAMS-33. In these cell lines, Oil Red O stained lipid droplets were smaller than those seen in AD-2 cells. Fat accumulation by UAMS-33 cells could also be stimulated by dexamethasone (not shown).

The cell lines could also be distinguished by their ability, or lack thereof, to provide stromal support for the development of myeloid cells and osteoclasts. As shown in Figure 2A, myelopoiesis occurred when nonadherent hematopoietic murine bone marrow progenitors were incubated for 3 weeks with UAMS-33 cells, but few myeloid cells were observed in co-cultures of nonadherent marrow cells and AD-2 or OB-6 cells. Moreover, UAMS-33 cells, but not the other two cell lines, supported the formation of osteoclastic cells in the presence of $1,25(OH)_2D_3$ (Fig. 2B).

The stability of the phenotype of OB-14 (another osteoblastic cell line similar to OB-6), AD-2 and UAMS-33 cells was established by subcloning and reanalyzing them for their ability to differentiate into osteoblastic or adipocytic cells. As shown in Table I, 83–90% of subclones displayed the same differentiation capability as the parental cell line. Practically identical results were obtained when one of the subclones of UAMS-33 retaining the mixed phenotype was subjected to a second subcloning analysis (data not shown). These observations demonstrate that the phenotype displayed by UAMS-33 cells is not due to the presence of contaminating OB or AD type cell lines. However, in view of the fact that a few monopotential cells did arise in cultures of UAMS-33 cells, subsequent experiments were performed with

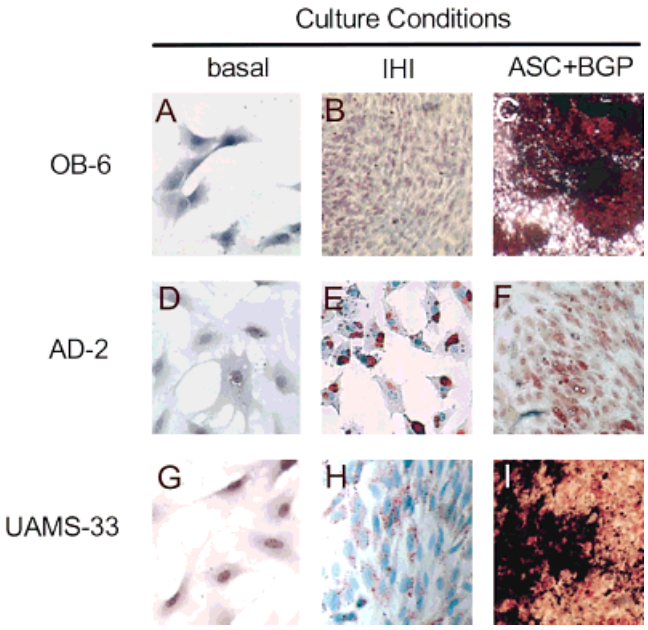

Fig. 1. Characterization of marrow-derived cell lines. OB-6 cells (**A–C**), AD-2 cells (**D–F**), or UAMS-33 cells (**G–I**) were cultured under basal conditions (A,D,G), or cultured with IHI to stimulate adipogenesis (B,E,H), or cultured with ASC + BGP to stimulate osteoblastogenesis (C,F,I) as described under Materials and Methods. Cells were stained with Oil Red O to visualize fat droplets (red) (B,E,H), or with von Kossa reagent to stain mineral (black) (C,F,I), and counterstained with hematoxylin (A–D,F,G,I) or methyl green (E,H). A,D,G: ×400; B,C,E,F,H,I: ×200.

early passage cells to minimize the possibility that monopotential cells would contribute to the results.

Comparison of the Expression of Genes Associated With Osteoblast Differentiation

When maintained under basal conditions, collagen secretion by UAMS-33 cells was 2- to 3-fold lower than OB-6 cells, and 10-fold higher than AD-2 cells (Fig. 3A). The addition of ascorbic acid and β-glycerophosphate stimulated collagen secretion and deposition of calcium in both UAMS-33 and OB-6 cells (Fig. 3A, B). Alkaline phosphatase (AP) activity was 4-fold higher in UAMS-33 cells than in OB-6 cells; but was barely detectable in AD-2 cells (Fig. 3C). Stimulation of osteoblast differentiation had no effect on AP activity of the above cell lines, but adipogenic stimulation of UAMS-33 cells caused an approximately 50% increase in AP activity, similar to that seen previously in another bone marrow-derived stromal cell line [Dorheim et al., 1993]. Osteocalcin secretion, a hallmark of osteoblastic cells, was similar in UAMS-33 and OB-6 cells, but this protein was not produced by AD-2 cells (Fig. 3D). Adipogenic stimulation of these cells had no effect on the synthesis of this protein. However, media from OB-6 or UAMS-33 cultures did not contain osteocalcin after mineralization had occurred.

The steady-state level of osteocalcin and α1(I) procollagen mRNA were lower in UAMS-33 than in OB-6 cells; however the level of each of these mRNAs had declined when mineralized cultures were analyzed (Fig. 3E). The latter finding is similar to the in vitro behavior of primary cultures of rat osteoblastic cells in the late stages of mineralization [Pockwinse et al., 1992]. Adipogenic stimulation of these cells had no effect on osteocalcin, or α1(I) procollagen mRNA. Consistent with their osteoblast phenotype, both UAMS-33 and OB-6 cells exhibited PTH-stimulated cAMP production, indicating that they expressed a PTH receptor (Table II), whereas AD-2 cells did not respond to the hormone.

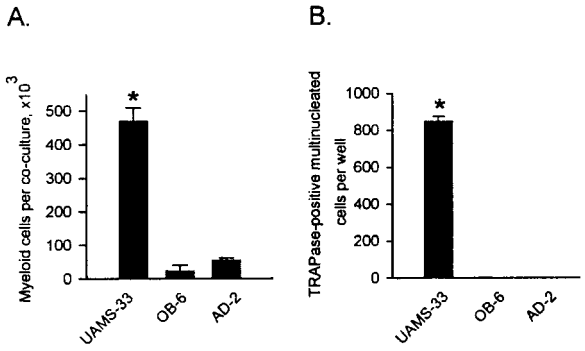

Fig. 2. Analysis of stromal cell function. Co-cultures consisting of nonadherent murine marrow cells and either UAMS-33, OB-6, or AD-2 cells were established, and myelopoiesis and osteoclastogenesis were determined as described under Materials and Methods. **A:** Myelopoiesis. The data shown are mean (±SD) cumulative number of myeloid cells produced during 21 days of culture. **B:** Osteoclastogenesis. Cultures were maintained in the presence of 10 nM $1,25(OH)_2D_3$. The data shown is mean (±SD) number of TRAPase-positive cells containing three or more nuclei. *$P < 0.05$ vs. OB-6 and AD-2.

Comparison of the Expression of Genes Associated With Adipocyte Differentiation

Lipoprotein lipase (LPL) activity was 5-fold lower in UAMS-33 as compared with AD-2 cells, and was barely detected in OB-6 cells (Fig. 4A). Stimulation with pro-adipocytic agents increased LPL activity by more than 2-fold in both UAMS-33 and AD-2 cells, but there was no effect of these agents on LPL activity in OB-6 cells. LPL activity was unchanged after induction of osteoblastogenesis in cultures of UAMS-33 cells or OB-6 cells. Similarly, Northern blot analysis indicated that LPL mRNA transcripts increased with adipogenic stimulation of UAMS-33 and AD-2 cells, but no transcript was detected in OB-6 cells (Fig. 4B). Expression of LPL mRNA was significantly higher in stimulated AD-2 cells, as compared with stimulated UAMS-33 cells. In contrast to AD-2 cells, however, UAMS-33 did not express transcripts for the adipocyte-specific protein adipsin, or for aP2, a fatty acid binding protein,

TABLE I. Phenotype of Subclones of Marrow-Derived Cell Lines

Cell line	No. of subclones analyzed	No. of subclones exhibiting phenotype (%)			
		Adipocytic and osteoblastic	Adipocytic	Osteoblastic	Neither phenotype
UAMS-33	23	83	9	9	0
AD-2	29	10	90	0	0
OB-6	45	4	4	89	2

either under basal conditions or after addition of adipogenic stimuli (Fig. 4B).

Expression of Osteoblast- and Adipocyte-Specific Transcription Factors

When maintained under basal conditions, UAMS-33 cells and OB-6 cells contained similar levels of transcripts for Osf2/Cbfa1 (Fig. 5A), an osteoblast transcription factor that stimulates the expression of osteocalcin and α1(I) procollagen mRNA [Ducy et al., 1997]. However, after the addition of ASC + BGP, Osf2/Cbfa1 mRNA levels were decreased at mineralization.

Transcripts for C/EPBα, a factor involved in adipogenesis, were present in both UAMS-33 and AD-2 cells maintained under basal conditions, and its steady state level was significantly elevated following stimulation with IHI (Fig. 5B). PPARγ1 transcripts were detected in UAMS-33 and OB-6 cells, while PPARγ2 was present at very low levels, if at all. By contrast, PPARγ2 mRNA was highly expressed in AD-2 cells. The addition of IHI did not affect the expression of either isoform in UAMS-33 or OB-6 cells but did stimulate expression of PPARγ2 in AD-2 cells. The addition of BRL49653, a thiazolidinedione activator of both PPARγ1 and PPARγ2 [Lehmann et al., 1995], stimulated adipogenesis only in AD-2 cells as evidenced by increased levels of LPL activity (Table III) as well as the appearance of Oil Red O staining (data not shown). By contrast, BRL49653 failed to stimulate LPL activity in cultures of UAMS-33 and OB-6 cells.

TABLE II. bPTH(1–34)-Stimulated cAMP Production by Bone Marrow-Derived Cell Lines

Cell line	pmoles cAMP per μg protein	Fold stimulation over control
UAMS-33	760.8 ± 27.1	11.5*
OB-6	1893.5 ± 467.0	32.6*
AD-2	7.97 ± 3.4	1.0

*$P < 0.01$ versus cultures not stimulated with bPTH(1–34).

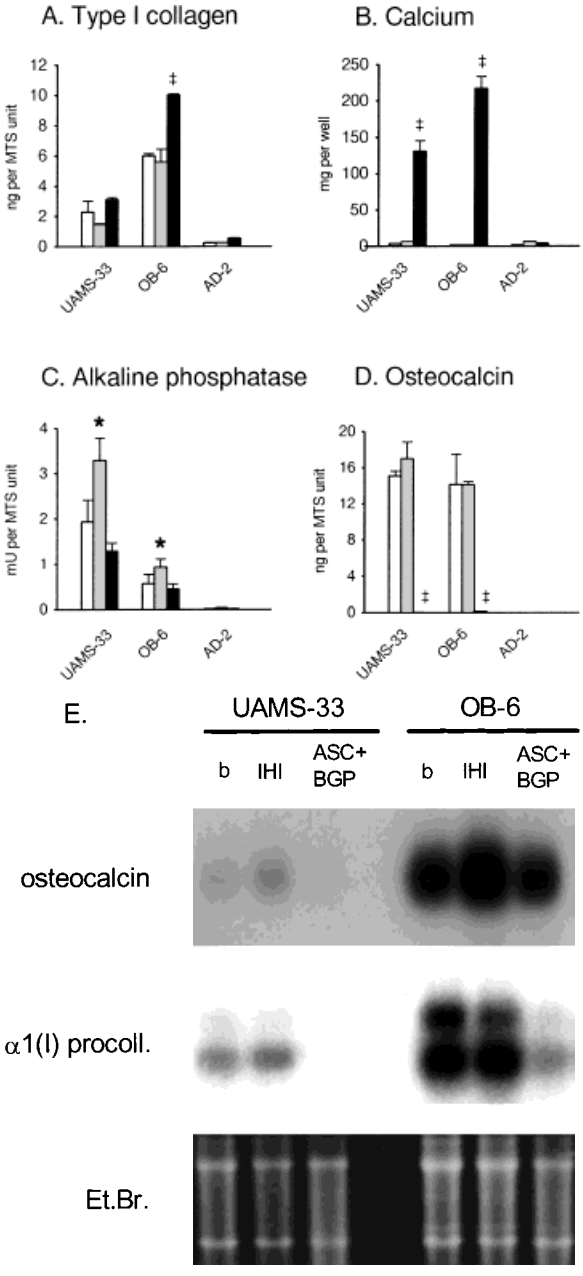

Fig. 3. Analysis of osteoblast markers. UAMS-33, OB-6, and AD-2 cells were maintained under basal conditions (open bars), cultured with IHI to stimulate adipogenesis (gray bars), or cultured with ASC + BGP to stimulate osteoblastogenesis (filled bars) as described under Materials and Methods. After 6 days, collagen I **(A)**, calcium deposition **(B)**, cellular alkaline phosphatase activity **(C)**, and osteocalcin content in the medium **(D)**, was determined. In a separate experiment **(E)** poly(A)$^+$ RNA was prepared from cells maintained for 6 days in basal conditions (b), or after stimulation of adipogenesis (IHI), or after stimulation of osteoblastogenesis (ASC + BGP). Each lane contained 10 μg of poly(A)$^+$RNA. After electrophoresis, the same blot was probed with ^{32}P-labeled cDNA for α1(I) procollagen and osteocalcin. Equivalence of loading was assessed by ethidium bromide staining of the original gel. A–D: Data shown are the mean (±SD) of three to four replicate wells, from the same experiment. Similar results were obtained in two additional experiments. *$P < 0.05$ IHI vs. basal conditions. ‡$P < 0.05$ ASC + BGP vs. basal conditions.

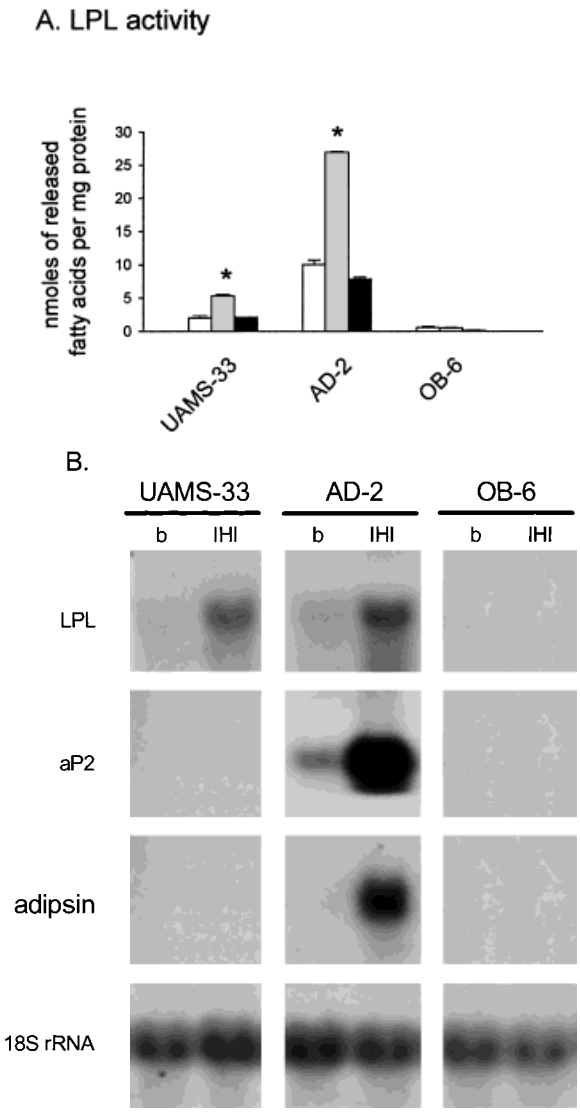

Fig. 4. Analysis of adipocyte markers. UAMS-33, OB-6, and AD-2 cells were maintained under basal conditions, cultured with IHI to stimulate adipogenesis, or cultured with ASC + BGP to stimulate osteoblastogenesis as described under Materials and Methods. **A:** Lipoprotein lipase (LPL) activity. Cells were maintained under basal (open bars), adipogenic (gray bars), or osteoblastogenic conditions (filled bars). The data shown are the mean (±SD) of three replicate wells. $*P < 0.05$ vs. basal. **B:** Northern blot analysis of expression of adipocyte gene markers. A total of 15 µg of total RNA from cells maintained under basal conditions ("b") or adipogenic conditions (IHI) was loaded in each lane. After electrophoresis, the same blot was probed with ^{32}P-labeled cDNA for LPL, aP2, and adipsin. Equivalence of RNA loading was determined by hybridization with a cDNA probe for 18S rRNA. Northern blots of LPL mRNA in AD-2 cells were exposed for 1 day and, in the case of UAMS-33 and OB-6 cells, for 5 days.

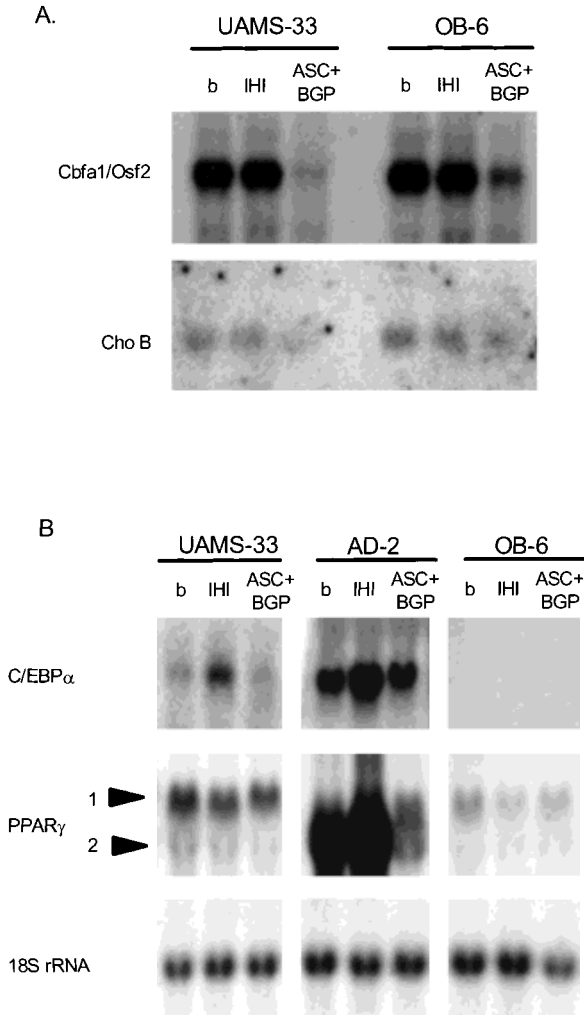

Fig. 5. Northern blot analysis of expression of osteoblastic (**A**) and adipocytic (**B**) transcription factors. Poly(A)$^+$RNA (A), or total RNA (B) was isolated from UAMS-33, AD-2, and OB-6 cells maintained under basal ("b"), adipogenic (IHI) or osteoblastic (ASC + BGP) conditions as described in Fig. 3. After electrophoresis, the same blot was probed with ^{32}P-labeled cDNA for Osf2/Cbfa1, C/EBPα, and PPARγ. A total of 10 µg of poly(A)$^+$RNA and 15 µg of total RNA was loaded on each lane. Equivalence of RNA loading was determined by hybridization of the same blots with cDNA probe for ChoB (A), or for 18S rRNA (B).

Demonstration of Reversible Expression of the Adipocytic Phenotype in UAMS-33 Cells

The data presented in Figure 3 show that UAMS-33 cells do not lose osteoblast phenotypic markers after stimulation with adipogenic agents. This behavior contrasted with that of other models of multipotential cells in which terminal differentiation toward a specific lineage is associated with loss of the ability to express phenotypic markers of any of the alternative lineages [Hu et al., 1997; Diduch et al.,

TABLE III. LPL Activity of UAMS-33 and AD-2 Cells After Treatment With BRL49653

Cells	LPL activity[a] (nmoles fatty acid released per mg protein)	
	Basal	5 μM BRL 49653
UAMS-33	1.3 ± 0.5	1.6 ± 0.9
OB-6	0.4 ± 0.3	0.1 ± 0.1
AD-2	4.4 ± 2.2	20.6 ± 1.88*

[a]Cells were maintained in culture for 6 days under basal conditions or in the presence of BRL 49653; LPL activity was then determined. Data shown represent the mean (±SD) from 3–4 replicate wells.
*$P < 0.01$ vs AD-2 cells maintained under basal conditions.

1993; Thompson et al., 1998], strongly suggesting that the UAMS-33 cells were unable to undergo terminal adipocyte differentiation. To examine this issue further, we determined whether induction of the lipogenic phenotype in UAMS-33 cells influenced their ability to subsequently differentiate into mineralizing osteoblastic cells. To do this, adipogenesis was stimulated with dexamethasone. Then, lipid-containing cells were isolated by flow cytometry using the nontoxic lipophilic dye Nile Red, and placed into medium containing ASC + BGP to stimulate osteoblastogenesis. As shown in Figure 6A, the sorted lipid-containing UAMS-33 cells lost lipid droplets and formed a mineralized matrix after stimulation with ASC + BGP, but they retained the adipocytic phenotype when maintained in the presence of dexamethasone. In a second experiment, UAMS-33 cells were cultured in IHI to stimulate adipogenesis, as indicated by Oil Red O staining in parallel cultures (not shown). Then, cultures were refed with ASC + BGP medium to stimulate osteoblastogenesis. LPL activity and mRNA expression were analyzed in parallel cultures at the time of medium change and 6 days after medium was changed. The elevated LPL activity and mRNA expression in UAMS-33 cells after adipogenic stimulation declined during subsequent culture in osteoblastogenic conditions (Fig. 6B), indicating that expression of the adipocytic phenotype of UAMS-33 cells was reversible.

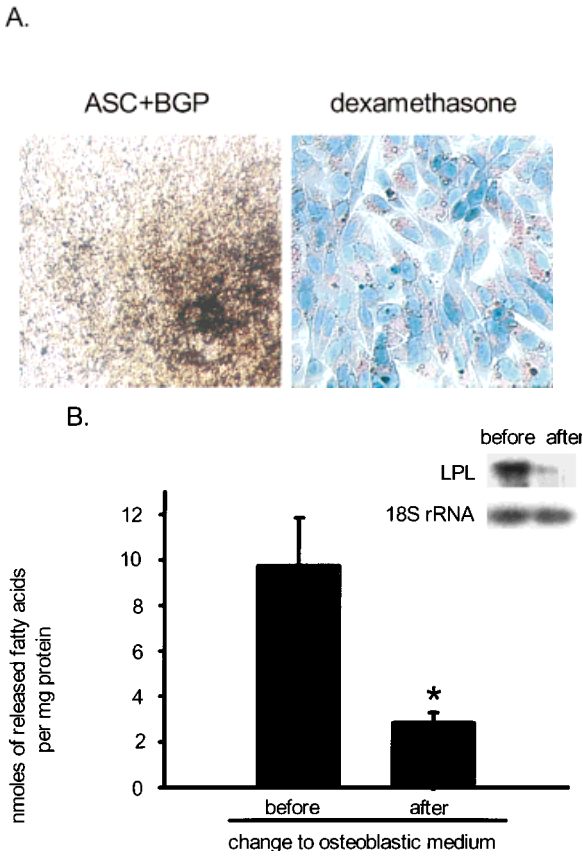

Fig. 6. Conversion of UAMS-33 cells from adipocytic to the osteoblast phenotype. **A:** Histology. Cells were maintained in the presence of 0.5 μM dexamethasone for 5 days. Cells were then detached using trypsin-EDTA, stained with Nile Red, and sorted by fluorescence activated cell sorting (FACS). Nile Red-positive cells were then cultured for 2 weeks in ASC + BGP medium to stimulate osteoblastogenesis and stained with von Kossa reagent (left, macrophotograph), or in medium containing dexamethasone, to stimulate adipogenesis and stained with Oil Red O (right, ×200). **B:** lipoprotein lipase (LPL) activity. Adipogenesis was stimulated with IHI. One-half of the cultures were analyzed for LPL activity and LPL mRNA expression ("before"). In the remaining cultures, medium was changed for ASC + BGP for the next 3 days and then analyzed for LPL activity and mRNA expression ("after"). The data shown for LPL activity are the mean (±SD) of triplicate cultures. *$P < 0.01$ vs. "before." Inset: Total RNA (10 μg per lane) was subjected to Northern blotting and probed for LPL mRNA and 18S rRNA.

Role of PPARγ2 in Induction of Terminal Adipocyte and Osteoblast Differentiation

To examine the possibility that lack of PPARγ2 in UAMS-33 cells is responsible for their failure to express the full repertoire of adipocyte-specific genes and to irreversibly commit to the adipocyte lineage, they were stably transfected with a PPARγ2 expression construct. Clones of UAMS-33 cells expressing this construct were isolated and screened for expression of functional PPARγ2 by their adipogenic response to BRL49653, as determined by Oil Red O staining. Of the eight clones that accumulated lipid after five days of treatment with this agent, three (designated γ2.27, γ2.28, and γ2.45)

were chosen for more detailed studies. Western blot analysis indicated that each of these clones, like AD-2 cells, expressed PPARγ2 protein, whereas parental UAMS-33 cells did not (Fig. 7A). Northern blot analysis of PPARγ2 mRNA expression confirmed this result (data not shown). Activation of PPARγ2 with 5 μM BRL49653 stimulated adipocyte differentiation of γ2.28 cells as indicated by Oil Red O staining (Fig. 7B) and expression of aP2 and adipsin mRNA (Fig. 7C), whereas a control cell line (γc18) transfected with empty vector did not exhibit these responses. The same results were obtained using clones γ2.27 and γ2.45 (data not shown).

Both UAMS-33 cells transfected with PPARγ2 and cells transfected with an empty vector formed a mineralized extracellular matrix when cultured in the presence of ASC + BGP (Fig. 8A). However, after 5 days of culture with 5 μM BRL49653, PPARγ2-transfected cells (but not empty vector controls) failed to form a mineralized matrix when subsequently cultured with ASC + BGP, but lipids were still present as evidenced by staining with Oil Red O (not shown). Basal levels of Osf2/Cbfa1 and α1(I) procollagen mRNA, as well as AP activity, were similar in UAMS-33 transfectants and empty vector controls (Fig. 8B, D). Osteocalcin mRNA and protein levels, however, were significantly lower in PPARγ2 transfected cells compared with empty vector controls (Fig. 8B,C). The addition of BRL49653 significantly reduced the steady-state levels of Osf2/Cbfa1 and α1(I) procollagen mRNA, as well as AP activity, in PPARγ2 transfectants, but had no effect on empty vector controls. Interestingly, osteocalcin mRNA levels, as well as protein production, were strongly inhibited by BRL49653 in both control and PPARγ2 transfected cells.

DISCUSSION

Using clonal cell lines isolated from the murine bone marrow, we have demonstrated here that PPARγ2 can convert stromal cells from a plastic osteoblastic phenotype that reversibly expresses adipocyte characteristics to terminally differentiated adipocytes. Moreover, PPARγ2 suppresses the expression of Osf2/Cbfa1, and thereby osteoblast specific genes. Although a role of PPARγ2 in terminal adipocyte differentiation was expected, to our knowledge, this evidence constitutes the first demon-

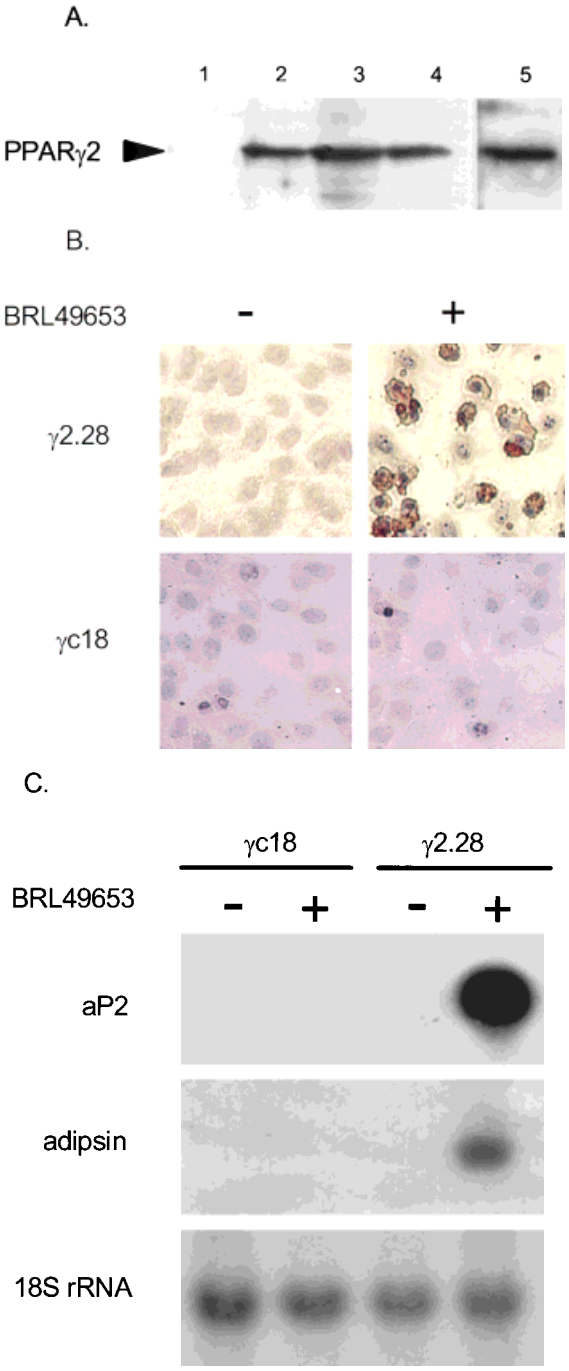

Fig. 7. Effect of ectopic expression and activation of PPARγ2 on the phenotype of UAMS-33 cells. **A:** Western blot analysis. Each lane contained 5 μg of total proteins extracted from: UAMS-33 **(lane 1)**, γ2.27 **(lane 2)**, γ2.28 **(lane 3)**, γ2.45 **(lane 4)**, and AD-2 **(lane 5)** cells. After electrophoresis, the blot was probed with an antibody against PPARγ2 as described under Materials and Methods. **B:** Histology. γ2.28 cells transfected with a PPARγ2 expression vector, and γc18 cells transfected with empty vector, were cultured in the absence or presence of 5 μM BRL49653 for 5 days. ×200. **C:** Northern blot analysis. Cells were cultured as in A and 15 μg of total RNA was subjected to Northern blot analysis and probed for aP2 and adipsin mRNAs, and 18S rRNA.

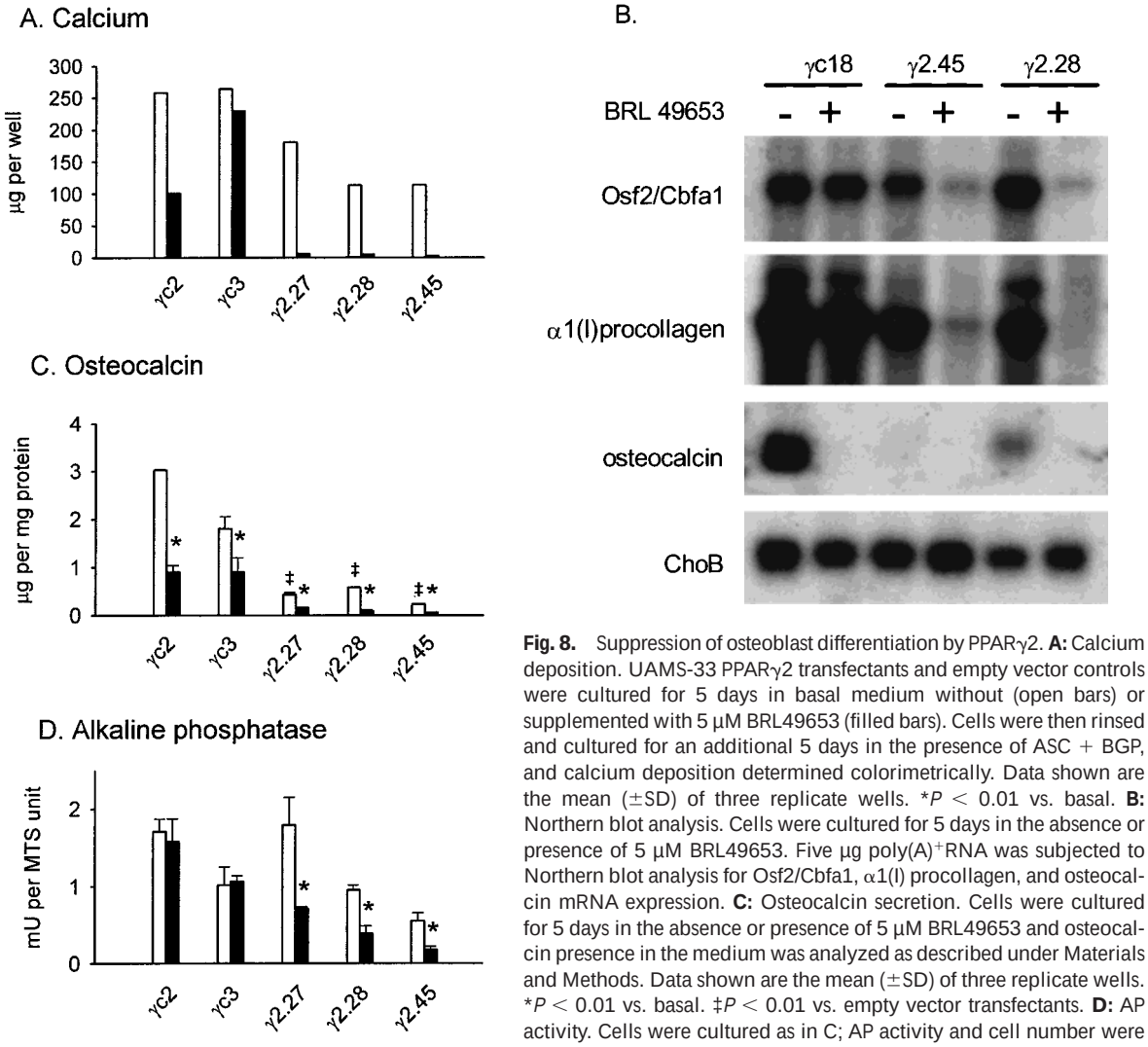

Fig. 8. Suppression of osteoblast differentiation by PPARγ2. **A:** Calcium deposition. UAMS-33 PPARγ2 transfectants and empty vector controls were cultured for 5 days in basal medium without (open bars) or supplemented with 5 μM BRL49653 (filled bars). Cells were then rinsed and cultured for an additional 5 days in the presence of ASC + BGP, and calcium deposition determined colorimetrically. Data shown are the mean (±SD) of three replicate wells. $*P < 0.01$ vs. basal. **B:** Northern blot analysis. Cells were cultured for 5 days in the absence or presence of 5 μM BRL49653. Five μg poly(A)$^+$RNA was subjected to Northern blot analysis for Osf2/Cbfa1, α1(I) procollagen, and osteocalcin mRNA expression. **C:** Osteocalcin secretion. Cells were cultured for 5 days in the absence or presence of 5 μM BRL49653 and osteocalcin presence in the medium was analyzed as described under Materials and Methods. Data shown are the mean (±SD) of three replicate wells. $*P < 0.01$ vs. basal. $‡P < 0.01$ vs. empty vector transfectants. **D:** AP activity. Cells were cultured as in C; AP activity and cell number were then determined colorimetrically. $*P < 0.05$ vs. basal.

stration of a suppressive effect of PPARγ2 on osteoblast differentiation. Similar to the inhibitory effect of PPARγ2 on the osteoblast phenotype, it was previously shown that the combination of PPARγ and C/EBPα suppressed the muscle cell phenotype when transfected into G8 myoblastic cells [Hu et al., 1995]. Taken together with the results of the present report, these findings strongly suggest that PPARγ2 plays a hierarchically dominant role in the determination of the fate of mesenchymal progenitors, due to its ability to inhibit the expression of other lineage-specific transcription factors.

Inhibition of Osf2/Cbfa1 expression by PPARγ2 following activation with BRL49653 is likely responsible for the decrease in α1(I) procollagen, alkaline phosphatase, and osteocalcin synthesis. A similar inhibitory effect of BRL49653 on AP activity has been reported previously in the multipotent stem cell line C3H10T1/2 [Paulik and Lenhard, 1997]. However, we noted that BRL49653 also inhibits osteocalcin expression in cells lacking PPARγ2 without affecting the expression of Osf2/Cbfa1 or the ability to form a mineralized matrix. This observation suggests the existence of one or more additional thiazolidinedione-activated transcription factors that inhibit osteocalcin synthesis. In view of our finding that PPARγ1—the other isoform of PPARγ [Zhu et al., 1995]—is expressed in osteoblastic cells, and evidence that thiazolidinediones activate both isoforms [Lehmann et al., 1995], it is possible that BRL49653 specifically inhibits osteocalcin expression via PPARγ1 activation, even in the presence of Osf2/Cbfa1.

The factors that determine the adipogenic properties of UAMS-33 cells are unclear. In view of evidence for a role of C/EBPα in adipogenesis [Freytag et al., 1994], and the increased expression of this factor in UAMS-33 cells after adipogenic stimulation, it seems likely that it facilitates the subsequent increase in LPL expression and fat accumulation. The failure of osteoblastic OB-6 cells to express either LPL or C/EBPα upon adipogenic stimulation is consistent with this notion. On the other hand, PPARγ1 does not seem to be sufficient for adipocyte differentiation because even though UAMS-33 cells express this factor, they do not differentiate into adipocytes upon exposure to BRL49653.

UAMS-33 cells are distinct from other known multipotential cell lines, such as 3T2 and 7F2, which differentiate into either matrix synthesizing osteoblasts or into terminally differentiated adipocytes expressing aP2 and adipsin upon exposure to appropriate pro-differentiation factors [Thompson et al., 1998; Chen et al., 1998]. In addition, adipocyte differentiation in these other cell lines is accompanied by loss of the ability to differentiate into osteoblastic cells. Our findings indicate that the lack of PPARγ2 is responsible for the distinctive behavior of UAMS-33 cells as compared to that of multipotential cell lines isolated by others. Thus, the introduction of PPARγ2, a key regulator of adipogenesis, changed UAMS-33 cells into truly bipotential cells that either form a mineralized matrix in the absence of a PPARγ activator or irreversibly differentiate into adipocytes when activated by its ligand. Nevertheless, Chen et al. [1998] recently demonstrated that bone morphogenetic protein receptor 1A (BMPR-1A) and BMPR-1B signaling mediate adipocyte and osteoblast differentiation, respectively, in 3T2 cells. Therefore, it is possible that appropriate BMPR signaling as well as PPARγ2 transcriptional activity interact to determine cell fate in the marrow stroma. Indeed, in studies not shown here, we have found that the osteoblastic features of UAMS-33 cells are determined in large part by autocrine production of BMP-2 and BMP-4 [Abe et al., 1998].

Besides the ability to synthesize lipid and to form a mineralized extracellular matrix, UAMS-33 cells also support the formation of myeloid cells and osteoclasts. In studies reported elsewhere we have established that each one of the other 9 cell lines with properties similar to UAMS-33 shares the ability to support osteoclast formation, but none of the purely osteoblastic or adipocytic cell lines are able to do so [Gubrij et al., 1997]. These findings are in agreement with evidence indicating that the cells that support osteoclast formation are closely related to the osteoblast lineage. Thus, mice lacking osteoblasts due to Osf2/Cbfa1 deficiency also lack osteoclasts [Komori et al., 1997], and bone marrow cells from SAMP6 mice—a strain with defective osteoblastogenesis—exhibit decreased osteoclastogenesis [Jilka et al., 1996]. Interestingly, some of the previously described murine cell lines that support osteoclast formation have been characterized as preadipocytic, but they also express some osteoblast markers [Kelly et al., 1998; Dorheim et al., 1993; Udagawa et al., 1989]. In view of evidence that stimulation of adipogenesis of bipotential BMS-2 stromal cells enhanced their ability to support osteoclast formation [Kelly et al., 1998], it is possible that some of the same genes involved in the activation of lipogenesis are also involved in support of osteoclastogenesis.

The evidence presented in this article raises two additional issues that merit special attention. First, extensive evidence indicates that the increased marrow fat and decreased bone formation rate associated with aging are due to an increase in the number of adipocyte progenitors and a reciprocal decrease in osteoblast progenitors [Rozman et al., 1989; Gimble et al., 1996b; Tavassoli, 1989; Parfitt, 1990; Jilka et al., 1996; Kajkenova et al., 1997]. Our new findings tempt us to suggest that these changes are caused by increased expression and/or activation of PPARγ2 in marrow stromal cells, resulting in loss of stromal cell plasticity and consequently the development of age-related osteopenia.

The second issue is the potential adverse effect of thiazolidinediones on bone metabolism. During the past few years, thiazolidinediones have been widely used for controlling hyperglycemia in patients with type 2 diabetes [Nolan et al., 1994]. The finding that BRL49653 inhibits the differentiation of progenitors expressing PPARγ2 into osteoblasts is consistent with evidence that administration of the thiazolidinedione Pioglitazone to rats for 28 days caused a reduction in osteoblast progenitors and loss of bone mass [Jennermann et al., 1995]. Individuals with type 2 diabetes have a low incidence of osteopenia or osteoporosis com-

pared with the general population [van Daele et al., 1995]. Hence, future studies to determine whether long-term thiazolidinedione use in diabetics will result in the loss of this apparent protective effect are warranted.

ACKNOWLEDGMENTS

The authors thank Catherine Smith for excellent technical assistance; J. Gimble (University of Oklahoma, Oklahoma City, OK) for the PPARγ2 expression construct; P.A. Kern for murine LPL cDNA and B. McGehee for aP2 and adipsin cDNAs (University of Arkansas for Medical Sciences, Little Rock, AR); S.C. McKnight (Tularik, Inc., South San Francisco, CA) for C/EBPα cDNA; G. Karsenty (Baylor University, Houston, TX) for murine Osf2/Cbfa1 cDNA; G. Stein and J. Lian (University of Massachusetts, Worchester, MA) for rat osteocalcin cDNA; and D.C. Morris (Glaxo Wellcome, Research Triangle Park, NC) for PPAR gamma ligands. This work was supported by the National Institutes of Health RO3 AG15605-01 (to B.L.-C.), PO1-AG13918 (to S.C.M. and R.L.J.), and 5KO7 AG00718-03 (to D.A.L.), the Department of Veterans Affairs (to S.C.M., R.L.J., and D.A.L.), and the Donald W. Reynolds Foundation (to D.A.L.).

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