Journal of Cellular Biochemistry 98:1457–1470 (2006)

Functional Characterization of Human Mesenchymal Stem Cells That Maintain Osteochondral Fates

Marina Romero-Prado,[1] Cristina Blázquez,[2] Carmen Rodríguez-Navas,[1] Jaime Muñoz,[1] Isabel Guerrero,[3] Emilio Delgado-Baeza,[2] and Josefa P. García-Ruiz[1]*

[1]Departamento de Biología Molecular, Centro de Biología Molecular, ''Severo Ochoa,'' Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain
[2]Hospital Universitario ''La Paz,'' Universidad Autónoma de Madrid, 28029 Madrid, Spain
[3]Centro de Biología Molecular ''Severo Ochoa,'' Cantoblanco, 28049 Madrid, Spain

Abstract Adult stem cells are essential for tissue renewal, regeneration and repair, and their expansion in defined culture medium is on focus for regenerative medicine and genetic pathologies. The bone marrow has been shown to be very rich is pluripotent mesenchymal stem cells (MSCs) capable of forming bone, cartilage and also may give rise, to neurons and astrocytes in vivo and in vitro. MSCs can be isolated and expanded in culture, but human cells cannot be verified for a cartilage or a bone fate by transfer experiments. Accordingly, here we used different approaches to characterize hMSCs osteoblastic differentiation in vitro. hMSCs grown in culture in the presence of fetal bovine serum (FBS) expressed the bone-specific transcription factor Runx2/AML3. When cells were incubated in osteoblastic differentiation medium, cells expressed transcripts belonging to the signaling of Indian HH-PTHrP axis, GLI transcription factors, and bone target genes including osteopontin. The HH pathway proved to be functional since it induced cells to grow. Cells growing or differentiating to osteoblasts presented the Runx2/AML3 transcription factor, its partner CBFB, and Smad2/3 at the nuclei associated with the nuclear matrix. Furthermore, Runx2/AML3 was observed to co-localize with SC35 to the nuclear intermediary filaments. These data support the notion that hMSCs isolated from human bone are or become bone progenitor cells upon culture. In the absence of FBS and in the presence of insulin or prolactin, cells show cytoskeletal organization and an AP-1 transcription site activity resembling proliferative osteochondrocytes while cells in the presence of dexamethasone and added prolactin or TGF-β resembled differentiated osteoblasts. These specific cellular conditions match those observed during endochondral bone formation. J. Cell. Biochem. 98: 1457–1470, 2006.

Key words: Indian HH signaling; Runx2/AML3; bone marrow-derived; mesenchymal stem cells; bone progenitor cells

Mesenchymal stem cells (MSCs) are a subset of bone marrow cells that are capable of self-renewal and of forming chondrocytes, osteocytes, and adipocytes (multipotential) [Caplan, 1987; Lennon et al., 1996; Pittenger et al., 1999; Jiang et al., 2002]. The characterization of these cells holds significant promise for basic science in cell biology, for understanding developmental/regeneration processes, and also in clinical medicine [Aggarwal and Pittenger, 2005; Le Blanc et al., 2005]. However, the lack of distinctive characteristics makes it very difficult to study stem cells in their natural context in tissues. It has been shown that skin and hair follicles depend on the self-renewal capacity of long-lived stem cells. Regeneration is accomplished by a bi-potent cell that can be induced to migrate downwards to the hair follicle as well as outwards to resurface at the epidermis in response to wounding [Morris et al., 2004; Tumbar et al., 2004]. These results were obtained by different approaches to overcome that epithelial stem cells cannot be tested readily in vivo for functional competence. The best characterized stem cells in the mammalian

Grant sponsor: Comunidad Autónoma de Madrid, Fondo de Investigación Sanitaria; Grant sponsor: The Ramón Areces Foundation.

*Correspondence to: Josefa P. García-Ruiz, Departamento Biología Molecular-Centro de Biología Molecular "Severo Ochoa," Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain.
E-mail: pgarcia@cbm.uam.es

Received 29 November 2005; Accepted 30 November 2005
DOI 10.1002/jcb.20778

blood-forming system are the clonogenic, multipotent hematopoietic stem cells (HSC) that have been prospectively isolated from bone marrow and demonstrated at the single-cell level the capacity to regenerate the entire hematopoietic system [Kondo et al., 2003]. However, human MSCs are less known and given the serious implications of the possible contribution to neuronal lineages and cancer [Beachy et al., 2004; Massengale et al., 2005], a rigorous functional characterization of hMSCs is needed before recommending their use in clinical medicine. What it is becoming clear is that stem cells isolated from adults divide infrequently and that their fate is influenced by specialized microenvironments or "niches" that house and regulates the stem cell pool. After an in vivo insult or a culture-expansion, cells divide to give rise to more proliferative daughter cells that eventually lose their proliferative capacity and undergo terminal differentiation.

According to Calvi et al. [2003] and Zhang et al. [2003], in the bone marrow of adult animals HSC are located in niches where osteoblasts synthesize many molecules of the extracellular matrix together with regulatory proteins. These two groups used different genetic strategies to increase the size of the osteoblast pool and this caused a parallel increase in the HSC population. Recently, it has been identified osteopontin as the component synthesized by osteoblasts in the HSC niche that negatively regulates the HSC pool size [Stier et al., 2005]. Although the exact localization of MSCs in the bone marrow of adult humans is unknown it is well demonstrated that MSCs are responsible for the development of the axial and appendicular skeleton in vertebrates. To this end, MSCs first condense and form a cartilaginous template of the bones that is surrounded by several layers of fibroblast-like cells conforming the perichondrium [Long et al., 2004]. In most condensations, cells become chondrocytes with proliferative capacity. Chondrocytes at the center of the cartilaginous template stop proliferating and become hypertrophic chondrocytes, directing adjacent perichondral cells to become osteoblasts forming the bone collar, vascularization, and bone mineralization. In long bones, the growth of chondrocytes continues at the growth plate, which in humans disappears at the time of adolescence while in experimental animals, this is not the case. This causes difficulties to extrapolate skeletal development/repair data from experimental animal to human, as well as the fact that hMSCs, as occurs with epithelial stem cells, cannot be verified for functional competence in vivo.

We approach the characterization of hMSCs that maintain osteochondral fate by experiments of osteoblastic differentiation in vitro. We determined the expression of the regulatory loop Hedgehog (Hh)-Paratohormone-related peptide (PTHrP) and bone target genes. Cells express Indian hedgehog (IHh), one of the three mammalian homologs of the *Drosophila* Hedgehog (hh) protein, that is a key regulator of the endochondral skeleton [Vortkamp et al., 1996; St-Jacques et al., 1999]. IHh is expressed by pre- and early hypertrophic chondrocytes, where it is a potent positive regulator of chondrocyte proliferation and controls chondrocyte hypertrophy via PTHrP [Karp et al., 2000; Long et al., 2004]. IHh cellular signaling is mediated by the Ptc and Smo proteins and the Gli zinc-finger transcription factors [Mullor et al., 1997; Palma and Ruiz i Altaba, 2004]. This work shows that during hMSC osteoblastic differentiation the cells express transcripts of the regulatory loop Indian HH-PTHrP and bone transcripts such as osteopontin and osteocalcin. Results show that hMSCs express the bone- and cartilage-specific transcription factor Runx2/AML3/Cbfa. In addition, this work shows the organization of the cytoskeleton, the presence of CBFB, Smad2/3 at the nucleus and the how Runx2/AML3 co-localized with the SC35 splicing factor at the nuclear matrix. These probes characterize human bone marrow-derived MSCs, verifying that they are osteochondro progenitor cells after in vitro expansion.

MATERIALS AND METHODS

Cell Culture

Two to four milliliters of human bone marrow samples from healthy donors were provided by Dr. Rañada from the *Hospital Universitario La Princesa* (Madrid, Spain). The isolation and culture expansion of hMSCs from 36 healthy donors were carried out, as previously described [Lennon et al., 1996; Ogueta et al., 2002]. Cells were collected by centrifugation on 70% Percoll gradient and seeded at 200,000/cm^2 in DMEM-LG supplemented with 10% FBS. hMSCs osteoblastic differentiation was performed following

the methodology used in previous work [Pittenger et al., 1999]. Briefly, aliquots of 8×10^5 hMSCs were plated and incubated with DMEM-LG plus 10% FBS during 14–16 h. Adhered cells were then washed with PBS and incubated in differentiation medium (C), DMEM-LG with ITS (6.25 μg/ml insulin; 6.25 μg/ml transferrin; 6.25 μg/ml selenous acid) (Collaborative Research), 1 mM pyruvate (Gibco); 37.5 μg/ml ascorbate (WAKO), at 37°C in 5% CO_2. Cells were treated with 0.1 μM dexamethasone (Decadran, Merck), 0.06 ng/ml TGF-β (R&D System), 40 ng/ml human recombinant PRL (hrPRL) (generously provided by Dr. Martial, Belgium), or a combination thereof for 1 week. The medium was replaced twice per week.

Proliferation Assays

Proliferation was determined by measuring the incorporation of (^{3}H)-thymidine to hMSCs. Cells (15×10^3) were seeded in M24 culture dishes and cultured in DMEM-LG supplemented with 10% FBS for 16 h. Then, cells were starved for 48 h and pulsed with 1 μCi of (^{3}H)-thymidine for the last 8 h. The functionality of the hh signaling pathway was assessed by the addition or not to cells of rmSonic hh at 0.3 μg/ml from Ontogeny or 10% FBS, or retinoic acid at 100 nM (Sigma-Aldrich) as positive controls. Each treatment was assayed in triplicate. Cells were harvested, after two of freeze-thaw cycles, with an automatic collector and radioactivity was measured in a beta scintillation counter. The results are expressed in cpm. Values are means of three separate experiments ± SEM (error bars).

In Situ Immunofluorescence and Digital Microscopy

hMSCs were plated on 0.5% gelatine-coated coverslips (40×10^3 cells/slip) (bovine skin, Sigma), cultured in medium C and treated or not with the above described factors over 1 week. Cells were processed as described previously [Javed et al., 2000], with some modifications. Briefly, for whole cell preparations cells were rinsed twice with ice-cold PBS and fixed in 3.7% formaldehyde in PBS during 15 min at RT. Then, cells were permeated in 0.1% Triton X-100 in PBS and blocked for immune staining. For cytoskeleton (CSK) preparations, cells were incubated with 0.5% Triton in CSK buffer containing 10 mM pipes, pH 6.8, 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 0.3 M sucrose, and 1 mM PMSF for 30 min on ice. Then, proteins were extracted by washing with CSK buffer. For nuclear matrix preparations, DNA was digested. To this end, cell preparations were depleted of soluble proteins as described above, and incubated with 1% NP-40, 0.5% deoxycholate acid twice for 2 min. They were then washed with 0.3 mM imidazole in H_2O, incubated at room temperature with DNase I (50 IU/ml) in CSK buffer, twice for 30 min, and cell preparations were washed with CSK containing 0.25 M $(NH_4)_2SO_4$. To detect NM-IF, the procedure was as with the NM preparations but protein washing was accomplished using 2 M NaCl in CSK buffer during 5 min. The preparations were then fixed with 3.7% formaldehyde. For cytoskeleton, nuclear matrix and NM-IF, the preparations were blocked with 1% BSA-0.1% Triton X-100 in PBS (PBSA) for 2 h at RT and exposed to Runx2/AML3 antibody (1/500, Oncogene), CBFB and Smad2/3 antibodies (1/200, Santa Cruz), phalloidin (1/100, Molecular Probes), serum from bGH-Tg mice (1/100) [Ogueta et al., 2000], and anti-SC35 [Fu and Maniatis, 1990] (anti-speckles monoclonal antibody (1/50) provided by Dr. I. Correas from the Severo Ochoa Molecular Biology Centre for 1 h. Cover slips were washed four times with PBSA. Secondary antibodies were either Alexa488 goat anti-rabbit or Alexa594 goat anti-mouse (Molecular Probes, Eugene, OR). These antibodies were used at 1/500 dilution. When required, nuclei were stained with Hoestch (1/5,000) for 5 min and then washed three times with PBS 0.1% Triton X-100. Immune staining of cell preparations was detected using a laser scanning confocal microscope LSM510 META coupled to an inverted Axiovert 200 (Zeiss) microscope from the CBMSO Microscopy Service.

Reverse Transcription (RT)-PCR

RNA from 1.5×10^6 cells was extracted and used for RT reactions as previously described [Ogueta et al., 2002]. The conditions for each PCR determination, gene, primer sequences (GeneBank), temperature, number of cycles and DNA-band sizes were as follows: Indian HH, gaggagtccctgcattatga (111–130) and caggaaaatgagcacatcgc (431–412) (XM_050846), 54°C, 35, 321-bp. Sonic Hh, gaagatctccagaaactcc (343–361), and tcgtagtgcagagactcc (576–558) (NM_000193), 57°C, 35, 234-bp. PTC, tttggactgcttctgggaaggg (681–702) and tttttgt-

tgggggctgtggc (923–904) (U43148), 57°C, 35, 243-bp. SMO, caggacatgcacagctacatcg (543–564) and ccacaaagaagcacgcattgac (887–865) (AF114821), 53°C, 35, 345-bp. GLI1, tgcagccaatacagacagtgg (1449–1469) and cagtataggcagagctgatgc (1741–1721) (NM-005269), 57°C, 35, 293-bp. GLI2, cacaagcgcagcaaggtcaag (701–722) and cagcctcctgcttacagtcat (827–807) (AB007296), 57°C, 35, 127-bp. GLI3, cagctccacgaccactga (72–89) and gtccatggcaaacaccgt (390–373) (M57609), 60°C, 35, 319-bp. PTHrP cttggaagaggtacctgctttc (2193–2215) and ggaaagttgattccacacaccc (4863–4842) (M57293), 53°C, 35, 507-bp. Sox9, cgatctgaagaaggagagcga (533–553) and cgttcttcaccgacttcctcc (912–892) (Z46629) 54°C, 35, 380-bp. Col1A1, aatcacctgcgtacagaacgg (230–250) and cctccggttgatttctcatca (640–620) (NM_000088), 53°C, 35, 411-bp. OP-1, ctaggcatcacctgtgccatacc (139–161) and cttgtggctgtgggtttcagc (789–769) (J04765), 59°C, 35, 651-bp. Runx2/AML3, cgtattcctgtagatccgagc (4–24) and ggggaggatttgtgaagacgg (583–563) (NM_004348), 56°C, 35, 582-bp. OC, agcagggtgcagcctttgt (110–128) and acaggtagcgcctgggtct (178–160) (NM-000711), 54°C, 35, 69-bp. β-Act, gcatggagtcctgtggcatccacg (844–867) and gggtgtaacgcaactaagtcatag (1198–1175) (X00351), 60°C, 35, 357-bp.

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared as described previously [Olazabal et al., 2000]. TGF-β-responsive element, TβRE, [Zaidi et al., 2002] 5′-caccacagccagaccacaggcagaca-3′ (sense) 5′-tgtctgcctgtggtctggctgtggtg-3′ (antisense), or the mutants M1, 5′-caggacagccagaccacaggcagaca-3′ (sense), 5′-tgtctgcctgtggtctggctgtcctg-3′ and M2, 5′-caccacagccagaccacaggcatcca-3′ (sense) and 5′-tggatgcctgtggtctggctgtggtg-3′ (antisense). AP-1 binding site activity was determined as described earlier [Olazabal et al., 2000]. Twenty nanograms of ssDNA probe was labeled using T4-PNK enzyme (New England Biolab) following the manufacturer's indications. After labeling, ^{32}P-ssDNA probes were annealed with their respective complementary antisense DNA, and filtered in a sephadex-G-50 column equilibrated with 10 mM Tris-HCl, pH 7.4. DNA-protein-binding reactions were conducted in a 20 μl volume. Reaction mixtures were composed of 0.25 μg poly (dI-dC) (Sigma, St. Louis, MO), 2.5 μg nuclear protein extracts, 0.25 μg denatured salmon sperm DNA, 4 μg BSA, 0.15 ng ^{32}P-probe ($100–150 \times 10^3$ cpm), and 4 μl (5×) binding reaction buffer. The binding buffer was composed of 20 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 8% glycerol, 0.1 mM PMSF, 1% NP-40, 1 mM DTT, and a mixture of protease inhibitor from Roche. Binding reactions were carried out at 4°C during 30 min. For supershift analysis, 1 μg of the following antibodies was used: Runx2/AML3 (FL-182), Smad2/3 (N-19), *c*-Jun (H-79), and c-Fos (K-25) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies were added after the first 15 min of incubation after which samples were left at room temperature for 30 min. Reactions were resolved on 4% non-denaturing polyacrylamide gel and Tris-glycine buffer (prerun at 30 V for 20 min) using a PowerPac300 (BioRad). The loaded gels were run at 60 V during 60 min, dried, and exposed on X Omat films (Eastman Kodak, Rochester, NY).

RESULTS

Transcripts of the Indian Hh-PTHrP Regulatory Loop Are Expressed During Osteoblastic Differentiation of hMSCs

Eight different batches hMSCs maintained in culture were assayed for the expression of Sonic HH (SHH) and IHH by RT-PCR and the results were negative. We then assessed whether hMSC osteoblastic differentiation bears any relationship with the endogenous expression of genes involved in endochondral bone formation. Thus, we determined the transcripts of the HH-PTHrP signaling pathway and bone target genes by comparative RT-PCR. The effect of PRL, Dex, or a mixture thereof was also assessed and compared with the effect of TGF-β plus Dex. To this end, $1–1.5 \times 10^6$ hMSCs grown to 70%–80% confluence were deprived of FBS and incubated in DMEM-low glucose medium, ITS (insulin, transferrin, selenium) and ascorbic acid over 1 week (control medium, C). Aliquots of these cells were treated with TGF-β plus Dex (T + D) [Pittenger et al., 1999], Dex (D), prolactin (P), or prolactin plus Dex (P + D) [Ogueta et al., 2002]. We determined the transcripts of: (i) members of the HH family (Indian and Sonic), Paratohormone-related peptide (PTHrP); PTC receptor, SMO and GLI1, GLI2 and GLI3 (Fig. 1A); and (ii) bone target genes such as collagen Col1A1, osteopontin (OPN), and osteocalcin (OC), and bone transcription factors such as Runx2/AML3 and Sox 9 (Fig. 1B). Determinations of the transcripts of β-actin were carried out in all

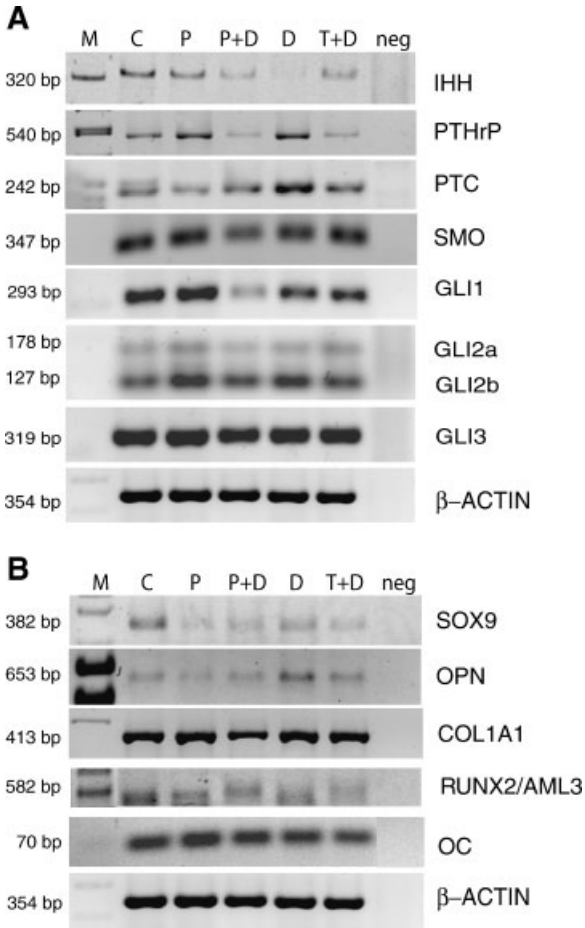

Fig. 1. Expression of IHH and bone genes in hMSCs differentiating to bone by RT-PCR. Total RNAs were isolated from 1×10^6 cells incubated for 1 week in chemically defined medium in the absence (C) or in the presence of the indicated regulatory factors: 50 ng/ml prolactin (P); 100 nM dexamethasone (D); and 0.06 ng/ml TGF-β (T). Negative image of ethidium bromide-stained agarose gels showing the expected PCR products. **A**: *HH* genes and (**B**) bone target genes. Lane neg shows a control of RT reactions. The specific primers used are described in Methods section.

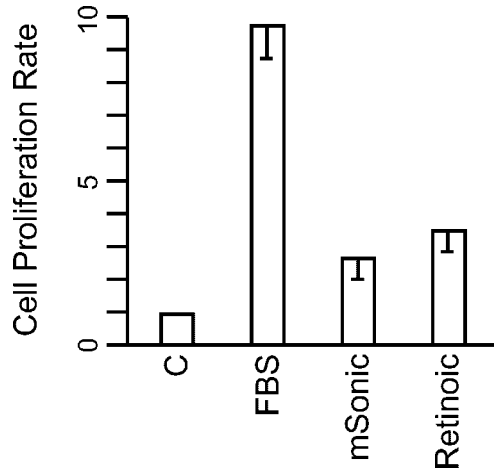

Fig. 2. Activation of Hedgehog signaling pathway induces proliferation of hMSCs. Cells (1.5×10^4) were seeded, cultured for 16 h, starved over the following 48 h and treated or not with 10% FBS, recombinant mSonic (0.3 ng/ml) or Retinoic acid (100 nM). Cells were pulsed for the last 16 h of incubation time with ^{3}(H) thymidine μCi/ml), harvested, and quantified in a liquid scintillation counter. Results are expressed as rates of cell proliferation with respect to controls. Values are means of three separate experiments ± SEM (error bars).

samples and experiments for normalization as well as RT negative control (lane neg). It was observed that either the composition of C medium or the endogenous ligands produced by the cells were sufficient to induce the expression of the transcripts of the IHH (Sonic was not found)-PTHrP regulatory loop, PTC, SMO, GLIs, and bone target genes. Interestingly, only small, non-significant differences were detected by cell treatment with P, PD, D, or TD. The results were in agreement with our previous data on chondrogenesis [Ogueta et al., 2002] and suggest that bone marrow-derived hMSCs would osteochondroprogenitor cells. In addition, our present data indicate that PRL, Dex, TGF-β, or mixtures thereof do not alter cell specification.

In order to determine whether the IHH signaling pathway was active cells were treated with rmShh. As shown in Figure 2, SHh addition to cells opened the HH signaling pathway and duplicated the proliferation rate of hMSCs. This result is consistent with the functionality of the IHH pathway for triggering the activation of the GLI transcription factors.

Electrophoretic Mobility Shift Analysis of Active TβRE and AP-1 Sites

It has been established that Runx2/CBFA1/AML3 transcription factor is required for bone development and osteoblast differentiation [Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Nakashima et al., 2002; Stein et al., 2004]. Runx proteins interact through their C-terminal end with Smads and recruit these transcription factors to the active transcription sites [Zaidi et al., 2002]. Runx2/AML3-Smad complexes interact with the TGF-β-responsive element (TβRE), which comprises two Runx2/AML3 and two Smad consensus sequences. Accordingly, we next tested the binding activity of these transcription factors to

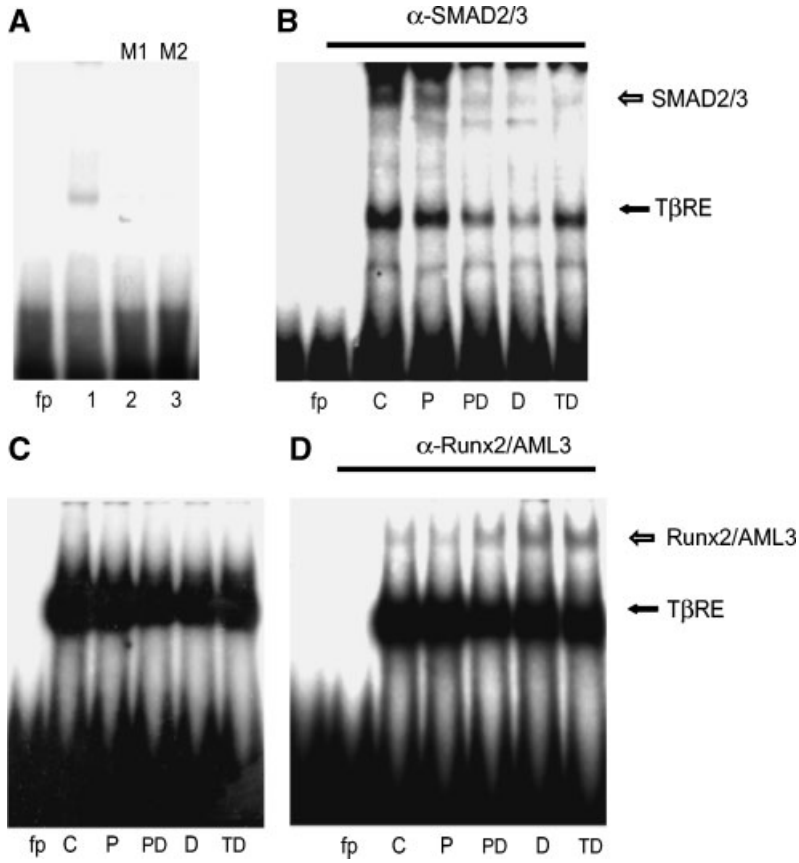

Fig. 3. Transcriptional activity of TβRE sites in osteoblastic differentiating cells. Nuclear extracts were prepared from cells incubated for 1 week under conditions C, P, PD, D, and TD as in Figure 1. **A**, **C**: Each nuclear extract (2 μg) was analyzed by EMSA using a ^{32}P TβRE probe (**C**) or M1 and M2 mutated probes (**A**). **B**, **D**: nuclear extracts were analyzed by supershift assays using anti-Smad2/3 (B) and anti-Runx2/AML3 (D) antibodies respectively. An equal amount of a non-specific antibody (0.2 mg) was used and analyzed in the first lane of B and D. Arrows indicate retarded complexes. The figure shows a representative experiment out of a total of three performed.

TβRE site in nuclear extracts from hMSCs-differentiating osteoblasts by EMSA. As can be observed in Figure 3A (lane 1), a single complex was retarded from control nuclear extracts incubated with the TβRE probe while incubation with the M1 (Fig. 3A, lane 2, and the M2 (Fig. 3A, lane 3) probes partially blocked their respective complex-binding activity. We observed that the binding activity in nuclear extracts from C- and P-treated cells was higher than in those from PD, D, or TD treated cells (Fig. 3C). The participation of both Runx2/AML3 and Smad2/3 in the retarded complexes was determined by supershift analysis using antibodies directed against Runx2/AML3 or Smad2/3. As shown in Figure 3D, the addition of Runx2/AML3 antibody to the binding reaction mixture containing TβRE probe did not produce a super-retarded band while the presence of nuclear extracts from osteoblastic differentiating cells caused a supershifted band of decreased mobility. The activity of super-retarded bands was higher in lanes PD, D, and TD than in C and P. These results suggest that glucocorticoids affect either the composition of the TβRE retarded complex, or alter antigenic domains exposed in the complex, or both. Interestingly, Smad2/3 supershift analysis revealed a reduction of TβRE retarded band and a major super-retarded complex in nuclear extracts from C and P, Figure 3B. These results suggest that in the presence of glucocorticoids other proteins must participate in the complex. Since many proteins, such as the transcription co-activator, p300 or NCo-140, associate with this transcription factor [Stein et al., 2004] further work is needed.

Several lines of evidence have shown that in addition of Runx2 other transcription factors from the AP-1 family [Kenner et al., 2004] [Eferl

et al., 2004] play an important role in bone development. Additionally, it is also known that PRL, TGF-β and Dex regulate the activity of AP-1 transcription sites [Olazabal et al., 2000; Palcy et al., 2000]. Thus, we considered it of interest to explore the activity of AP-1 sites in nuclear extracts of osteoblastic differentiating hMSCs by EMSA and supershift assays. A specific band was retarded, corresponding to the AP-1 complex in nuclear extracts of C cells (Fig. 4A), which disappeared upon incubation with a 50-fold molar excess of unlabeled probe. It was observed that AP-1 activity was higher in nuclear extracts of cells maintained in basal differentiation medium C or in cells treated with prolactin P (Fig. 4A, lanes C and P) while decreased activity was detected when Dex was present in the incubation medium (Fig. 4A lanes, PD, D, and TD). We also observed that c-fos was the major component of the AP-1 retarded complex (Fig. 4B), while c-Jun was only detected in nuclear extracts from C and P (Fig. 4C). These results add new evidence for the osteoblastic cell lineage of bone marrow-derived hMSCs.

Cytoskeletal Organization

The ability of TGF-β to modulate cell morphology and the actin cytoskeleton has been reported previously for a variety of cell types [Moustakas and Stournaras, 1999]. It is also known that dexamethasone causes the reorganization of the cell tight junctions, preventing the formation of F-actin [Guan et al., 2004]. Thus, we decided to study the organization of the cytoskeleton during hMSCs osteochondral differentiation, as well as the effect of PRL. To accomplish this, we determined actin cytoskeleton rhodamine–phalloidin staining by direct fluorescence. We also determined the organization of microtubules/microtubule-associated proteins by indirect fluorescence using the serum from bGH-transgenic mice [Ogueta et al., 2000]. To this end, hMSCs were seeded on gelatin and aliquots were differentiated using the experimental conditions shown in Figure 1 or were maintained with FBS (F) for 1 week (Fig. 5). It was observed that cells treated with PRL (P) showed a fibroblastic morphology, with abundant actin stress fibers as in C- and F-treated cells. Treatment of hMSCs with dexamethasone (D) resulted in an alteration of the overall cell shape, which became rounded, spindle-shaped and flat cells. D-treated cells show actin stress fibers emanating from abundant focal adhesions, which were supported by concomitant changes in the organization of the actin cytoskeleton. These D-induced alterations

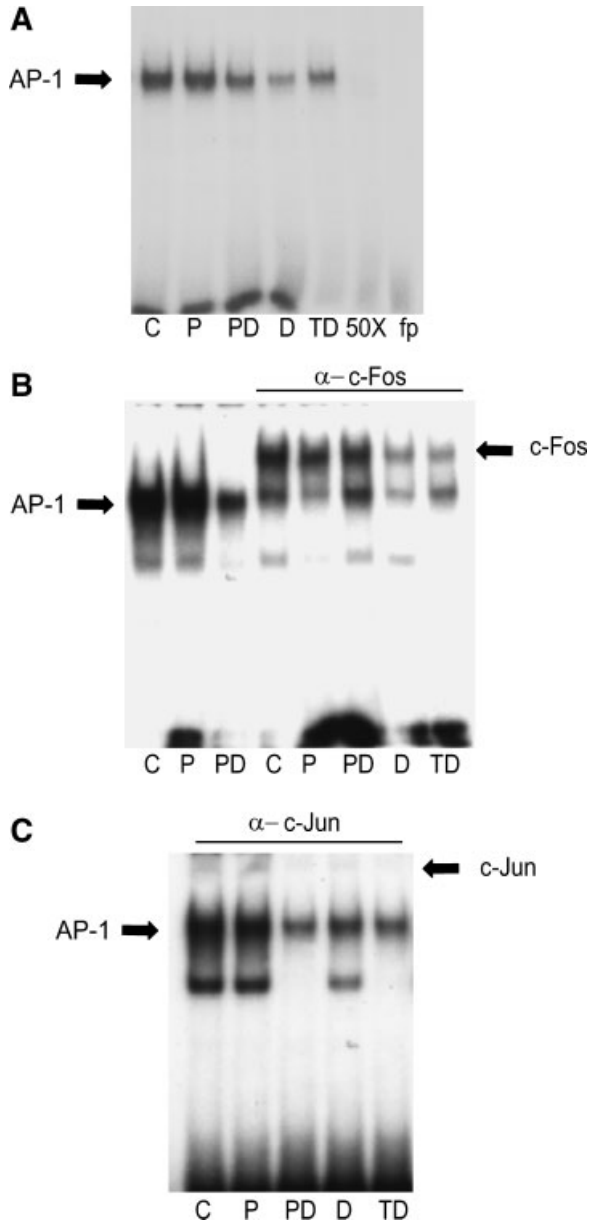

Fig. 4. Transcription activity of AP-1 sites in osteoblastic differentiating cells. Nuclear extracts (2 μg) were also analyzed by EMSA using ^{32}(P)-AP-1 probe. A 50-fold excess of unlabeled AP-1 consensus probe was used as specific competitor. **A**: The retarded complex of a representative experiment is shown in lanes C, P, PD, D, TD, Fp (free probe), and 50× (specific competitor). **B**: nuclear extracts were analyzed by supershift assays using anti-c-Fos and anti-*c*-Jun **C**. Arrows indicate the retarded AP-1 complex and the supershifted complexes. Three independent experiments were performed.

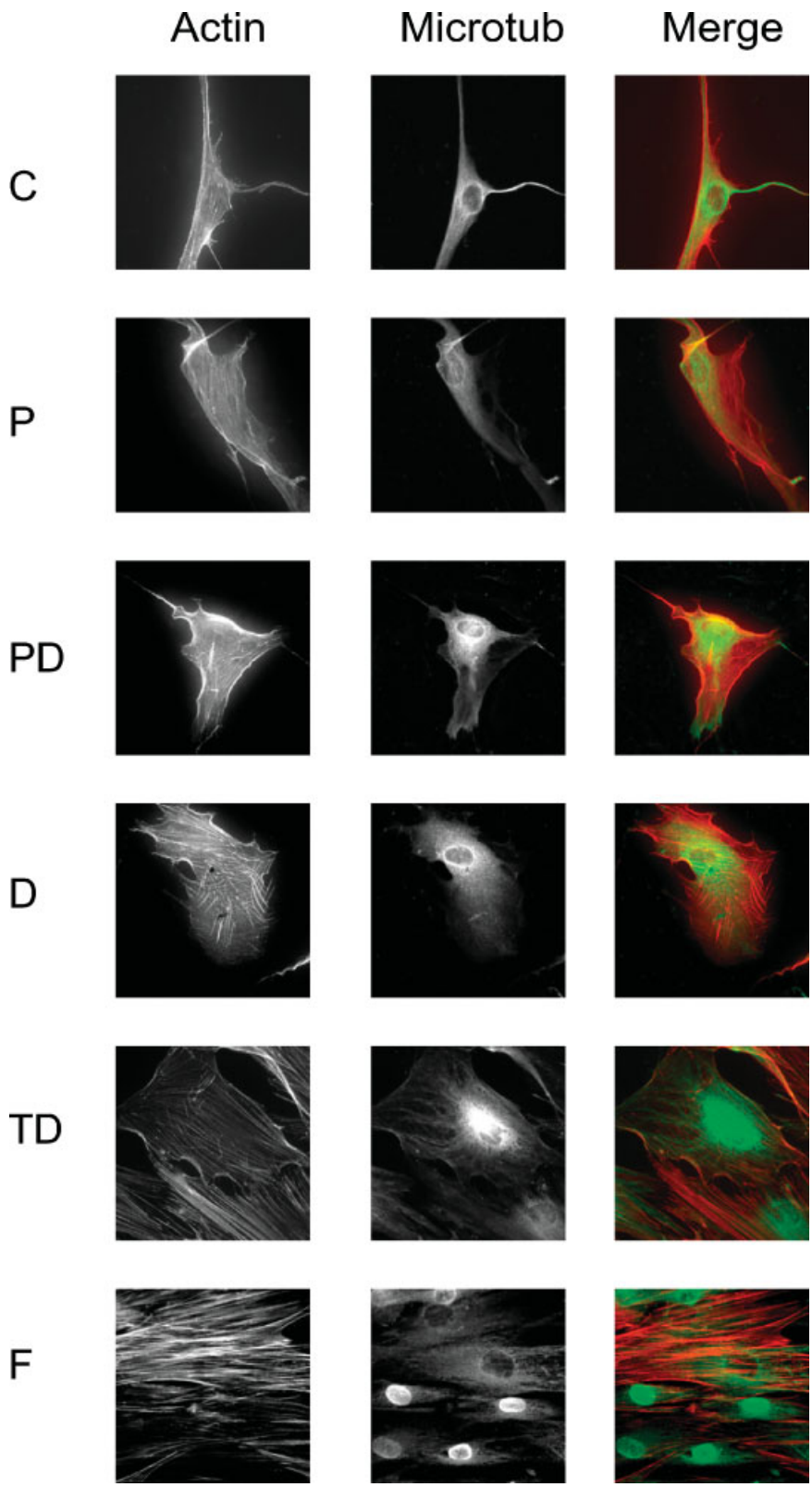

Fig. 5. Cytoskeletal reorganization induced by osteoblastic differentiating hMSCs. Cells (40×10^3) were seeded on 0.5% gelatin-coated coverslips and cultured for 24 h in the presence of FBS. Cells were incubated in triplicate in medium C, P, PD, D, TD, and F (FBS) for 1 week as described in Materials and Methods. They were then fixed in 3.7% paraformaldehyde in PBS and permeabilized with 0.5% Triton. Actin was detected by direct fluorescence using rhodamine–phalloidin and tubulin-associated organization by indirect fluorescence, using serum for bGH-Tg mice and a secondary antibody labeled with Alexa-488.

persisted in cells treated with PD and TD. The results also showed that in the presence of D, microtubules/microtubule-associated proteins undergo changes to be adapted to an increase in cell volume. These changes in the cytoskeleton were consistent with those caused by TGF-β and Dex described in previous works [Moustakas and Stournaras, 1999; Rodriguez et al., 2004]. Up to this point, the results suggest that under C and P osteoblastic differentiation conditions hMSCs have the characteristic cellular cytoskeleton of proliferating and non-terminal differentiated cells. However, in the presence of D, the reorganization of the cytoskeleton resembles a cellular stage of terminal differentiation.

Runx2/AML3, CBFB, and Smad2/3 Are Associated With the Nuclear Matrix of hMSCs

The Runx2/AML3 transcription factor is essential for MSC differentiation into chondrocytes, and chondrocyte proliferation and maturation during skeletal development [Yoshida et al., 2004]. It has been shown that Runx factors are transcriptional effectors of Smad signaling by targeting Smads to specific active foci within the nucleus [Viereck et al., 2002; Zaidi et al., 2002; Maeda et al., 2004]. We wondered whether the three well characterized transcription factors of bone cells-Runx2/AML-3, Cbfb, and Smad2/3- might be associated with the nuclear matrix of hMSCs incubated in growth or osteoblastic differentiation conditions. To this end, hMSCs were incubated in growth medium or in osteoblastic differentiation conditions during a week and fixed. Transcription factors were detected by indirect immunofluorescence using anti-Runx2, anti-Cbfb and anti-Smad2/3 antibodies in cell preparations in which cytosolic soluble proteins, the cytoskeleton, DNA and most nuclear proteins had been extracted. Figure 6 shows that the three nuclear factors were present in the cell nuclei, associated with the nuclear matrix, both during hMSC growth and under osteoblastic differentiating conditions. These results agree with those obtained in cells with osteoblastic lineage where it has been demonstrated that Runx2/AML3 targets Smad proteins to the nucleus. Therefore, our results confirm that hMSCs isolated from adult bone marrow are bone progenitors cells. This commitment is preserved both in growth condition and under cell treatments with PRL, TGF-β, Dex, or mixtures thereof.

Runx2/AML3 Co-Localizes With the SR Splicing Factor Family

There is increasing evidence supporting the notion that gene transcription should not be viewed simply as a process that turns on a gene, but as a process of enormous complexity within a specific sub-nuclear organization [Kosak and Groudine, 2004]. We were thus prompted to study whether Runx2/AML3 could co-localize with the splicing factor SC35 as a probe for the active transcriptional function of Runx2 in hMSCs during osteoblastic differentiation. With this in mind, hMSCs were seeded on gelatine, treated and processed as above, but more exhaustive cell extractions were performed to retain nuclear matrix intermediate filaments and associated proteins. Then, cells were incubated with anti-Runx2/AML3 and anti-SC35 and secondary antibodies. Figure 7 shows an example of the results obtained from two separate experiments. The results show that Runx2/AML3 and the splicing factor SC35 co-localize at active nuclear foci, (yellow dots), in both growing hMSCs and osteoblastic differentiating cells. The number of yellow dots detected was similar in cells from different treatments and were few in comparison with numerous orange or red dots. Cells have bone active transcription foci with Runx2/AML3 transcription factors and this is not altered by insulin, ascorbic acid of C medium, and nor by the addition of PRL, Dex TGF-β nor mixtures thereof. These results strongly support the notion that human MSCs are osteochondro progenitor cells.

DISCUSSION

With increases in life expectancy, the need for a better understanding of skeletal biology and associated pathologies such as osteoporosis and arthritis is crucial. The hope for tissue repair and regeneration in patients suffering from a broad spectrum of degenerative and genetic diseases has led to the study of the cellular and molecular bases of endochondral bone formation processes using hMSCs as target cells. This work reports different approach used to characterize adult human bone marrow-derived MSCs under growth and differentiation conditions to overcome that they cannot be tested

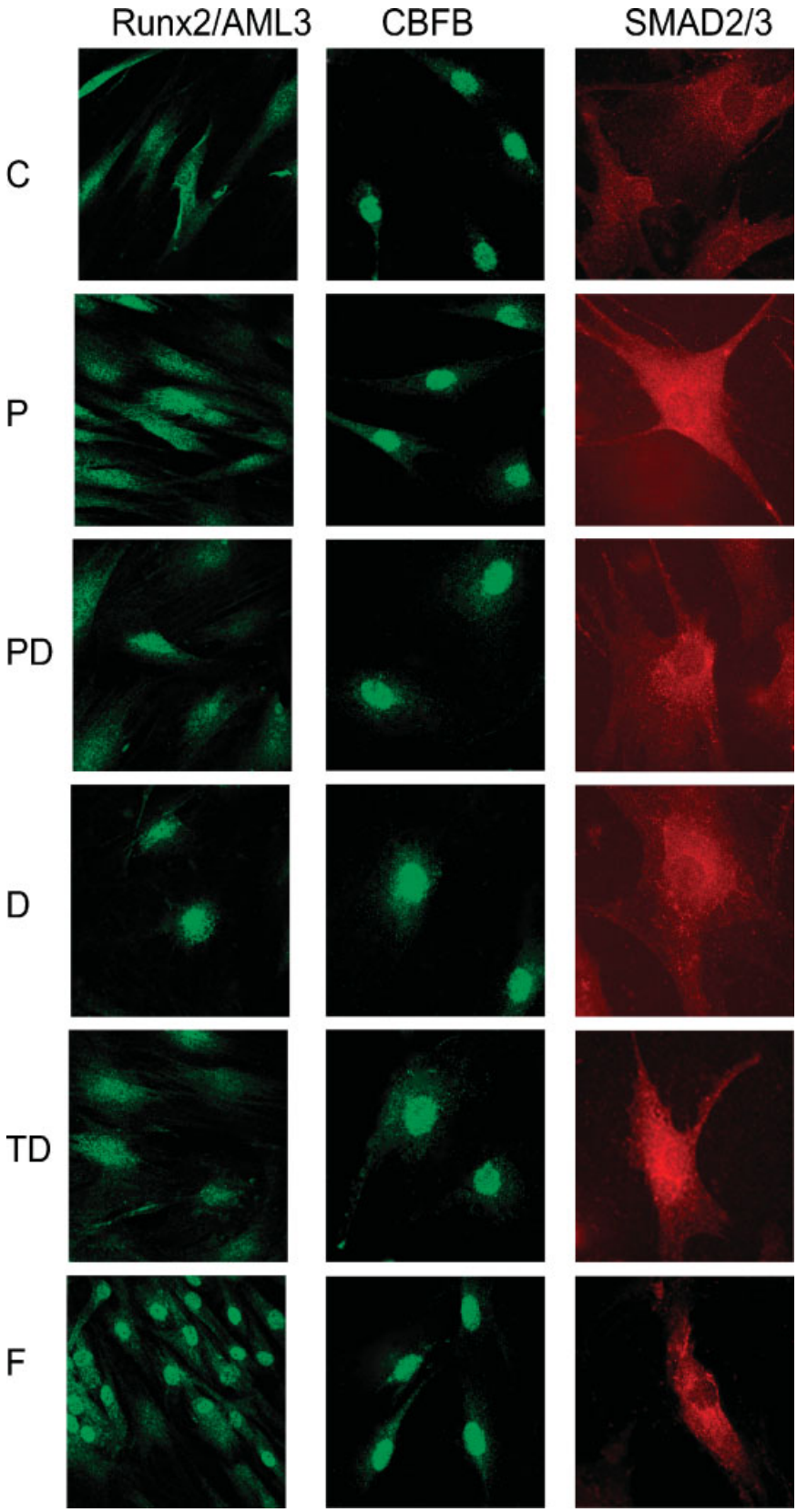

Fig. 6. Nuclear matrix targeting of bone specific transcriptional factors in osteoblastic differentiating hMSCs. Cells were induced to differentiate as indicated on gelatin-coated coverslips for 1 week as in Figure 5. Then, cell preparations were treated with DNAase as indicated in Materials and Methods. We analyzed the presence of Runx2/AML3, CBFB and Smad2/3 using specific antibodies and fluorescent secondary antibodies. Cells did not show background fluorescence when no primary antibody was added (results not shown).

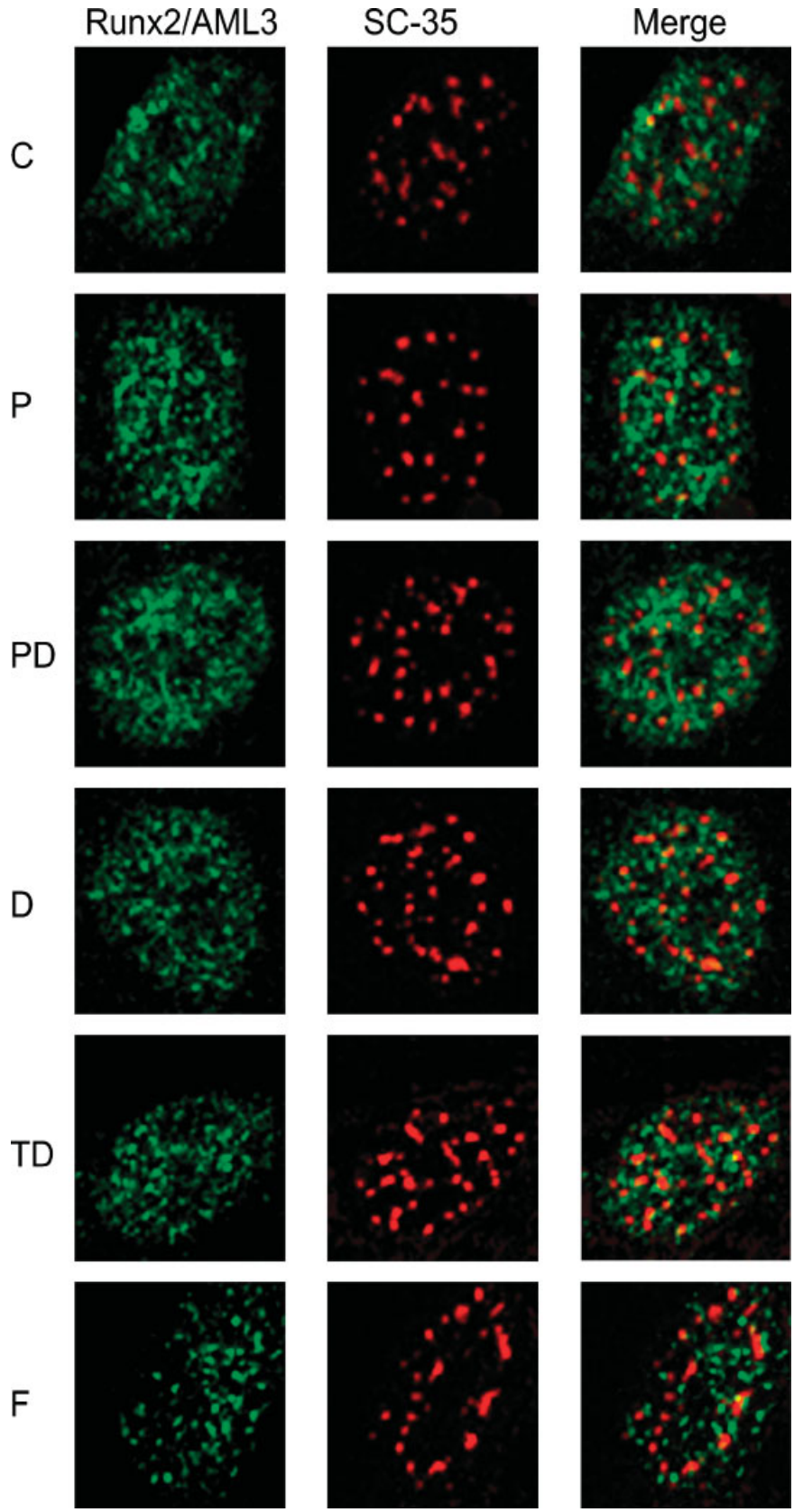

Fig. 7. hMSCs and osteoblastic differentiating cells present Runx2/AML3 co-localized with SC35 at nuclear foci. Examples of nuclear confocal images from osteoblastic differentiating cells in the conditions: C, P, PD, D, TD, and F. Images are 100×. Preparations of nuclear matrix intermediate filaments were obtained after an imidazole and 2 M NaCl wash. Runx2/AML3 is shown in green fluorescence, SC35 in red, and the completed overlap between both proteins in yellow.

in vivo for functional competence. The work strongly supports the notion that hMSC expanded in vitro are committed to becoming osteochondro progenitor cells.

During development, cells respond to microenviromental factors through specific phenotypic alterations that underlie epithelial mesenchymal transition or mesenchymal epithelial transition. These changes are the ultimate steps in cell plasticity. However, in adult organisms plasticity may be less frequent and subject to tighter restrictions. It has been suggested that the phenotypic plasticity responsible for all types of regenerative process is never completely lost from cells from the adult organism [Prindull and Zipori, 2004]. The gradual reduction in plasticity observed after the completion of development insures the stability of previously formed tissues and organs. This restrictive model of cell organization has been proposed to account for events within the hematopoietic microenvironment or HSC niche [Zipori and Barda-Saad, 2001]. The results of the present work support the notion that this restrictive model should be extended to bone regeneration process from MSCs. Interestingly, osteoblasts are a major constituent of HSC niche and the osteopontin produced by these cells is a negative regulatory element of the HSC pool size [Calvi et al., 2003; Zhang et al., 2003; Stier et al., 2005].

Results indicate that bone marrow-derived hMSCs isolated from adults are osteochondro progenitor cells that are induced to grow in the presence of serum or by regulatory factors present in the microenvironment [Lemischka and Moore, 2003]. This explains why hMSCs express the Runx2/AML3 transcription factor, as occurs in the primitive bone template during development. In fact, Runx2/AML3 is expressed in mesenchymal condensations of developing bones and the mRNA has been shown to increase in osteochondro tissues during embryogenesis. Interestingly, it has been reported that Runx2/AML3$^{-/-}$ mice die shortly after birth as the result of a failure to undergo osteogenesis, although they do form a cartilaginous precursor skeleton [Komori et al., 1997; Otto et al., 1997]. Furthermore, it has been demonstrated that Runx2/AML3 is able to become equally distributed in parallel with the chromosomal partitioning by which cells sustain a balanced expression of phenotypic genes post-mitosis [Zaidi et al., 2003]. Significantly, the myogenic contributions from marrow stromal cells observed [Dezawa et al., 2005] required in vitro induction, first by culturing the cells with certain growth factors and then by ectopic expression of constitutively active Notch-1.

Our results are consistent with the notion that hMSCs deprived of serum or treated with prolactin resemble cells at the stage of proliferative chondrocytes in the endochondral bone formation program. There are many reasons to support this hypothesis: (i) cells started to express IHH, which was not detected in hMSCs; (ii) cells expressed the IHH-PTHhP regulatory loop, the IHH cellular signaling components, and bone target genes; (iii) the activation of HH cellular signaling caused an increase in cell proliferation. These results are in agreement with those obtained for vertebrate skeletal development [Vortkamp et al., 1996; St-Jacques et al., 1999; Long et al., 2004]. Furthermore, the cells showed a cytoskeleton organization with abundant actin stress fibers, suggesting an active state of cell growth. Interestingly, as seen to occur in hMSCs the transcription factors Runx2/AML3, CBFB, and Smad2/3 were detected in the nuclei. Furthermore the presence of PRL, which opens the JAK-STAT signaling pathway, may help to preserve cell commitment as shown for LIF [Clevenger et al., 2003]. This action might be redundant with other members of the hematopoietic family of cytokines but could accelerate the bone regeneration detected after transplanting hMSC and platelet-rich plasma during distraction osteogenesis [Kitoh et al., 2004].

The results obtained on incubating cells in the presence of glucocorticoids can be interpreted as the cellular situation of an osteoblast differentiated cell due to the endochondral bone formation process. Major changes were detected in cytoskeletal organization, which afforded the overall cell shape of being rounded, spindle-shaped, and with a flat morphology as a consequence of actin stress fibers emanating from abundant focal adhesions. It would be interesting to explore whether the cellular changes are reversible and also why in the presence of dexamethasone the TβRE-retarded complex is either Runx2/AML3-enriched or free for antibody access. Regardless of the interest of the above issues, the most interesting result is the constant presence of Runx2/AML3 in hMSCs, proliferating cells, or osteoblastic differentiating cells, which agrees with earlier results obtained in bone-derived cell lineages. The Runx2/AML3 tran-

scription factor plays a unique role in skeletal development [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Yoshida et al., 2004]. In addition to its natural partner CBFB, Runx2/AML3 interacts with TLE proteins, the nuclear matrix, and many transcription factors activated by different ligands such as TGF-β. Consequently, active foci are detected under all cellular conditions analyzed in hMSCs. It appears that active transcription centers represent a nuclear compartment where the transcripts from groups of genes are processed and are found at the periphery of nuclear speckles/interchromatin.

The results strongly support the notion that human bone marrow-derived stem cells become bone progenitor cells upon culture. The results can be considered promising in terms of cell therapy for a large number of genetic and degenerative diseases affecting the human skeleton. However, before recommending clinical application, we strongly advise that studies should be carried out to elucidate whether hMSCs are able to go through epithelial transitions, and whether a single cancer cell is present in the pool of progenitor cells.

ACKNOWLEDGMENTS

We are in debt to Dr. Arnold I. Caplan, Donald P. Lennon and Margie Harris for their help in MSC isolation and culture. We thank E. Blázquez, J.A. Reig, and E. Rañada for encouraging this work. We also thank María de los Ángeles Muñoz for technical assistance with the confocal microscopy.

REFERENCES

Aggarwal S, Pittenger MF. 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105(4):1815–1822.

Beachy PA, Karhadkar SS, Berman DM. 2004. Tissue repair and stem cell renewal in carcinogenesis. Nature 432(7015):324–331.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425(6960):841–846.

Caplan AI. 1987. Bone development and repair. Bioessays 6(4):171–175.

Clevenger CV, Furth PA, Hankinson SE, Schuler LA. 2003. The role of prolactin in mammary carcinoma. Endocr Rev 24(1):1–27.

Dezawa M, Ishikawa H, Itokazu Y, Yoshihara T, Hoshino M, Takeda S, Ide C, Nabeshima Y. 2005. Bone marrow stromal cells generate muscle cells and repair muscle degeneration. Science 309(5732):314–317.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89(5):747–754.

Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M, Wagner EF. 2004. The Fos-related antigen Fra-1 is an activator of bone matrix formation. EMBO J 23(14):2789–2799.

Fu XD, Maniatis T. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature 343(6257):437–441.

Guan Y, Rubenstein NM, Failor KL, Woo PL, Firestone GL. 2004. Glucocorticoids control beta-catenin protein expression and localization through distinct pathways that can be uncoupled by disruption of signaling events required for tight junction formation in rat mammary epithelial tumor cells. Mol Endocrinol 18(1): 214–227.

Javed A, Guo B, Hiebert S, Choi JY, Green J, Zhao SC, Osborne MA, Stifani S, Stein JL, Lian JB, van Wijnen AJ, Stein GS. 2000. Groucho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. J Cell Sci 113(Pt 12):2221–2231.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49.

Karp SJ, Schipani E, St-Jacques B, Hunzelman J, Kronenberg H, McMahon AP. 2000. Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. Development 127(3):543–548.

Kenner L, Hoebertz A, Beil T, Keon N, Karreth F, Eferl R, Scheuch H, Szremska A, Amling M, Schorpp-Kistner M, Angel P, Wagner EF. 2004. Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. J Cell Biol 164(4):613–623.

Kitoh H, Kitakoji T, Tsuchiya H, Mitsuyama H, Nakamura H, Katoh M, Ishiguro N. 2004. Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis—A preliminary result of three cases. Bone 35(4):892–898.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5):755–764.

Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, Shizuru JA, Weissman IL. 2003. Biology of hematopoietic stem cells and progenitors: Implications for clinical application. Annu Rev Immunol 21:759–806.

Kosak ST, Groudine M. 2004. Form follows function: The genomic organization of cellular differentiation. Genes Dev 18(12):1371–1384.

Le Blanc K, Gotherstrom C, Ringden O, Hassan M, McMahon R, Horwitz E, Anneren G, Axelsson O, Nunn J, Ewald U, Norden-Lindeberg S, Jansson M, Dalton A, Astrom E, Westgren M. 2005. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. Transplantation 79(11):1607–1614.

Lemischka IR, Moore KA. 2003. Stem cells: Interactive niches. Nature 425(6960):778–779.

Lennon DP, Haynesworth SE, Bruder SP, Jaiswasl N, Caplan AI. 1996. Human and animal mesenchymal progenitor cells from bone marrow: Identification of serum for optimal selection and proliferation. In Vitro Cell Dev Biol 32:602–611.

Long F, Chung UI, Ohba S, McMahon J, Kronenberg HM, McMahon AP. 2004. Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. Development 131(6):1309–1318.

Maeda S, Hayashi M, Komiya S, Imamura T, Miyazono K. 2004. Endogenous TGF-beta signaling suppresses maturation of osteoblastic mesenchymal cells. Embo J 23(3): 552–563.

Massengale M, Wagers AJ, Vogel H, Weissman IL. 2005. Hematopoietic cells maintain hematopoietic fates upon entering the brain. J Exp Med 201(10):1579–1589.

Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G. 2004. Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22(4):411–417.

Moustakas A, Stournaras C. 1999. Regulation of actin organisation by TGF-beta in H-ras-transformed fibroblasts. J Cell Sci 112(Pt 8):1169–1179.

Mullor JL, Calleja M, Capdevila J, Guerrero I. 1997. Hedgehog activity, independent of decapentaplegic, participates in wing disc patterning. Development 124(6):1227–1237.

Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. 1997. Mutations involving the transcription factor Cbfa1 cause cleidocranial dysplasia. Cell 89(5):773–779.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1):17–29.

Ogueta S, Olazabal I, Santos I, Delgado-Baeza E, Garcia-Ruiz JP. 2000. Transgenic mice expressing bovine GH develop arthritic disorder and self-antibodies. J Endocrinol 165(2):321–328.

Ogueta S, Munoz J, Obregon E, Delgado-Baeza E, Garcia-Ruiz JP. 2002. Prolactin is a component of the human synovial liquid and modulates the growth and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. Mol Cell Endocrinol 190(1–2):51–63.

Olazabal I, Munoz J, Ogueta S, Obregon E, Garcia-Ruiz JP. 2000. Prolactin (PRL)-PRL receptor system increases cell proliferation involving JNK (c-Jun amino terminal kinase) and AP-1 activation: Inhibition by glucocorticoids. Mol Endocrinol 14(4):564–575.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89(5):765–771.

Palcy S, Bolivar I, Goltzman D. 2000. Role of activator protein 1 transcriptional activity in the regulation of gene expression by transforming growth factor beta1 and bone morphogenetic protein 2 in ROS 17/2.8 osteoblast-like cells. J Bone Miner Res 15(12):2352–2361.

Palma V, Ruiz i Altaba A. 2004. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development 131(2):337–345.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284(5411): 143–147.

Prindull G, Zipori D. 2004. Environmental guidance of normal and tumor cell plasticity: Epithelial mesenchymal transitions as a paradigm. Blood 103(8):2892–2899.

Rodriguez JP, Gonzalez M, Rios S, Cambiazo V. 2004. Cytoskeletal organization of human mesenchymal stem cells (MSC) changes during their osteogenic differentiation. J Cell Biochem 93(4):721–731.

St-Jacques B, Hammerschmidt M, McMahon AP. 1999. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 13(16):2072–2086.

Stein GS, Lian JB, van Wijnen AJ, Stein JL, Javed A, Montecino M, Zaidi SK, Young D, Choi JY, Gutierrez S, Pockwinse S. 2004. Nuclear microenvironments support assembly and organization of the transcriptional regulatory machinery for cell proliferation and differentiation. J Cell Biochem 91(2):287–302.

Stier S, Ko Y, Forkert R, Lutz C, Neuhaus T, Grunewald E, Cheng T, Dombkowski D, Calvi LM, Rittling SR, Scadden DT. 2005. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 201(11):1781–1791.

Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M, Fuchs E. 2004. Defining the epithelial stem cell niche in skin. Science 303(5656):359–363.

Viereck V, Siggelkow H, Tauber S, Raddatz D, Schutze N, Hufner M. 2002. Differential regulation of Cbfa1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. J Cell Biochem 86(2):348–356.

Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273(5275):613–622.

Yoshida CA, Yamamoto H, Fujita T, Furuichi T, Ito K, Inoue K, Yamana K, Zanma A, Takada K, Ito Y, Komori T. 2004. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. Genes Dev 18(8):952–963.

Zaidi SK, Sullivan AJ, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2002. Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. Proc Natl Acad Sci USA 99(12):8048–8053.

Zaidi SK, Young DW, Pockwinse SM, Javed A, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2003. Mitotic partitioning and selective reorganization of tissue-specific transcription factors in progeny cells. Proc Natl Acad Sci USA 100(25):14852–14857.

Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425(6960):836–841.

Zipori D, Barda-Saad M. 2001. Role of activin A in negative regulation of normal and tumor B lymphocytes. J Leukoc Biol 69(6):867–873.