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Reciprocal Control of Osteoblast/Chondroblast and Osteoblast/Adipocyte Differentiation of Multipotential Clonal Human Marrow Stromal F/STRO-1$^+$ Cells

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Abstract The regulation of human bone marrow stromal precursor cell differentiation toward the chondrocyte, osteoblast or adipocyte lineages is not known. In this study, we assessed the lineage-specific differentiation and conversion of immortalized clonal F/STRO-1$^+$ A human fetal bone marrow stromal cells under the control of dexamethasone (Dex), indomethacin/insulin (Indo/Ins) and linoleic acid (LA). Under basal conditions, F/STRO-1$^+$ A cells expressed markers mRNAs or proteins of the osteoblast lineage [CBFA1, osteocalcin (OC), alkaline phosphatase (ALP), type 1 collagen], of the chondrocyte lineage (aggrecan, types 2, 9 and 10 collagen), and of the adipocyte lineage (PPARγ2, C/EBPα, aP2, G3PDH, lipoprotein lipase, leptin). Treatment with Dex increased CBFA1, OC and ALP mRNA and protein levels. Exposure to LA enhanced expression of adipocytic genes and cytoplasmic triglycerides accumulation, and suppressed the Dex-induced stimulation of osteoblast marker genes. Indo/Ins stimulated the synthesis of aggrecan and type 2 collagen and increased types 9 and 10 collagen mRNA levels, and suppressed both basal and Dex-promoted expression of osteoblast markers. Conversely, stimulation of osteoblastogenesis by Dex suppressed both basal and Indo/Ins-stimulated chondrocyte genes. Thus, the clonal human fetal bone marrow stromal F/STRO-1$^+$ A cell line is a lineage-unrestricted common progenitor that expresses tripotential adipocyte, osteoblast or chondrocyte characteristics. Our data also show that differentiation towards one pathway in response to Dex, Indo/Ins and LA restricts expression of other lineage-specific genes, and provide evidence for a controlled reciprocal regulation of osteoblast/chondroblast and osteoblast/adipocyte differentiation of clonal F/STRO-1$^+$ human bone marrow stromal cells. J. Cell. Biochem. 81:23–38, 2001. © 2001 Wiley-Liss, Inc.

Key words: clone; STRO-1; chondroblast; osteoblast; adipocyte; human

Pluripotent mesenchymal stem cells in the bone marrow stroma are believed to undergo lineage-commitment and differentiate towards the osteoblast, chondrocyte or adipocyte lineage pathways under appropriate environmental cues [Owen and Friedenstein, 1988; Aubin et al., 1995; Gimble et al., 1996; Triffitt, 1996; Prockop, 1997]. There is evidence for the existence of bipotential cells that can differentiate into adipocytes or osteoblasts in rat [Beresford et al., 1992; Benayahu et al., 1997], mouse [Thompson et al., 1998; Lecka-Czernik et al., 1999] and human bone marrow stroma [Hicok et al., 1998; Houghton et al., 1998; Nuttall et al., 1998]. Very recent studies suggest that tripotent bone marrow stromal cells can differentiate into adipocytes or bone- and cartilage-forming cells in mice [Dennis et al., 1999; Negishi et al., 2000] and humans [Pittenger et al., 1999; Muraglia et al., 2000]. The differentiation toward these lineages is under the control of hormonal and local factors. Dexamethasone (Dex), bone morphogenetic proteins (BMPs) and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] can induce rat and human marrow stromal cells to differentiate into osteoblastic cells [Beresford et al., 1994; Rickard et al., 1994; Cheng et al., 1996; Fromigué et al., 1998; Gori et al., 1999], as shown by the expression of osteoblast-specific factor 2/Core-binding factor-a1 (Osf2/Cbfa1), osteocalcin

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(OC), alkaline phosphatase (ALP) and type 1 collagen. Transforming growth factor-β (TGF-β) and Dex [Johnstone et al., 1998; Dennis et al., 1999; Pittenger et al., 1999] can induce chondrogenic differentiation of mouse or human bone marrow stromal cells, as judged by expression of aggrecan and types 2, 9 and 10 collagen. Rabbit serum, polyunsaturated fatty acids, 3-isobutyl-1-methylxanthine (IBMX), insulin, indomethacin and dexamethasone induce adipogenic differentiation, characterized by the expression of the adipocyte-specific transcription factors peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and CCAAT-enhancer binding proteins (C/EBP), the adipocytic differentiation-related genes adipocyte lipid-binding protein (aP2), lipoprotein lipase (LPL) and glycerol-3-phosphate dehydrogenase (G3PDH), and cytoplasmic accumulation of neutral lipids [Beresford et al., 1992; Diascro et al., 1996; Dennis et al., 1999; Pittenger et al., 1999]. However, most knowledge concerning the lineage differentiation of bone marrow stromal cells derive from heterogenous stromal cells or bipotential cell lines, and very little is known on the control of human tripotential bone marrow stromal cells [Pittenger et al., 1999; Muraglia et al., 2000]. It is important to characterize the regulation of human tripotential bone marrow stromal cells because the processes of endochondral bone formation [Pechak et al., 1986], fracture repair [Ham and Harris, 1971] and bone formation [Caplan, 1991] are strongly dependent on the ability of precursor cells in the marrow stroma to differentiate into the appropriate lineage pathway.

The isolation and characterization of human clonal cells that progress from progenitors to mature osteoblasts, chondrocytes and adipocytes are paramount to identifying factors that regulate the controlled development of mature cells. Recently, we isolated and partially characterized a clonal osteogenic cell line (F/STRO-1^+ A) from fetal human bone marrow stroma [Oyajobi et al., 1999]. This clonogenic stromal cell line is a homogeneous population and therefore provides a valid tool for the study of human stromal cell lineages. In this study, we identify the F/STRO-1^+ A cell line as a tripotent progenitor cell line, and we show that the differentiation of clonal tripotential common precursor F/STRO-1^+ A cells into osteoblasts, chondroblasts and chondrocytes is controlled by restricted and reciprocal mechanisms.

MATERIALS AND METHODS

Reagents

Culture media was from Eurobio (Les Ulis, France) and fetal calf serum (FCS) was from Deutcher (Paris, France). Tissue culture (Falcon) plasticwares were purchased from Becton Dickinson (Le Pont de Claix, France). Dexamethasone (Dex), indomethacin, insulin, rabbit serum, linoleic acid (LA), PGE_2, retinoic acid, recombinant human fibroblast growth factor-2 (rhFGF-2) and human PTH (1-34) were from Sigma (St. Louis, MI). Recombinant human BMP-2 was from Genetics Institute (Cambridge, MA). Reagents for molecular analysis were from Promega (Charbonierre, France). Rabbit affinity-purified polyclonal antibodies against PPARγ2, type 2 collagen (COL2A1) or human β-actin were from Sigma. Mouse Osf2 antiserum (provided by Dr. G. Karsenty) was prepared in rabbits using a synthetic peptide having the following sequence: SFFWDPSTS-RRFSPPS, amino acids 84–99 of the Osf2 sequence [Xiao et al., 1998]. Reagents for Western blot analysis were from Boehringer (Mannheim, Germany).

Clonal F/STRO-1^+ A Cell Line

The clonogenic human fetal stromal F/STRO-1^+ A cell line was established as previously described [Oyajobi et al., 1999]. Briefly, fresh fetal human bone marrow stromal cells enriched by a two-stage procedure (density, non-adherence and lectin-binding) were immunoselected by magnetic activated cell sorting (MACS) using a murine monoclonal antibody, STRO-1 [Simmons and Torok-Storb, 1991] which recognizes all stromal precursors, including osteoprogenitors [Gronthos et al., 1994]. The immunoselected cell population was subsequently electroporated with an origin-defective mutant of the SV-40 large tumor antigen (Tag) and cloned. The most immature clone, as defined by the lowest ALP activity was selected and designated clone F/STRO-1^+ A. Expression of the STRO-1-binding epitope and nuclear large T-antigen in the cloned cells was confirmed immunocytochemically [Oyajobi et al., 1999]. The cells have been maintained at 37°C in 5% CO_2 in DMEM with 10% heat-inactivated FCS, 1% penicillin/streptomycin, with medium change every 2 days. To assess the expression of markers under basal conditions, confluent F/STRO-1^+ A cells were rinsed with phosphate

buffer (PBS), and biochemical and molecular analyses of specific markers for the three lineages were performed, as described below.

In preliminary studies, we optimized culture conditions to identify factors capable of promoting terminal differentiation into cells with distinct phenotypes. We tested factors previously reported to support osteogenic, chondrogenic and/or adipogenic differentiation of marrow stromal cells. The end points chosen for these initial experiments were increased ALP activity, Alcian blue-staining of extracellular matrix and cytoplasmic Oil Red O-staining for mature osteoblasts, chondrocytes and adipocytes, respectively. Optimal culture conditions that consistently promoted phenotypic differentiation towards one particular cell lineage were then selected as follows. For osteoblast differentiation, cells were cultured as monolayers and treated with Dex (0.1 μM) in DMEM supplemented with 5% FCS. For chondrogenic differentiation, the cells were cultured as aggregates. To this end, 10^6 F/STRO-1$^+$ A cells were passaged onto bacteriological-grade plastic culture dishes which allowed cellular aggregation [Oyajobi et al., 1999] and formation of a cartilage-like tissue [Xu et al., 1996]. After 24 h, the cells were cultured in DMEM with 5% FCS and treated with indomethacin or insulin at the indicated concentrations. For adipocytic differentiation, monolayer cultures were incubated with 10% rabbit serum or linoleic acid (100 μM) in DMEM supplemented with 5% FCS for 1–14 days. To assess temporal and quantitative changes in mRNA and protein levels of various markers, confluent F/STRO-1$^+$ A cells were treated with either Dex, indomethacin, insulin, rabbit serum, LA, or various combinations of these at the indicated doses or with the respective vehicles, and mRNA and protein levels were determined by RT-PCR and Western blotting, respectively.

Biochemical Analyses

ALP levels were determined colorimetrically, as previously described [Oyajobi et al., 1999], and the specific activities were calculated by correcting for total cellular protein. The amount of cartilage-specific sulfated glycosaminoglycans (GAGs; chondroitin and keratan sulfate) was measured by the amount of extractable dye as described [Asahina et al., 1993]. Briefly, Alcian blue-stained aggregates were extracted with 6 M guanidine–HCl for 2 h at room temperature, optical densities of the extracted dye were measured at 650 nm and corrected for total cellular protein content. Adipocytic differentiation of F/STRO-1$^+$ A cells to mature adipocytes was followed by observing the accumulation of triacylglycerol in Oil Red O (or Sudan Black)-staining cytoplasmic vesicles [Gimble et al., 1996]. After counterstaining with toluidine blue, cells were visualized by bright-field microscopy and the number of Oil Red O-positive cells was counted and expressed as percentage of total number of cells with a minimum of 1,000 cells counted in 3 wells/culture.

Purification of Messenger RNAs, RT-PCR and Southern Blotting Analyses

The steady-state expression of marker genes for the osteoblast, chondrocyte or adipocyte phenotypes in F/STRO-1$^+$ A cells as well as expression of these genes in cells treated with indicated agents was determined by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis and confirmed in all cases by Southern blotting. We opted to use RT-PCR/Southern blotting rather than Northern analysis to avoid the possibility of false negatives which may arise due to the relative insensitivity of the latter for detecting messages constitutively expressed at very low levels. For osteoblast markers, we evaluated ALP, pro-α1(1) collagen (COL1A1), CBFA1 [Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997], osteocalcin (OC), and PTH/PTHrP 1 receptor (PTH-R1). The chondrocyte markers examined were aggrecan (AGC), collagen α1(2A) (COL2A1A), collagen α1(2B) (COL2A1B), collagen α2(9) (COL9A2), collagen α1(10) (COL10A1), Indian hedgehog (IHH), matrix gla protein (MGP), SOX9, CART-1 and BMP-6 [Hale et al., 1988; Gerstenfeld and Landis, 1991; Sandell et al., 1991; Zhao et al., 1993; Vortkamp et al., 1996; Grimsrud et al., 1999; Healy et al., 1999]. The adipocytic markers examined were PPARγ, C/EBPα, LPL, G3PDH, leptin and aP2 [MacDougal and Lane, 1995; Wu et al., 1999]. Total cellular RNA was isolated from F/STRO-1$^+$ A cells using Extract-All reagent according to the manufacturer's protocol (Eurobio, France). In preliminary experiments, optimization of RT-PCR was performed, the optimal cycle number within the linear range of the amplification reactions (23–30 cycles) was determined, and the experiments

were conducted in these appropriate conditions. Briefly, 10 μg RNA from each culture was reverse-transcribed at 37°C for 1 h in a 20 μl reaction mixture containing 200 U MMLV reverse transcriptase (Life Technologies, France), 1 mM dNTPs, 40 U RNase inhibitor, 0.1 M DTT and 100 pmol of oligodT primers. PCR amplification was carried out using cDNA from the same reverse transcriptase reaction to avoid possible variations which may occur from tube to tube. cDNA samples were then divided into aliquots and amplified using specific oligonucleotide primer pairs. The PCR was performed in 50 μl volume reaction mixture containing 20 pmol of sense and antisense primers, 2 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Eurobio) in PCR buffer for 23–30 cycles consisting in 1 min at 94°C, 1 min at 60°C and 1 min at 72°C, followed by a final extension step at 72°C for 10 min before cooling at 4°C. Primer sequences for the osteoblast markers Col 1A1, ALP, OC and GAPDH were previously reported [Fromigué et al., 1998]. Sequences for other markers are:

The predicted PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. The identities of the amplicons and the specificity of the amplifications were confirmed in all cases by Southern blotting. Autoradiographic signals were quantified using a scanning densitometer (Transydine General Corporation, Ann Arbor, MI). The signal for each gene was corrected for the ubiquitously expressed housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control sequence. The data presented in graphs represent the mean ± SEM of values obtained in three independent experiments.

Western Immunoblot Analyses

The expression of crucial functional and transcription factor genes was confirmed at the protein level by Western blot analysis. F/STRO-1^+ A cell extracts were prepared by adding 500 μl of (ice cold) lysis buffer containing 10 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM

CBFA1	5′-TACCAGACCGAGACCAACAGAG-3′	Sense
	5′-CACCACCGGGTCACGTCGC-3′	Antisense
PTH-R1	5′-TTCACAGTCTTCGGCTGG-3′	Sense
	5′-TTGAAGTCCAGTGCCAGT-3′	Antisense
SOX9	5′-CTACGACTGGACGCTGGTGC-3′	Sense
	5′-CGATGTCCACGTCGCGGAAG-3′	Antisense
CART-1	5′-TCATGGGCCATGTAACATAC-3′	Sense
	5′-AAACAGGGTTTGTGGAGACT-3′	Antisense
IHH	5′-GGCTATCTCGGTGATGAACC-3′	Sense
	5′-AATGAGCACATCGCTGAAGG-3′	Antisense
MGP	5′-AAACCATGAAGAGCCTGATCC-3′	Sense
	5′-ACAGGCTTCCCTATTGAGCTCG-3′	Antisense
AGC	5′-CCATGCAATTTGAGAACT-3′	Sense
	5′-ACAAGAAGAGGACACCGT-3′	Antisense
COL2AIA/B	5′-CTGCTCGTCGCCGCTGTCCTT-3′	Sense
	5′-AAGGGTCCCAGGTTCTCCATC-3′	Antisense
COL9A2	5′-CGGCAAGGACGGCGAGAAGG-3′	Sense
	5′-ACTGCCAGACTCTCCTTTGG-3′	Antisense
COL10A1	5′-GCCCAAGAGGTGCCCCTGGAATAC-3′	Sense
	5′-CCTGAGAAAGAGGAGTGGACATAC-3′	Antisense
BMP-6	5′-AGAGTGACCGCGCCGCGACT-3′	Sense
	5′-TGAGCAGTCCCCGCCACCTC-3′	Antisense

C/EBPα	5′-ACTGTATGCCTTCAGCATTGC-3′	Sense
	5′-AGAAGAGAACCAAGCCGTCC-3′	Antisense
PPARγ2	5′-GAATGTCGTGTCTGTGGAGA-3′	Sense
	5′-TGAGGAGAGTTACTTGGTCG-3′	Antisense
LPL	5′-GATCCAGCTGGACCTAACTT-3′	Sense
	5′-AAAGGCCTGATTGGTATGGG-3′	Antisense
G3DPH	5′-TTGCAACTGTTTTAGGACTTT-3′	Sense
	5′-AGCATTGGGAAATGTTCAAGG-3′	Antisense
aP2	5′-GTACCTGGAAACTTGTCTCC-3′	Sense
	5′-GTTCAATGCGAACTTCAGTCC-3′	Antisense
LEPTIN	5′-TGTCCAAGCTGTGCCCATCC-3′	Sense
	5′-CCTTCAAGGCCTCAGCACCC-3′	Antisense

NaF, 1 mM Na_3VO_4, 10% glycerol and a cocktail of protease inhibitors (Boehringer). Lysates were clarified by centrifugation at 12,000*g* for 10 min at 4°C and the protein content of supernatants was determined using the DC Protein Assay (Bio-Rad Laboratories, CA). Equal aliquots of protein lysates were electrophoresed in on 4–15% SDS-polyacrylamide gradient gels (Biorad) under reducing conditions for detection of CBFA1, PPARγ2 or collagen type 2. β-actin was used as an internal control for protein loading. Electrophoresed proteins were transferred onto PVDF membranes (Hybond-P, Amersham) in buffer containing 20% methanol. The membranes were incubated overnight with 1% blocking buffer in TBS (50 mM Tris HCl, 150 mM NaCl) containing 0.1% Tween-20, and then reacted with either Osf2/Cbfa1 antiserum, affinity-purified polyclonal antibody raised in rabbit against PPARγ2, affinity-purified polyclonal antibody against type 2 collagen, or human β-actin antibody. After washing twice with TBS/0.1% Tween-20 and 0.5% blocking buffer, blots were incubated for 1 h with appropriate horse radish peroxidase (HRP)-conjugated secondary antibody. After three washes of the membranes in TBS/0.1% Tween-20, signals were visualized using chemiluminescence detection kits (Boehringer Mannheim) [Fromigué et al., 1998].

Statistical Analysis

All experiments were repeated three to four times. The data were expressed as mean ± SEM of 3–4 different experiments (RT-PCR) or cultures (biochemical data) and analyzed using the statistical package superANOVA (Macintosh, Abacus Concepts, Inc., Berkeley, CA) with $P < 0.05$ considered to be significant.

RESULTS

Expression of Osteoblast, Chondrocyte and Adipocyte Markers in F/STRO-1$^+$ A Cells

When maintained under basal culture conditions, F/STRO-1$^+$ A cells expressed several early and late markers of the osteoblast, chondroblast and adipocyte lineages (Fig. 1). After 2 days of culture, the RT-PCR Southern blot analysis showed expression of mRNAs for CBFA1, OC, ALP, COL1A1 and PTH-R1, all marker genes of the osteoblast lineage (Fig. 1A). Identical results were found after 7 days of culture (not shown). Concomitantly, the cells also expressed mRNAs for cartilage-specific transcription factors such as SOX9 and CART-1, for IHH and for early and late markers of the chondrocyte lineage such as MGP, AGC, COL2A1B, COL9A2, COL10A1 and BMP-6 at 2 days of culture (Fig. 1B). The pattern of expression after 7 days was essentially the same, except that COL2A1A mRNA was now also detectable (data not shown). Furthermore, mRNAs for several markers of the adipocyte lineage were constitutively expressed in F/STRO-1$^+$ A cells under basal conditions after 7 days of culture. This includes the adipocyte-specific transcription factors (C/EBPα and PPARγ) which are induced relatively early during adipocyte differentiation (42), as well as LPL, G3PDH, aP2, and LEPTIN which are late functional adipocyte differentiation-related genes [Wu et al., 1999] (Fig. 1C).

In parallel to these studies, immunoblot analysis was conducted to confirm the consti-

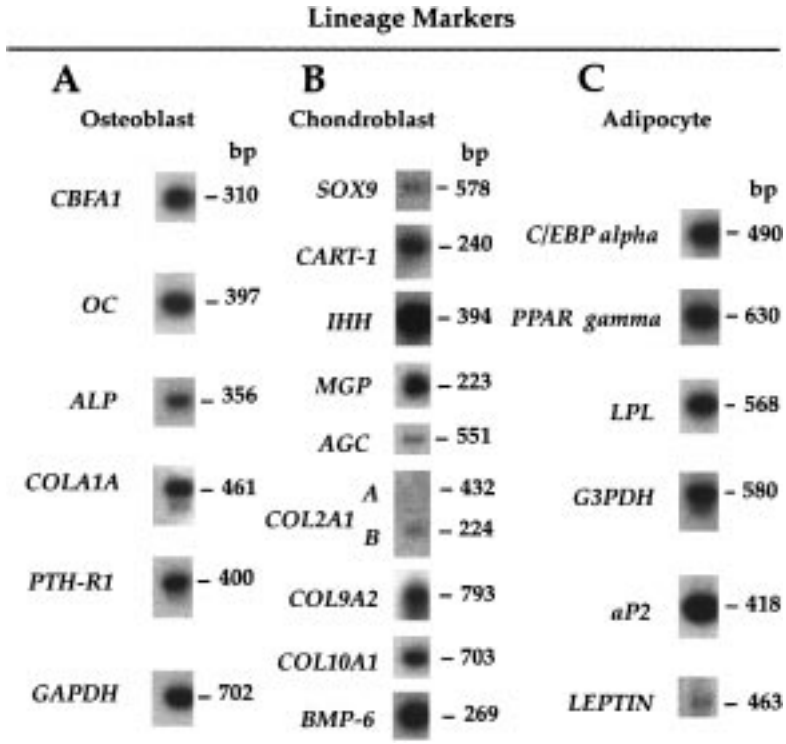

Fig. 1. Constitutive expression of osteoblast (**A**), chondrocyte (**B**) and adipocyte (**C**) marker genes in clonal human bone marrow stromal F/STRO-1^+ A cells under basal conditions. The cells were cultured to confluence in DMEM with 5% serum for 2 days (A, B) or 7 days (C) and the expression of lineage-specific genes was determined thereafter by RT-PCR analysis. Aliquots of cDNA synthesized from 10 μg total RNA were amplified in a 50 μl reaction mixture. Following Southern transfer, blots were probed with appropriate [^{32}P]-labeled oligomers for each amplicon (See Section 2).

tutive expression at the protein level of the genes detected in F/STRO-1^+ A cells. CBFA1, type 2 collagen and PPARγ2 proteins were clearly detectable in F/STRO-1^+ A cells (see below and Figs. 2, 4 and 5). We also performed immunolocalization studies of type 2 collagen and aggrecan, two cartilage-specific macromolecules to further define the phenotype of F/STRO-1^+ A cells. In addition to type 1 collagen expression which we had previously demonstrated [Oyajobi et al., 1999], we detected intracellular immunoreactivity for both aggrecan and type 2 collagen in F/STRO-1^+ A cells under steady-state conditions (not shown), confirming that the cells are actively producing and secreting these chondrocyte-specific molecules. Moreover, staining for cartilage proteoglycans with Alcian blue showed that the cells also synthesized cartilage-specific sulfated GAGs as indicated by the amounts of Alcian blue dye extracted from whole cell aggregates in the absence of exogeneous stimuli (see below). These data show that the clonal F/STRO-1^+ A cell line constitutively expresses characteristics of the osteoblast, chondroblast and adipocyte lineages in basal culture conditions.

Enhancement of Osteoblastic Differentiation by Dex in F/STRO-1^+ A Cells

We then determined whether F/STRO-1^+ A tripotential cells can be primed to differentiate further into more mature osteoblasts. Of the agents tested, only Dex increased ALP activity whereas rhFGF-2, rhTGF-β2, retinoic acid, PGE_2, hPTH, or rhBMP-2 had no effect at any of the doses tested (data not shown). Dose–response and kinetics analysis showed that Dex increased ALP activity about 2-fold with a maximal effect at 1 μM after 4–8 days ($P < 0.05$). Figure 2A shows that treatment of F/STRO-1^+ A cells with a suboptimal concentration of Dex (0.1 μM) for only 2 days was sufficient to increase expression of CBFA1 and OC. In contrast, an increase in ALP mRNA was evident only after exposure to Dex for 7 days, and COL1A1 mRNA was only slightly increased. These changes were validated by densitometric analysis of the Southern blot autoradiographs (Fig. 2B). Western blot analy-

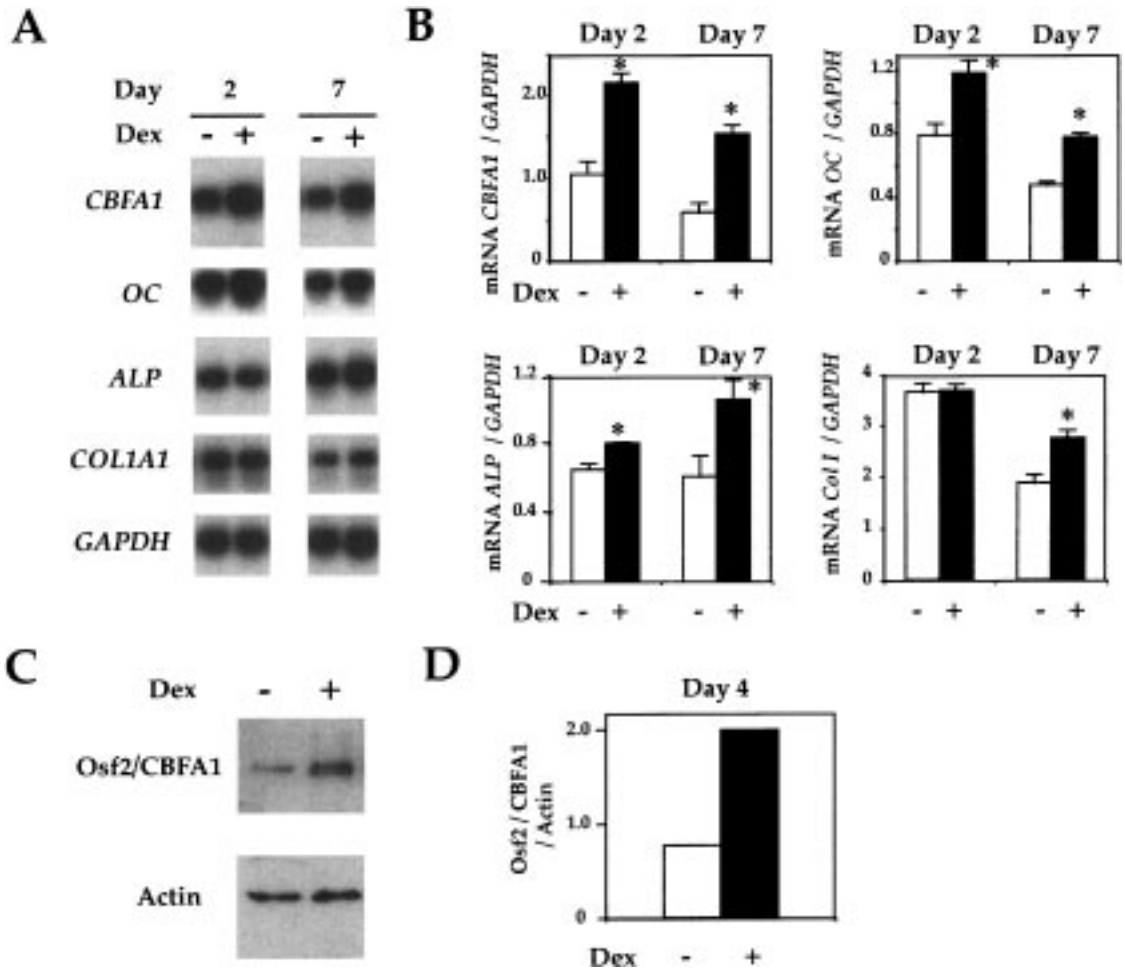

Fig. 2. Dexamethasone promotes osteoblast differentiation in F/STRO-1$^+$ A cells. Cells were cultured for 2 or 7 days in the presence of dexamethasone (Dex, 0.1 μM) or the vehicle. **A**: Total cellular RNA was reverse-transcribed and cDNA amplified by PCR to examine the expression of CBFA1, osteocalcin (OC), alkaline phosphatase (ALP), and pro-α1(1) collagen (COL1A1) transcripts followed by Southern blotting. **B**: Densitometric analysis of mRNA levels after corrrection for GAPDH (mean ± SEM of three independent experiments (*$P < 0.05$, significant differences from vehicle-treated control cells). **C**: Equal aliquots of total cell lysates were examined for Osf2/Cbfa1 protein levels on Western blot probed with Osf2/Cbfa1 antiserum and the levels were quantified by densitometry. Values are presented as arbitrary densitometric units after normalization to the corresponding β-actin band.

sis further showed that Dex (0.1 μM, 4 days) increased the protein level of Osf2/Cbfa1 more than two fold in F/STRO-1$^+$ A cells (Fig. 2C and D), confirming the data with RT-PCR/Southern blotting. Thus, Dex promotes the expression of osteoblast markers in F/STRO-1$^+$ A cells.

Promotion of Chondrocyte Differentiation by Indo/Ins in F/STRO-1$^+$ A Cells

We next investigated chondrogenic differentiation in F/STRO-1$^+$ A cells cultured in aggregates. Insulin induced a 2-fold increase in GAG synthesis as evaluated by staining in the cellular aggregates, compared to vehicle-treated controls (Fig. 3). Although indomethacin had no effect on its own, it had a synergistic effect (4-fold increase) on GAG synthesis when combined with insulin (Fig. 3). All other agents tested were ineffective in this regard (data not shown). Consistent with this, treatment of F/STRO-1$^+$ A aggregates with 10 μM indomethacin/insulin increased the expression of the cartilage-specific proteoglycan AGC as well as COL9A2 and COL10A1 mRNA levels after 2 or 7 days of treatment (Fig. 4A,B). Moreover, Western blot analysis further showed that indomethacin/insulin (10 mM, 7 days) increased protein levels of type 2 collagen by 5-fold

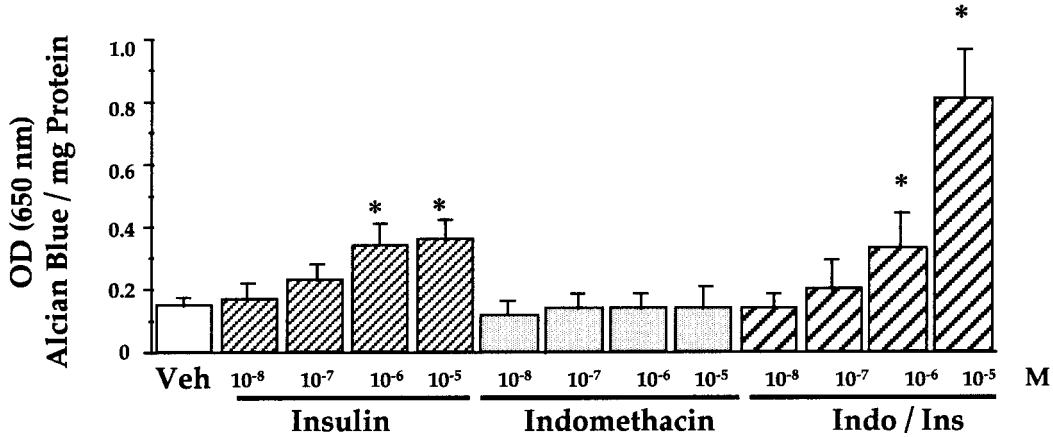

Fig. 3. Indomethacin/insulin enhances glycosaminoglycan synthesis in F/STRO-1$^+$ A cells. The cells were treated for 8 days with indomethacin, insulin or a combination (Indo/Ins) of the two, and Alcian blue incorporated into GAGs was quantified colorimetrically after extraction (see Section 2). The data are the mean optical density (OD) ± SEM of 3–4 replicates (*$P < 0.05$, significant differences from vehicle-treated control cells).

(Fig. 4C,D). Thus, Ind/Ins promotes chondrocyte differentiation in F/STRO-1$^+$ A cells.

Stimulation of Adipogenic Differentiation in F/STRO-1$^+$ A Cells

We then determined the effects of known adipogenic agents (IBMX, rabbit serum, oleic acid, linoleic acid, palmitic acid) on the differentiation of F/STRO-1$^+$ A cells into mature adipocytes. Rabbit serum increased PPARγ, G3PDH, and LEPTIN mRNA levels about 2-fold at 7 days of culture (Fig. 5A,B). In parallel to the above experiments, we determined the accumulation of neutral lipids. Under basal conditions, few F/STRO-1$^+$ A cells stained for Oil Red O-staining. Rabbit serum markedly increased the number of Oil Red O-positive stained cells (Fig. 5G), with about 85% of the cells being fat-laden. IBMX (1 mM) had no effect on lipid droplets formation in these cells (data not shown). Similar findings were observed when Sudan Black was used to stain the lipid vacuoles (not shown). As the effect of rabbit serum is thought to be due to the its high content of polyunsaturated fatty acids such as oleic acid, linoleic acid and palmitic acid [Diascro et al., 1996], we tested the effect of these fatty acids independently on cytoplasmic accumulation of neutral lipids. Linoleic acid was the most potent in inducing lipid droplets formation after 7 days of culture, with 100% of the cells containing fat-laden vacuoles (Fig. 5G). Accordingly, treatment with optimal concentrations of LA (100 μM) increased PPARγ, C/EBPα and LPL mRNA levels and this was confirmed by densitometric analysis (Fig. 5C, D). The LA-induced increase in the mRNA expression of PPARγ was associated with a five fold increase in PPARγ2 protein, a key transcription factor involved in commitment to the adipocytic phenotype, as shown by Western blot analysis (Fig. 5E,F). Thus, linoleic acid promotes the expression of early and late adipocyte markers and adipogenic differentiation of F/STRO-1$^+$ A cells.

We next investigated whether Dex and Indo/Ins could modulate adipocyte differentiation of F/STRO-1$^+$ A cells. As shown in Figure 6, treatment with Dex for 7 days increased the expression levels of PPARγ mRNA (2-fold) and G3PDH mRNA (five fold), and to a lesser extent, LPL mRNA level (Fig. 6A,B), indicating that Dex promotes early and some late markers of adipocyte differentiation. Dex also increased PPARγ2 protein levels in F/STRO-1$^+$ A cells (Fig. 6C,D) albeit at a lesser extent than with LA (Fig. 5F). Treatment with Indo/Ins for 7 days also increased mRNA levels of G3PDH and LPL (Fig. 6A,B), albeit the effect was less marked than seen with Dex treatment. Indo/Ins only slightly enhanced PPARγ2 protein levels after 7 days (Fig. 6C,D). This effect appears transient since PPARγ mRNA level in indomethacin/insulin-treated cultures was not different from those of vehicle-treated cultures after 7 days (Fig. 6A,B). Treatment with either Dex or Indo/Ins slightly increased the number of Oil Red O-positive lipid droplets in F/STRO-1$^+$ A cells. Combined treatment with Dex and indomethacin/insulin had an additive effect

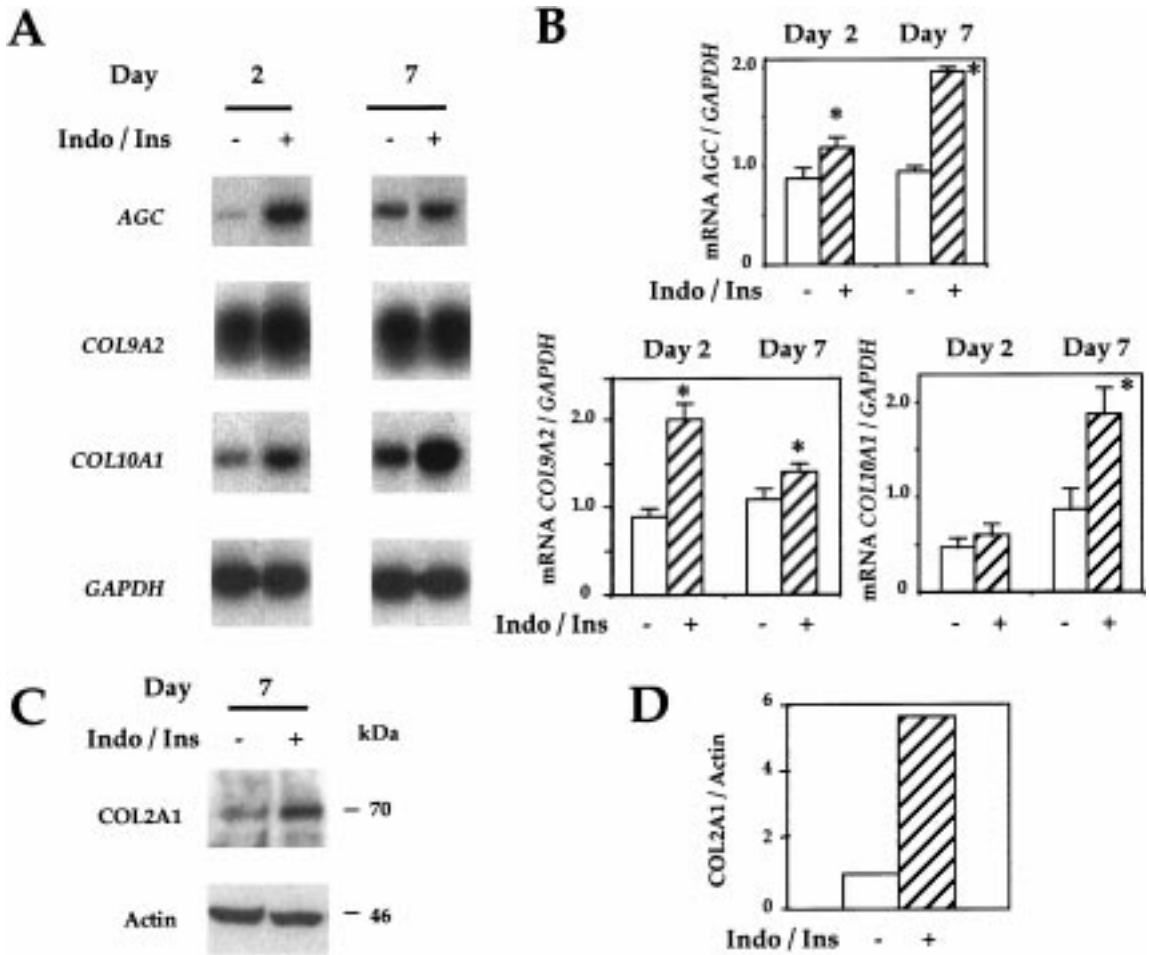

Fig. 4. Indomethacin/insulin enhances chondrocyte differentiation in F/STRO-1$^+$ A cells. The cells were treated with Indo/Ins (10 μM) for 7 days. **A**: Total cellular RNA was reverse-transcribed and cDNAs amplified by PCR to examine the expression of transcripts for aggrecan (AGC), types 9 (COL9A2) and 10 (COL10A1) collagen followed by Southern blotting. **B**: Densitometric analysis of mRNA levels after normalization with respective GAPDH. Values were expressed as the mean ± SEM of three independent experiments (*$P < 0.05$, significant differences from vehicle-treated control cells). **C**: Equal aliquots of total cell lysates were examined for type 2 collagen expression on a Western blot by probing with an anti-COL2A1 antibody, or with an anti-human β-actin antibody used as internal control. **D**: After detection, the levels were quantified by densitometry. Values are presented as arbitrary densitometric units after normalization to the corresponding β-actin band.

(Fig. 6E). Thus, Indo/Ins and Dex up-regulated early and late markers of adipocyte differentiation, but neither treatment was as effective as LA in inducing formation of oil-red stained adipocytes in F/STRO-1$^+$ A cells.

Indo/Ins and LA Repress Osteoblast Differentiation of F/STRO-1$^+$ A Cells

We then asked whether agents that enhance chondrocytic or adipocytic differentiation of F/STRO-1$^+$ A cells conversely modulate osteoblast differentiation. Treatment of F/STRO-1$^+$ A cells with either Indo/Ins or LA had negligible effects on constitutive expression of CBFA1, OC and ALP mRNAs (Fig. 7A,B). However, Indo/Ins completely abolished the stimulatory effect of Dex on expression of CBFA1, OC and ALP mRNA levels (Fig. 7A, B). Linoleic acid partially (50–80%) abrogated the enhancing effect of Dex on CBFA1, OC or ALP mRNA levels (Fig. 7A,B). These results were confirmed at the protein level. As shown in Figure 7C and D, both Indo/Ins and LA blocked the Dex-induced increase in Osf2/CBFA1 protein levels in F/STRO-1$^+$ A cells. These results show that Indo/Ins and LA repress the Dex-promoted osteoblast differentiation in F/STRO-1$^+$ cells at the time they

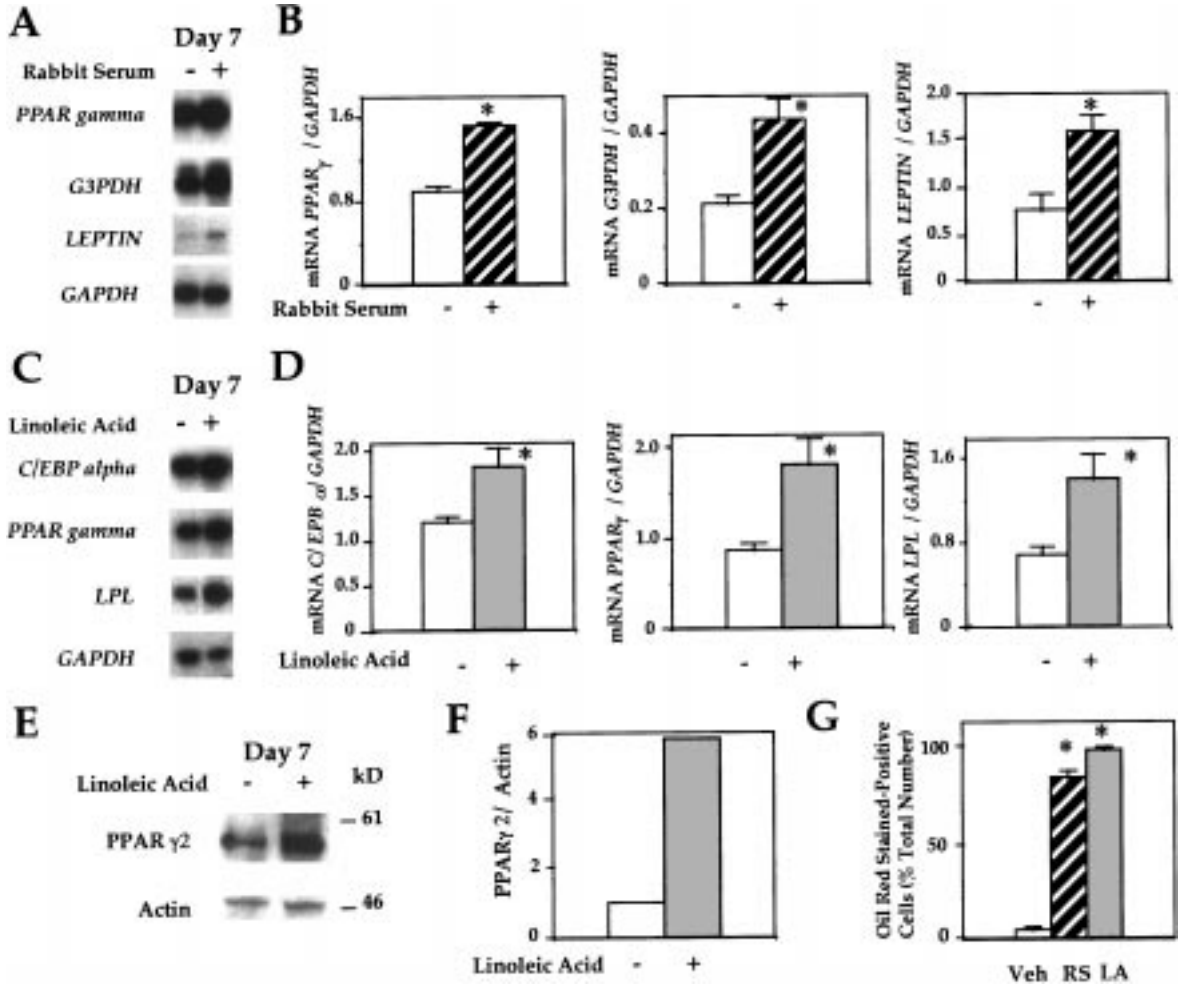

Fig. 5. Linoleic acid promotes adipogenesis and suppresses osteoblast differentiation in F/STRO-1^+ A cells. Cells were treated for 7 days with 10% rabbit serum (RS), linoleic acid (LA; 100 μM) or the vehicle (Veh). **A, C**, Total cellular RNA was reverse-transcribed and cDNAs amplified by PCR to examine the expression of PPARγ, G3PDH, LEPTIN, LPL or C/EBPα transcripts followed by Southern blotting. **B, D**: Graphs represent densitometric analysis of mRNA levels after correction for GAPDH. Values were expressed as arbitrary densitometric units and the mean ± SEM of three independent experiments is presented. **E**: Equal aliquots of total cell lysates were examined for PPARγ2 expression on a Western blot by probing with an anti-PPARγ2 antibody, or with an anti-human β-actin antibody used as internal control. **F**: After detection, the levels were quantified by densitometry. Values are presented as arbitrary densitometric units after normalization to the corresponding β-actin band. **G**: Cytoplasmic inclusions of neutral lipids were revealed by Oil Red O staining, the number of Oil Red O-positive stained cells was counted in three separate cultures and expressed as percentage (mean ± SEM) of total number of cells (>1000 cells). Asterisk indicates a significant difference with control vehicle-treated cells ($P < 0.05$).

promote chondrogenic and osteogenic differentiation, respectively.

Dex Suppresses Chondroblast Differentiation of F/STRO-1^+ A Cells

We also determined whether Dex or LA could likewise modulate chondrogenic differentiation of F/STRO-1^+ A cells induced by Indo/Ins. Treatment of F/STRO-1^+ A cells with Dex alone for 7 days completely abolished COL10A1 mRNA expression, a late marker of chondrocyte differentiation (Fig. 8A,B). In addition, Dex almost completely suppressed type 2 collagen protein levels produced by F/STRO-1^+ A cells in basal conditions (not shown). Furthermore, Dex completely inhibited the Indo/Ins-stimulated expression of COL10A1 mRNA (Fig. 7A,B), showing that Dex supressed late stages of chondrogenic differentiation in F/STRO-1^+ A cells. In contrast, LA had no effect on COL10A1 mRNA and did not prevent the inhibitory effect of Dex on COL10A1 mRNA expression (Fig. 7A,

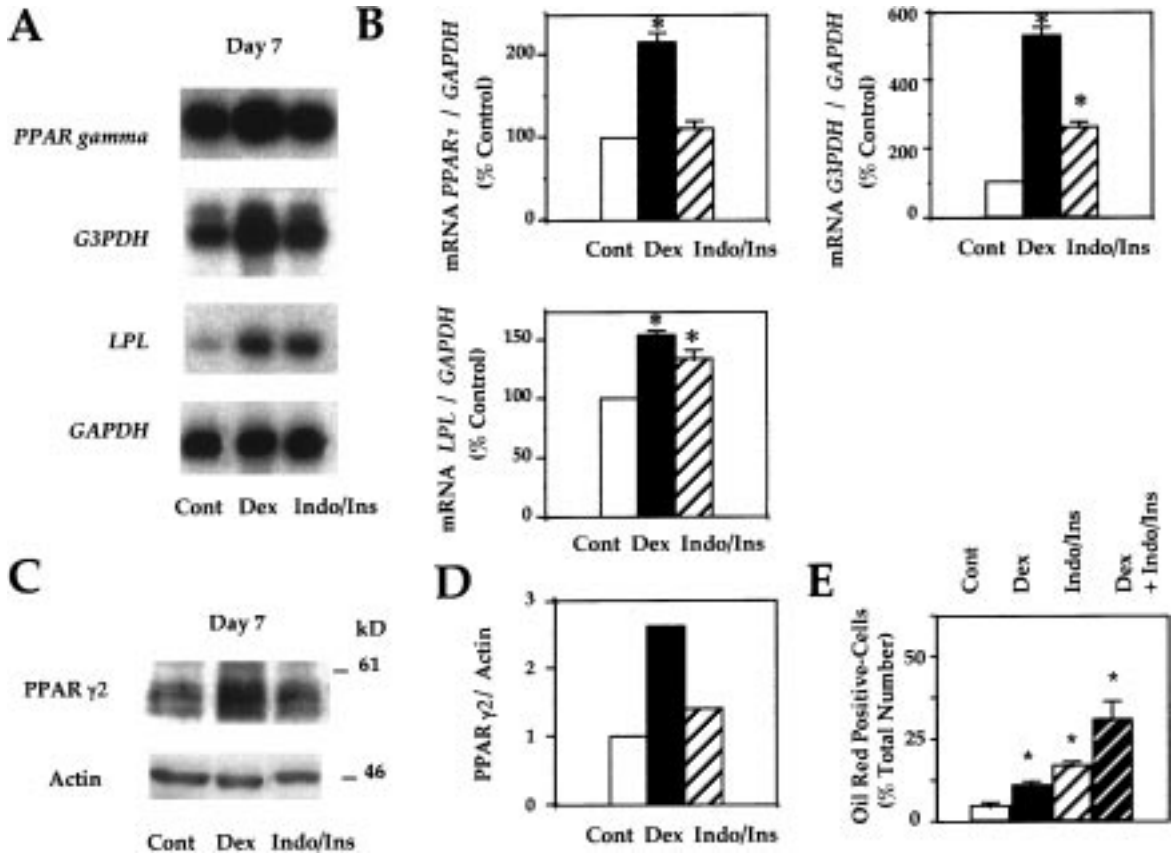

Fig. 6. Dexamethasone and indomethacin/insulin increase adipocyte differentiation markers in F/STRO-1$^+$ A cells. Cells were cultured for 7 days in the presence of dexamethasone (Dex) (0.1 μM), indomethacin/insulin (Indo/Ins) (1 μM) or the respective vehicles (Veh). **A**: Total cellular RNA was reverse-transcribed and cDNAs amplified by PCR to examine the expression of PPARγ, G3PDH and LPL transcripts followed by Southern blotting. **B**, Graphs represent densitometric analysis of mRNA levels after correction for GAPDH. Values were expressed as percentage of control (vehicle-treated cells) and the mean ± SEM of three independent experiments is presented. **C**: Equal aliquots of total cell lysates were examined for PPARγ2 expression on a Western blot by probing with an anti-PPARγ2, or with an anti-human β-actin antibody used as internal control. **D**: After detection, the levels were quantified by densitometry, and the values are presented as arbitrary densitometric units after normalization to the corresponding β-actin band. **E**: The number of Oil Red O-positive stained cells was counted and expressed as percentage of total number of cells (>1000 cells in three separate cultures; mean ± SEM). Asterisk indicates a significant difference with control cells ($P < 0.05$).

B). Thus, chondrocyte differentiation promoted by indomethacin/insulin is downregulated by Dex but not by LA. Altogether, these data provide evidence for a controlled reciprocal regulation of osteoblast/chondroblast, in addition to adipocyte/osteoblast differentiation in clonal F/STRO-1$^+$ A human marrow stromal cells.

DISCUSSION

In the present study, we show that the clonal human fetal bone marrow stromal F/STRO-1$^+$ A cell line is a lineage-unrestricted common progenitor that expresses tripotential characteristics and can differentiate toward the adipocyte, osteoblast or chondrocyte phenotypes in response to Dex, Indo/Ins and LA. We also demonstrate that the differentiation of this human clonal common progenitor cell towards one pathway restricts expression of other lineage-specific genes, and provide evidence that osteoblast and chondroblast pathways are reciprocally regulated.

A number of lines of evidence demonstrate the tripotentiality of F/STRO-1$^+$ A cells. Under basal conditions, F/STRO-1$^+$ A cells express CBFA1 and other genes that are markers of the osteogenic lineage. In addition, the cells formed a mineralized bone-like matrix when cultured as aggregates [Oyajobi et al., 1999], confirming the osteogenic potential of these cells. How-

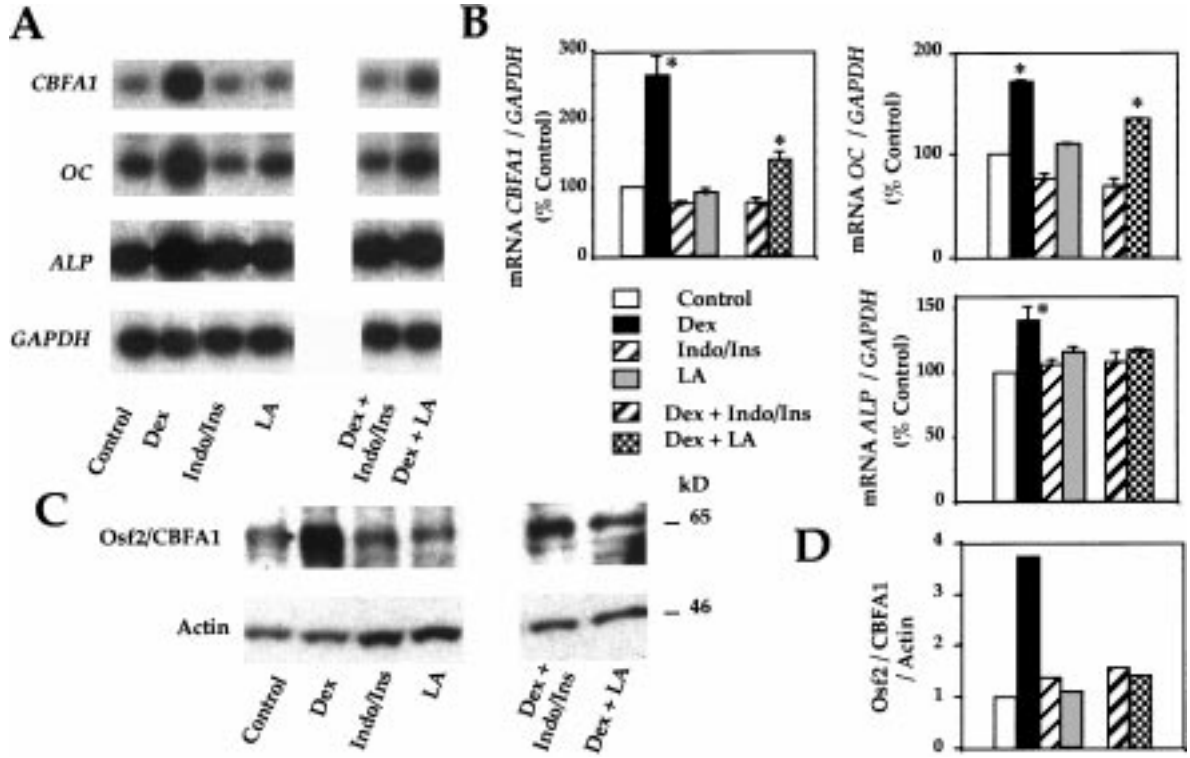

Fig. 7. Indomethacin/insulin and linoleic acid suppress osteoblast differentiation in F/STRO-1$^+$ A cells. Cells were cultured for 7 days in the presence of dexamethasone (Dex, 0.1 μM), indomethacin/insulin (Indo/Ins, 1 μM each) or linoleic acid (LA, 100 μM), or the combination of treatment. **A**: Total cellular RNA was reverse-transcribed and cDNA amplified by PCR to examine the expression of CBFA1, OC and ALP transcripts followed by Southern blotting. **B**: Graphs represent densitometric analysis of mRNA levels after correction for GAPDH. Values are expressed as percentage of controls (mean ± SEM of three independent experiments; $*P < 0.05$, significant difference from vehicle-treated control cells). **C**: Equal aliquots of total cell lysates were immunoblotted with an anti-Osf2/Cbfa1 antiserum or with an anti-human β-actin antibody used as internal control. **D**: After detection, the levels were quantified by densitometry, and normalized to the corresponding β-actin band.

ever, F/STRO-1$^+$ A cells are not osteogenic lineage-restricted precursor cells. We found that the cells express SOX9, a transcription factor necessary for chondrogenic commitment and cartilage gene expression [Bi et al., 1999; Sekiya et al., 2000]. The cells also expressed early markers of the chondrocyte lineage [Zhao et al., 1993; Vortkamp et al., 1996; Grimsrud et al., 1999; Healy et al., 1999] and functional cartilage specific genes [Hale et al., 1988; Gerstenfeld and Landis, 1991; Sandell et al., 1991], constitutively. The cells also synthesized type2 collagen as well as aggrecan and chondroitin and keratan sulfate GAGs, as judged by Alcian blue staining, confirming characteristics of cartilage cells. Furthermore, F/STRO-1$^+$ A cells showed characteristics of cells of the adipocyte lineage in basal conditions. In addition to express the transcription factors PPARγ2 and C/EBPα that are involved in preadipocyte differentiation and adipogenesis, the cells expressed aP2 and LPL, both of which are known PPARγ target genes, as well as G3PDH and LEPTIN which are late adipocyte differentiation-related genes [MacDougal and Lane, 1995; Wu et al., 1999]. Thus, this clonal cell line represents a human marrow stromal common osteoblast/chondroblast/adipocyte precursor cell. Although few tripotential cell lines derived from murine or human marrow stroma have been reported [Dennis et al., 1999; Pittenger et al., 1999; Muraglia et al., 2000; Negishi et al., 2000], the F/STRO-1$^+$ A cell line is the first immortalized clonal fetal human marrow stroma-derived cell line which expresses osteoblastic, chondrocytic and adipocytic phenotypes. Interestingly, Cbfa1, Sox9, and PPARγ2, which are important genes controlling osteoblast, chondrocyte and adipocyte differentiation, respectively [Komori et al., 1997; Healy et al., 1999; Wu et al., 1999], are co-expressed in F/STRO-1$^+$ A cells under steady-state con-

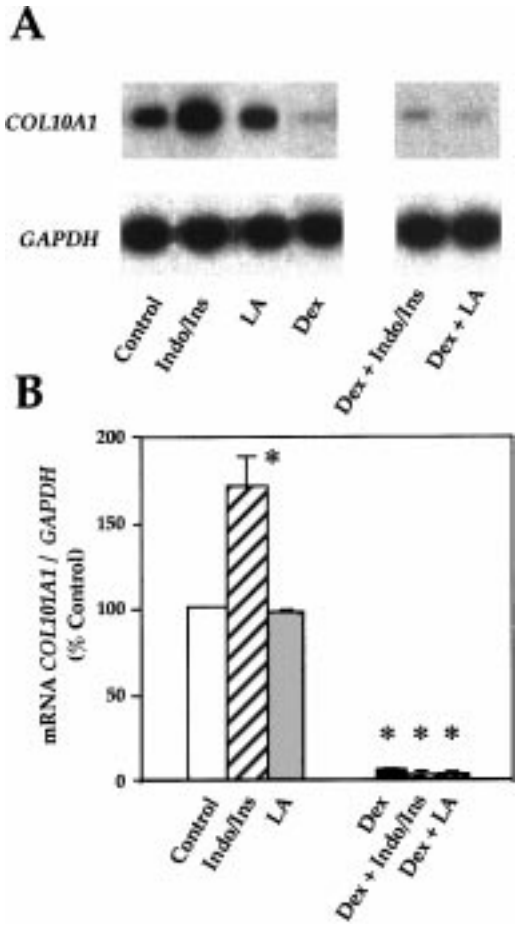

Fig. 8. Dexamethasone, but not linoleic acid, suppresses chondrocyte differentiation in F/STRO-1$^+$ A cells. Cells were cultured for 7 days in the presence of dexamethasone (Dex, 0.1 μM), linoleic acid (LA, 100 μM) or the combination of treatments. **A**: Total cellular RNA was reverse-transcribed and cDNA amplified by PCR to examine the expression of type 10 (COL10A1) collagen transcripts. **B**: Graph represents densitometric analysis of mRNA levels after correction for GAPDH. Values are expressed as percentage of controls (mean ± SEM of three independent experiments; $^*P < 0.05$, significant difference from vehicle-treated control cells).

ditions. Such multi-lineage 'priming' of gene expression ensures that genes of different lineages are co-expressed within cells of the same clone [Hu et al., 1997]. This lineage-promiscuous gene expression pattern observed in F/STRO-1$^+$ A cells provides evidence for their early progenitor status and a model to study the differentiation into distinct cell lineages in response to promoting agents.

Few agents were found to be able to promote the differentiation of F/STRO-1$^+$ A cells towards the distinct lineages. We found that Dex promoted osteoblastic differentiation, as shown by the increased expression of CBFA1, OC and ALP, which is consistent with prior studies in heterogenous rodent and human marrow stromal cell cultures [Beresford et al., 1994; Rickard et al., 1994; Cheng et al., 1996; Fromigué et al., 1998; Muraglia et al., 2000]. On the other hand, promotion of chondrocytic differentiation of F/STRO-1$^+$ A cells was achieved using a combination of indomethacin and insulin which enhanced the expression of both early and late chondrocyte marker genes, and increased type 2 collagen synthesis and sulfated GAG content of the ECM. In contrast to recent studies indicating that TGF-β or Dex can promote marrow stromal cell differentiation into cartilage-forming cells in vitro [Johnstone et al., 1998; Dennis et al., 1999; Pittenger et al., 1999], the differentiation of clonal common progenitor F/STRO-1$^+$ A cells into the chondrocyte lineage was strictly dependent on Indo/Ins stimulation. These methodological differences may result from species differences [Dennis et al., 1999] or may reflect distinct methodology to obtain human marrow stromal cell precursor cells [Johnstone et al., 1998; Pittenger et al., 1999]. Rabbit serum or linoleic acid are potent promoters of adipogenic differentiation in F/STRO-1$^+$ A cells, as shown by the increased expression of adipocytic-specific genes and massive accumulation of cytoplasmic Oil-Red O-staining lipid droplets. Although Indo/Ins and Dex also increased expression of adipocyte differentiation-related genes, adipogenic differentiation was incomplete, probably because other genes that are involved in mature adipogenic differentiation [MacDougal and Lane, 1995; Wu et al., 1999] were not activated by these treatments. Thus, complete differentiation of the common precursor F/STRO-1$^+$ A cell line towards at least three distinct lineages can be achieved under controlled conditions.

Of major interest is our finding that differentiation of F/STRO-1$^+$ A cells towards one lineage was associated with restricted expression of other lineage pathways. Although Dex promoted adipocyte genes, the expression of CBFA1 and osteocalcin still persisted, indicating that the moderate stimulation of adipocyte differentiation by Dex did not repress the expression of osteoblast markers. In contrast, forced differentiation of F/STRO-1$^+$ A cells into mature adipocytes by LA resulted in complete inhibition of CBFA1, OC and ALP expression with all of the cells becoming fat cells. Thus,

differentiation towards mature adipogenesis suppressed osteoblastogenesis in F/STRO-1^+ A stromal cells. This effect may be mediated by the increased expression of PPARγ2 which was found to repress CBFA1 expression and other osteoblast genes in PPARγ2-transfected murine bone marrow-derived cells [Hicok et al., 1998]. In addition to confirm the inverse relationship between osteoblast and adipogenic differentiation previously found in human and murine osteo-adipocytic progenitor cells [Beresford et al., 1992; Thompson et al., 1998; Gori et al., 1999], our data indicate that separation of the two lineages probably occurs at a late stage during adipogenic differentiation. We also demonstrate that, whilst promoting osteoblast differentiation in F/STRO-1^+ A cells, Dex inhibited steady-state COL10A1 mRNA expression and abrogated the Indo/Ins-induction of chondrocyte markers, showing that Dex negatively regulates chondrocyte maturation in F/STRO-1^+ A cells. Conversely, we found that, besides enhancing the expression of chondrocyte differentiation, treatment with Indo/Ins abolished the Dex-induced expression of CBFA1 and OC in F/STRO-1^+ A cells. Thus, Indo/Ins promotes chondrogenic differentiation whilst simultaneously restricting differentiation towards the osteoblastic lineage. This reciprocal regulation between chondroblast and osteoblast ontogeny complements the inverse relationship between osteoblast and adipocyte pathways. This dual reciprocal control of the osteoblast/adipocyte and osteoblast/chondrocyte differentiation of human fetal common precursor cells in the marrow stroma is likely to be of major importance in the control of skeletal growth and postnatal remodeling, because endochondral bone growth, bone formation and bone repair processes are all strongly dependent on the appropriate differentiation of marrow stroma-derived precursor cells into bone and cartilage lineage pathways [Ham and Harris, 1971; Pechak et al., 1986; Caplan, 1991].

In summary, we demonstrate here that the clonal fetal human bone marrow stromal F/STRO-1^+ A cell line is a lineage-unrestricted common progenitor that can differentiate into osteoblasts, chondrocytes or adipocytes in response to Dex, Indo/Ins or LA. Moreover, differentiation of this common tripotential precursor cell into one lineage restricts expression of other lineage specific genes. Our data also provide evidence for a dual controlled reciprocal osteoblast/chondroblast and osteoblast/adipocyte differentiation of clonal F/STRO-1^+ A common precursor cells in the human bone marrow stroma.

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