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Osteogenic Imprinting Upstream of Marrow Stromal Cell Differentiation

Kazuhito Satomura,[1] Paul Krebsbach,[2] Paolo Bianco,[3] and Pamela Gehron Robey[1]*

[1]Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892
[2]Department of Oral Medicine/Pathology/Surgery, University of Michigan, School of Dentistry, Ann Arbor, Michigan 48108
[3]Dipartimento di Medicina Sperimentale, Università dell'Aquila, L'Aquila, Italy

Abstract Five spontaneously transformed cell lines were established from a population of murine bone marrow stromal cells (BMSCs) and the expression profiles of phenotype-characteristic genes, patterns of in vitro differentiation, and osteogenic capacity after in vivo transplantation were determined for each. All the clones expressed stable levels of *cbfa1*, the osteogenic "master" gene, whereas the levels of individual phenotypic mRNAs were variable within each, suggestive of both maturational and phenotypic plasticity in vitro. Varying levels of collagen type I and alkaline phosphatase (AP) were expressed in all the clonal lines. The clonal lines with proven in vivo osteogenic potential (3 out of 5) had a high proliferation rate and expressed bone sialoprotein (BSP), whereas the two nonosteogenic clones proliferated more slowly and never expressed BSP. Bone nodules were only observed in 2 out of 3 of the osteogenic lines, and only 1 out of three formed cartilage-like matrix in vitro. There was no evidence of chondrogenesis in the nonosteogenic lines. By contrast, LPL was expressed in two osteogenic and in two nonosteogenic lines. These results demonstrate the presence of multipotential and restricted progenitors in the murine stromal system. *cbfa1*, collagen type I, and AP expression were common to all, and therefore presumably early, basic traits of stromal cell lines that otherwise significantly differ with respect to growth and differentiation potential. This finding suggests that an osteogenic imprinting lies upstream of diversification, modulation, and restriction of stromal cell differentiation potential. J. Cell. Biochem. 78:391–403, 2000.

Key words: bone marrow stromal cell; differentiation; phenotype; osteoblast; chondroblast; adipocyte

Continuous recruitment of progenitor cells is required for skeletal growth, remodeling and repair throughout postnatal life. According to the current hypothesis, the most likely source of the skeletal progenitor cells is the bone marrow stromal system, as first suggested by Friedenstein and Owen and coworkers [Friedenstein and Kuralesova, 1971; Owen, 1985; Owen et al., 1987]. When a single cell suspension of marrow, which contains bone marrow stromal cells (BMSCs) is cultured at low density in vitro, colonies are formed. Each of these is considered to be derived from a single, fibroblast-like clonogenic progenitor (colony forming unit-fibroblast, CFU-F) [Friedenstein, 1973; Friedenstein et al., 1970; Owen, 1985; Owen et al., 1987]. When ex vivo expanded BMSCs are transplanted using diffusion chambers in vivo, mature bone, cartilage, and adipose tissues of donor origin are simultaneously generated [Ashton et al., 1980, 1984; Bab et al., 1984a,b, 1986; Benayahu et al., 1989; Gundle et al., 1995; Maniatopoulos et al., 1988; Mardon et al., 1987]. If open transplantation systems that allow for ingress of host blood-borne cells are used, hematopoiesis of host origin develops upon hematopoiesis-supporting stromal cells of donor origin. These processes contribute to the generation of an ectopic "ossicle" directly and closely mimick the architecture of the normal intact bone/bone marrow organ [Friedenstein and Kuralesova, 1971; Goshima et al., 1991; Krebsbach et al., 1997, 1998; Kuznetsov et al., 1997; Martin et al., 1997].

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*Correspondence to: Pamela Gehron Robey, Building 30, Room 228, 30 Convent Drive MSC-4320, Bethesda, MD 20892. E-mail: probey@dir.nidcr.nih.gov

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Kazuhito Satomura's current address: First Department of Oral & Maxillofacial Surgery, School of Dentistry, Tokushima University 3-18-15 Kuramoto-cho Tokushima 770 Japan.

These observations have substantiated the now generally accepted view that the BMSC population contains multipotential progenitors capable of giving rise to multiple differentiated skeletal connective tissues. CFU-Fs are postulated to represent or comprise such a putative multipotent progenitor [Beresford, 1989; Bianco et al., 1999; Gehron Robey and Bianco, 1999; Gronthos and Simmons, 1996; Owen, 1985]. Data indicating a heterogeneous differentiation potential across different individual stromal progenitors have long been available [Barling et al., 1989; Benayahu et al., 1989; Cassiede et al., 1996; Dennis et al., 1999; Gronthos et al., 1994; Haynesworth et al., 1992; Kassem et al., 1991; Leboy et al., 1991; Lecka-Czernik et al., 1999; Luria et al., 1987; Mackay et al., 1998; Maniatopoulos et al., 1988; Owen, 1988; Owen and Friedenstein, 1988; Owen et al., 1987; Pittenger et al., 1999; Rickard et al., 1996; Satomura et al., 1991; Satomura and Nagayama, 1991]. Yet a clearer understanding of a potential hierarchy of progenitors with broader or more restricted potential has remained elusive.

Dissecting the origin, mutual positioning, and hierarchical layout of stromal progenitor cells requires (1) the identification of clonal stromal cell subsets with multiple or restricted potential, and (2) the definition of appropriate markers suited to establish clear-cut divergences, kinships, or lineage relationships among functionally heterogeneous stromal cell clones. In this study, we isolated 30 single colony-derived strains from the murine bone marrow stromal population, out of which we obtained 5 spontaneously immortalized clonal cell lines (C-3, C-4, C-8, C-10 and C-11). Each clonal line was then characterized with respect to its gene expression profile by RT-PCR analysis, in vitro differentiation, and in vivo osteogenic potential. We show here that stable and consistent expression of the osteogenic "master" gene, *cbfa1*, is found in murine clonal stromal cell strains otherwise noted for marked heterogeneity of differentiation potential across different strains, and phenotypic instability of individual strains in culture. These data suggest that a flexible osteogenic imprinting lies upstream of stromal cell diversification, providing a novel angle for interpreting the organization of the stromal cell system.

MATERIALS AND METHODS

Cell Cloning And Culture

All experiments were performed under an institutionally approved animal study protocol (#114-93). Bone marrow was obtained from the dissected femora and tibiae of 6–8-week-old male C57Bl/6 mice. The epiphyses were removed, and marrow tissue was flushed from the shaft using 0.5 ml of α-MEM (Life Technologies, Grand Island, NY) expelled from a syringe through a 25-gauge needle. A single cell suspension was obtained by gently aspirating the cells sequentially through 20- and 23-gauge needles, and finally a cell strainer (70 μm in sieve size, Becton Dickinson Labware, Franklin Lakes, NJ) to exclude tissue debris. The cells were washed with α-MEM and seeded into T-75 flasks (Falcon Labware, Meylan Cedex, France) at a cell density of 4.0×10^5 nucleated cells/cm^2, and cultured in α-MEM containing 20% lot selected fetal bovine serum (Atlanta Biologicals, Norcross, GA), containing 10^{-8} M dexamethasone (Sigma Chemicals Company, St. Louis, MO), 10^{-4} M L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 100 IU/ml penicillin and 100 μg/ml streptomycin.

After 1 week, the culture medium was changed for the first time. Thereafter, the medium was changed twice a week. After 2 weeks in culture, the adherent BMSCs were washed with Hanks' balanced salt solution, subcultured by treating with trypsin-EDTA (Life Technologies) and plated into 150-mm^2 tissue culture dishes (Falcon Labware, Meylan Cedéx, France) at a cell density of 1.1–5.7×10^2 cells/cm^2. Only one-half of the culture medium was changed twice a week, until distinct colonies were identified. Thereafter, the medium was completely changed twice a week. Two to 3 weeks later, discrete colonies well separated from one another were cloned using cloning cylinders (Sigma). After cloning, the cells were expanded in number by passaging and plating at a density 2×10^4 cells/cm^2 in successively larger tissue culture vessels. Based on their ability to proliferate continuously, five clonal strains (C-3, C-4, C-8, C-10, and C-11) appeared to be spontaneously immortalized and were further characterized as described below.

Growth Curve Analysis

The clonal lines were plated into 6-well culture plates (Falcon Labware, Meylan Cedéx, France) at a cell density of 2×10^3 cells/cm^2. Medium was changed every 2 days. Cells were harvested every day for 10 days by treatment with Trypsin-EDTA, suspension in serum-containing medium and counted using a Coulter Counter (Coulter Electronics, Hialeah, FL).

RNA Extraction, cDNA synthesis, and RT-PCR

After 30–35 passages, cells were plated into T-75 culture flasks at a cell density of 2.0×10^3 cells/cm^2 and medium was changed twice a week. Total RNA was extracted at 1, 2, 3, and 4 weeks after reaching confluence, and treated with 1 U of DNase I at room temperature for 15 min to digest any contaminating DNA. The reaction was terminated by the addition of 1 μl of EDTA followed by heating at 65°C for 10 min. cDNA was synthesized from 3 μg of total RNA in a 20 μl reaction mixture containing 1× reverse transcription buffer: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (Gibco-BRL, Grand Island, NY), 0.5 mM of dNTP (Perkin-Elmer, Foster City, CA), 200 U of SuperScript II® reverse transcriptase (Gibco-BRL), 0.5 μg of oligo$(dT)_{12-18mer}$ primer (Gibco-BRL). The reaction was performed for 50 min at 42°C.

Aliquots (5%) of the total cDNA was amplified in each PCR in a 50 μl reaction mixture containing 10 pmole of 5′ and 3′ primers specific for *cbfa1*, α1(I) procollagen, alkaline phosphatase (AP), bone sialoprotein (BSP), α1(II) procollagen, lipoprotein lipase (LPL), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table I), 1× PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (Perkin-Elmer), 0.5 mM of dNTP, 2.5 U of Taq polymerase (Perkin-Elmer), and 0.44 μg of TaqStart™ (Clontech, Palo Alto, CA). The amount of cDNA in each sample was confirmed by preliminary PCR in logarithmic phase for GAPDH. Amplification was performed in a GeneAmp Thermal cycler 2400 (Perkin Elmer) for 21–30 cycles after an initial denaturation at 94°C for 5 min. All the PCR reactions were terminated before the reaction reached the saturation phase. The reaction profile was 94°C for 30 s, 55-58°C for 2 min, and 72°C for 2 min for *cbfa1*, α1(I) procollagen, AP, BSP, α1(II) procollagen, LPL, and GAPDH (Table I).

TABLE I. Oligonucleotide Primers Used in RT-PCR

Cbfa1
sense: 5′-CCAAATTTGCCTAACCAGAATG-3′
antisense: 5′-GAGGCTGTGGTTTCAAAGCAC-3′
amplified fragment length: 458bp
α1(I) procollagen (GenBank Accession #X06753)
sense: 5′-TCTCCACTCTTCTAGTTCCT-3′
antisense: 5′-TTGGGTCATTTCCACATGC-3′
amplified fragment length: 269bp
Alkaline phosphatase (GenBank Accession #J02980)
sense: 5′-AAGACGTGGCGGTCTTTGC-3′
antisense: 5′-GGGAATCTGTGCAGTCTGTG-3′
amplified fragment length: 457bp
Bone sialoprotein (GenBank Accession #20232)
sense: 5′-GAAACGGTTTCCAGTCCAG-3′
antisense: 5′-GACTTTGGGCAAGTCTTCC-3′
amplified fragment length: 564bp
α1(II) procollagen (GenBank Accession #M65161)
sense: 5′-CACACTGGTAAGTGGGGCAAGACCG-3′
antisense: 5′-GGATTGTGTTGTTTCAGGGT-TCGGG-3′
amplified fragment length: 172bp
Lipoprotein lipase (GenBank Accession #J03302)
sense: 5′-TCCAGAGTTTGACCGCCTTC-3′
antisense: 5′-TTGGTCAGACTTCCTGCTACG-3′
amplified fragment length: 477bp
GAPDH (GenBank Accession #M32599)
sense: 5′-ACCACAGTCCATGCCATCAC-3′
antisense: 5′-TCCACCACCCTGTTGCTGTA-3′
amplified fragment length: 450bp

Reaction products were analyzed by electrophoresis of 5-μl aliquots in 6% nondenaturing polyacrylamide gels. The amplified DNA fragments were visualized by ethidium bromide staining. The semiquantitative nature of this procedure was confirmed by comparison of amplification to northern blot analysis for BSP. The relative intensities of the PCR products of BSP were comparable to the intensities of northern blot analysis (data not shown).

Differentiation of Marrow Stromal Cells in Culture

The clonal strains were passaged into T-75 culture flasks at a density of 2.0×10^3 cells/cm^2, and cultured for 4–5 weeks. Cultures were fixed briefly in 4% phosphate-buffered formalin (PBF) freshly prepared from paraformaldehyde at room temperature and stained

for alkaline phosphatase by the azo dye method, using naphthol AS-BI phosphate and fast blue BB salt as substrate (Sigma, kit 86-C). For the detection of mineralized matrix formation, the cultures were fixed in 4% PBF and stained with Alizarin Red S or by the von Kossa reaction. The presence of proteoglycans was identified by staining with Alcian blue (pH 2.5), and adipocytes with Oil Red O after fixation with 4% PBF.

Assessment of Osteogenic Potential of Mouse Stromal Cells In Vivo

Transplantation in immunocompromised mice. Confluent clonal strains (after 30–35 passages) and mouse skin fibroblasts (after 5 passages) were harvested with trypsin-EDTA and resuspended in 1 ml of culture medium. 3.0×10^6 cells were collected in sterile microtubes by centrifugation and absorbed to a 25–50-mm^3 Gelfoam sponge (Upjohn, Kalamazoo, MI) by incubation at 37°C for 30 min as described previously [Krebsbach et al., 1997, 1998]. NIH-BG-NU-XID immunocompromised mice (6–8-week-old females) were used as transplantation recipients.

Animals were anesthetized by an intraperitoneal injection of 0.015 ml of 2.5% Avertin per gram of body weight. One mid-longitudinal skin incision of ~1 cm in length was made on the dorsal surface of the cranium and the skin and periosteum were separated by blunt dissection. A 5-mm cranial defect in diameter was prepared with a trephine bur (Fine Science Tools, Foster City, CA) attached to an electric handpiece (Dremell, Racine, WI). Full-thickness calvarial bone was removed with minimal invasion of the dura mater. Gelfoam sponges with mBMSCs or skin fibroblasts were placed over the cranial defect, and the skin was sutured with 5-0 Vicryl suture (Ethicon, Johnson and Johnson, Somerville, NJ).

Four weeks later, the transplants were retrieved from the recipient mice and fixed in 4% PBF at 4°C for 2 days. After decalcification with 10% EDTA (pH 8.0), the tissues were dehydrated through ascending concentrations of ethanol, embedded in paraffin and sectioned at 5 μm in thickness. After deparaffinization, sections were stained with hematoxylin and eosin.

Demonstration of donor origin by polymerase chain reaction for a mouse y chromosome-specific repetitive sequence. Since the donor cells were of male origin, PCR for a mouse Y chromosome-specific repetitive sequence was performed to determine the origin of newly formed bone. The template DNA was isolated from the tissue sections by a modification of the method of Candeliere [Candeliere et al., 1997]. In brief, newly formed bone in the transplant, fibrous tissue around new bone, and host female bone were retrieved from the eosin-stained sections by microexcision, using a small scalpel under the microscope. To prevent any contamination of tissues, the sections were covered with Crystal Mount (Biomeda, Foster City, CA) and completely dried before microexcision, to facilitate the procedure. After washing excised tissue with water in order to remove Crystal Mount, the tissues were collected in microtubes by centrifugation and boiled in the presence of 10 μl of 50 mM NaOH for 10 min in order to remove contaminating RNA and protein. The solution was neutralized by the addition of 2 μl of 1 M Tris-HCl (pH 7.5), and the resulting solution was used for PCR amplification after clarification by centrifugation. The amplification was performed in a 50-μl reaction mixture containing 10 pmole of 5′ and 3′ specific primers (sense: 5′-GAATTCTGAACATGTTCTCACATAG-3′ and antisense: 5′-GAATTCATCCAGTATCCTTTCTCTTC-3′, GenBank Accession #L03314) for 50 cycles. The reaction profile was 94°C for 30 s, 60°C for 30 s and 72°C for 1 min after an initial denaturation at 94°C for 5 min. When the 30th cycle was completed, 2.5 U of Taq polymerase was added into the reaction mixture. 10% of the reaction product was analyzed by electrophoresis in 6% nondenaturing polyacrylamide gels. The amplified DNA fragment was visualized by ethidium bromide staining.

RESULTS

General characteristics and growth of murine clonal stromal cell lines

Of 30 clones initially isolated, 5 spontaneously transformed cell lines were established (C-3, C-4, C-8, C-10, and C-11). At low density, all these cell lines exhibited polygonal and relatively flat cell morphology in culture. After plating at a density of 2.2×10^3 cells/cm^2, the cell lines grew at different rates, reaching confluency between 5 and 7 days. C-3 exhibited the fastest rate of proliferation (doubling time of 24 h) and reached the highest confluent density. C-4 and C-8 proliferated at approximately one-half that rate (doubling times of 32 and

34 h, respectively) and were half as dense at confluency. C-10 and C-11 proliferated at an even slower rate (doubling times of 53 and 54 h, respectively) and were slightly less dense at confluency (Fig. 1A). After reaching confluency, C-3, C-4, and C-8 consisted of polygonal cells throughout the culture dish, and C-3 and C-8 formed multilayered clusters of cells. Interestingly, the clonal lines that were osteogenic in vivo (described below), had shorter doubling times than those that were nonosteogenic (Fig. 1B).

Gene Expression Profile in Murine Clonal Stromal Cell Lines

For each clonal line, a gene expression profile was defined by semiquantitative RT-PCR analysis of selected phenotype-characteristic mRNA species. Based on established patterns of tissue-specific expression in vivo, BSP was chosen as a marker of a mature osteoblast and also because of its high correlation with the initial phase of matrix mineralization [Bianco et al., 1993], α1(II) procollagen as a marker of the chondrogenic phenotype [Thorogood et al., 1986]; and LPL for the adipocytic phenotype [Kirchgessner et al., 1989]. α1(I) procollagen, expressed in osteoblastic and fibroblastic cells in vivo, and AP, expressed in osteoblastic and pre-adipocytic cells in vivo, were also measured.

All five lines expressed α1(I) procollagen mRNA and AP mRNA. BSP, a marker of osteoblastic maturation, was expressed in 3 out of 5 cell lines, all of which proved to be osteogenic in vivo. By contrast, the two BSP-silent lines failed to form bone in the same assay (described below). mRNA for α1(II) procollagen was expressed in 2 out of 3 of the BSP-expressing, osteogenic lines. LPL was expressed in 4 of 5 lines but failed to correlate to actual adipogenic capacity as detectable by histological analysis of long-term cultures (Fig. 2A) (see below).

Levels of expression of individual markers in individual cell lines changed with time in culture. In the clonal line C-3, mRNA for α1(I), AP, and BSP increased with time in culture, whereas mRNA for α1(II) and LPL declined to nearly undetectable levels (Fig. 2B). C-4 also expressed increasing amounts of proα1(I) and BSP mRNA with time, however at relatively lower levels compared with C-3. While initially high, AP levels decreased, and α1(II) and LPL started low and were undetectable with time

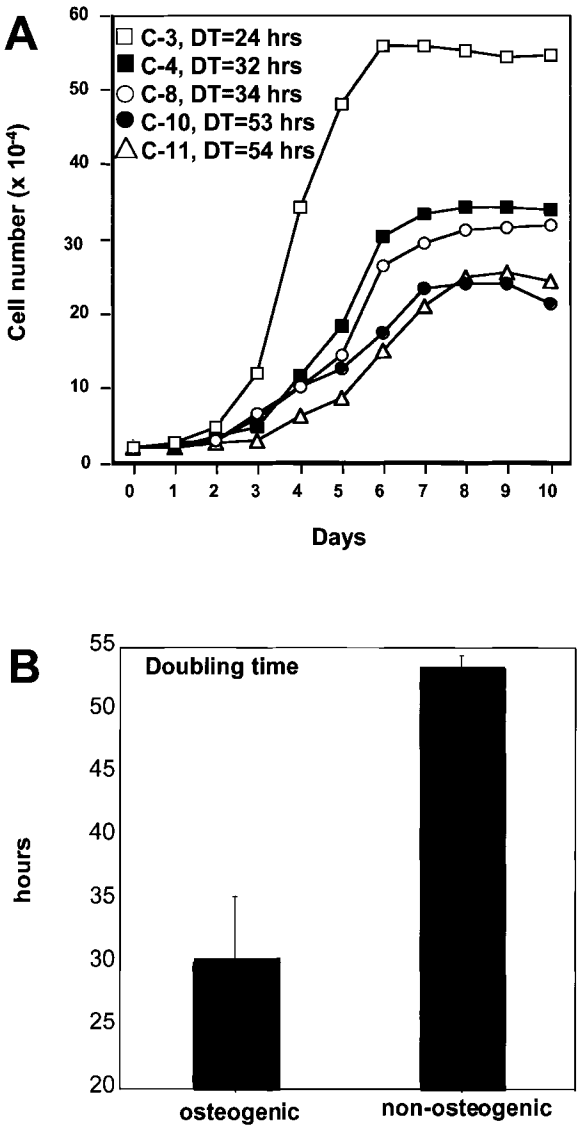

Fig. 1. **A:** Growth curves of 5 murine clonal stromal cell lines. Cells were inoculated into 6-well culture plates at a cell density of 2×10^3 cells/cm^2 and cultured in α-MEM containing 20% fetal bovine serum, 10^{-8} M, 10^{-4} M L-ascorbic acid phosphate magnesium salt, 100 IU/ml penicillin and 100 μg/ml streptomycin. Medium was changed every 2 days. Cells were retrieved every day until day 10 after inoculation by treatment with Trypsin-EDTA, suspended in serum-containing medium and counted using a Coulter counter. The clonal lines grew at different rates, and achieved different levels of confluent density. **B:** Based on demonstration of osteogenic capacity by in vivo transplantation (see text and Fig. 6), it was found that the osteogenic clonal lines (C-3, C-4, C-8) proliferated more rapidly (shorter doubling time) than nonosteogenic clonal lines (C-10 and C-11).

(Fig. 2C). C-8 was similar to C-3, but α(II) mRNA was expressed at high levels, and a low level of LPL was maintained throughout the culture period (Fig. 2D). C-10 expressed rela-

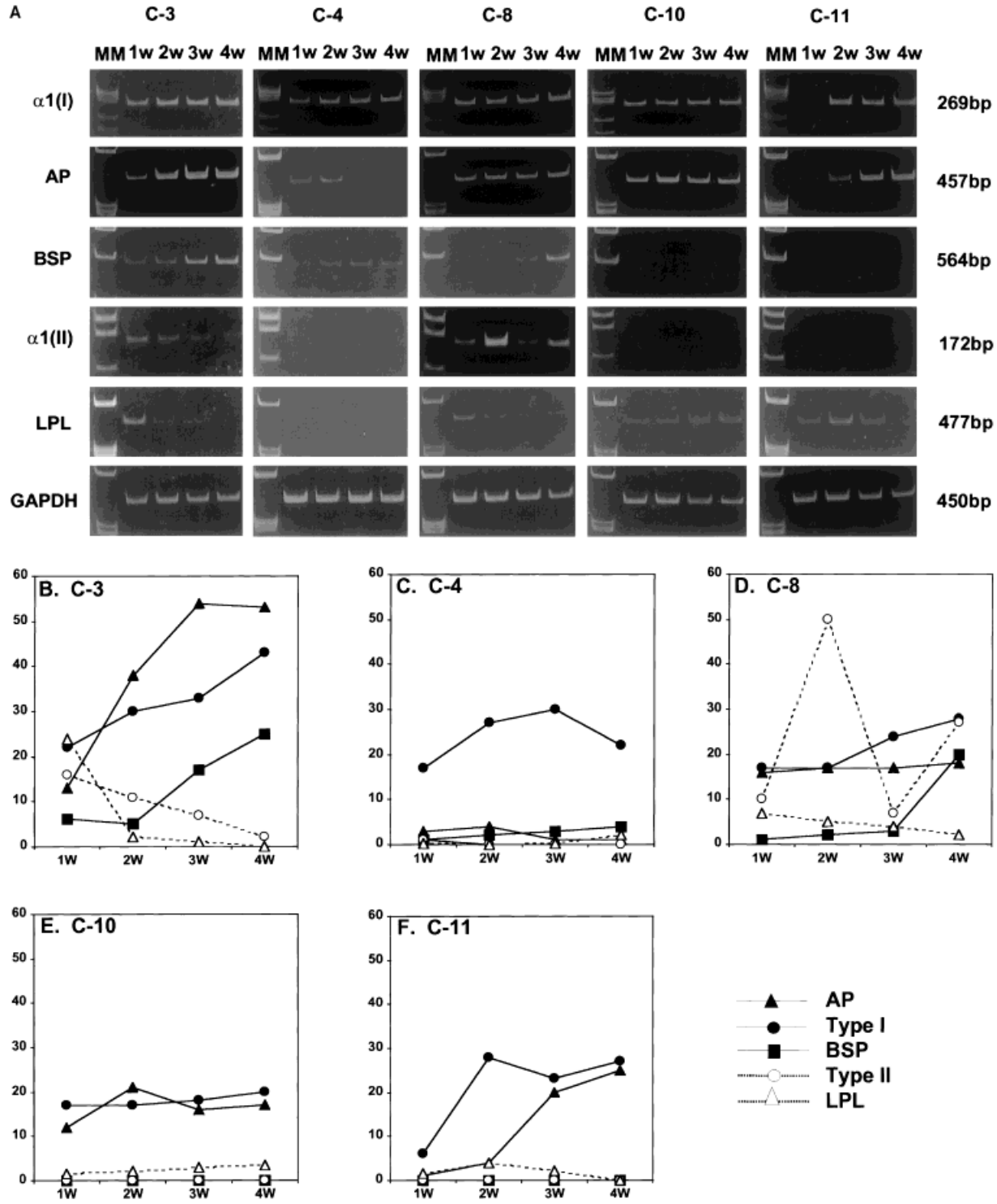

Fig. 2. **A:** Patterns of gene expression in murine clonal stromal cell lines as a function of time in culture. After reaching confluency, mRNA was harvested from the murine clonal BMSC lines at 1-week intervals and the levels of mRNA for α1(I) procollagen, AP, BSP, α1(II) procollagen, and LPL were determined by semiquantitative RT-PCR as described under Materials and Methods. BSP is a marker of osteoblastic differentiation, while α1(II) procollagen is indicative of chondrogenic differentiation, and LPL is indicative of adipocytic conversion. α1(I) procollagen is commonly expressed by many phenotypes, AP is expressed by osteogenic and preadipogenic cells. The levels of amplification product were quantitated by densitometric scanning and plotted on a relative scale for each murine clonal line **B:** C-3. **C:** C-4. **D:** C-8. **E:** C-10. **F:** C-11. Each line exhibited a characteristic pattern of mRNA expression, and the levels of the different markers varied within each line as a function of time. All the lines expressed α1(I) procollagen and AP at some point during the time course, whereas only three (C-3, C-4, C-8) expressed BSP, and two expressed α1(II) procollagen (C-3 and C-8), although only C-8 maintained it consistently. Interestingly, those clones that did not express BSP or α1(II) procollagen exhibited a low level of LPL expression, and maintained expression of α1(I) procollagen and AP.

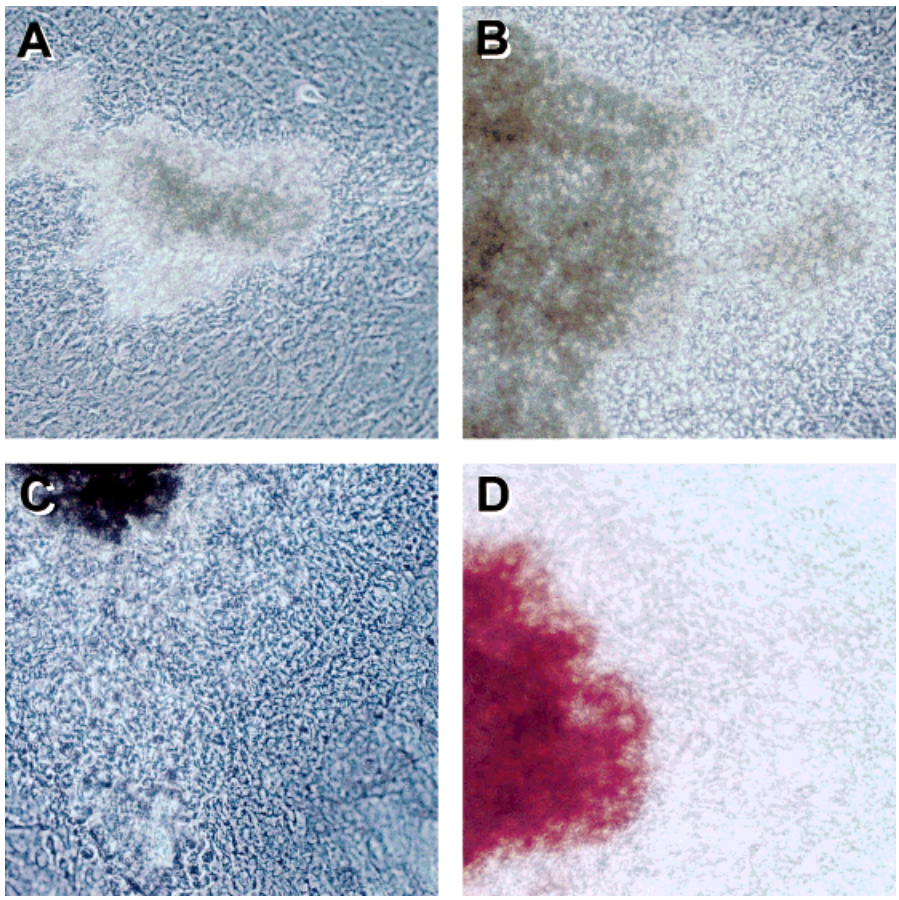

Fig. 3. Phase-contrast micrographs of C-3. At 3–4 weeks after confluency, the multilayered areas of the culture began to mineralize (**A**), and the mineralized area became larger with time (**B**). These nodules, as well as surrounding cells were positively stained for AP (**C**) and Alizarin Red S as a marker of calcium accumulation (**D**).

tively constant levels of α1(I) and AP mRNA, with a slight increase in LPL with time (Fig. 2E). C-11 expressed stable levels of mRNA for α1(I) and initially low levels of AP that increased with time, as well as low LPL that diminished with time (Fig. 2F).

In Vitro Differentiation of Clonal Stromal Cell Lines

Evidence of in vitro differentiation toward osteoblastic, chondrogenic, or adipogenic phenotypes was sought for each cell line at 4 weeks postconfluency, using established morphological/cytochemical criteria. Formation of "bone nodules" (dense cellular mineralizing foci, positive for AP, calcium, and phosphate) was taken as indicative of osteoblastic differentiation in vitro. Accumulation of chondroid-appearing, alcianophilic matrix was taken as evidence of chondrogenic potential, and intracellular accumulation of Oil Red O-stainable lipid vacuoles as evidence of adipogenesis in vitro.

Evidence of "bone nodule" formation in the absence of chondrogenic or adipogenic differentiation was obtained for line C-3 (Fig. 3). Evidence of multiple differentiation (osteogenic, chondrogenic, adipogenic) within the same culture was obtained for line C-8 (Fig. 4). No clear-cut evidence of any type of in vitro differentiation parameter was obtained for the other lines. Of note, "bone nodule" formation was not observed in line C-4, which proved to be osteogenic in vivo (see below).

cbfa1 Expression in Clonal Stromal Cell Lines

Cells were harvested at different time points after reaching confluency, and semiquantitative RT-PCR was performed for *cbfa1*, a transcription factor that has been shown to be essential for osteogenesis during development and postnatal bone formation [Ducy et al., 1997, 1999; Komori et al., 1997]. All lines consistently expressed *cbfa1*. The level of expression was stable over the 4-week culture period

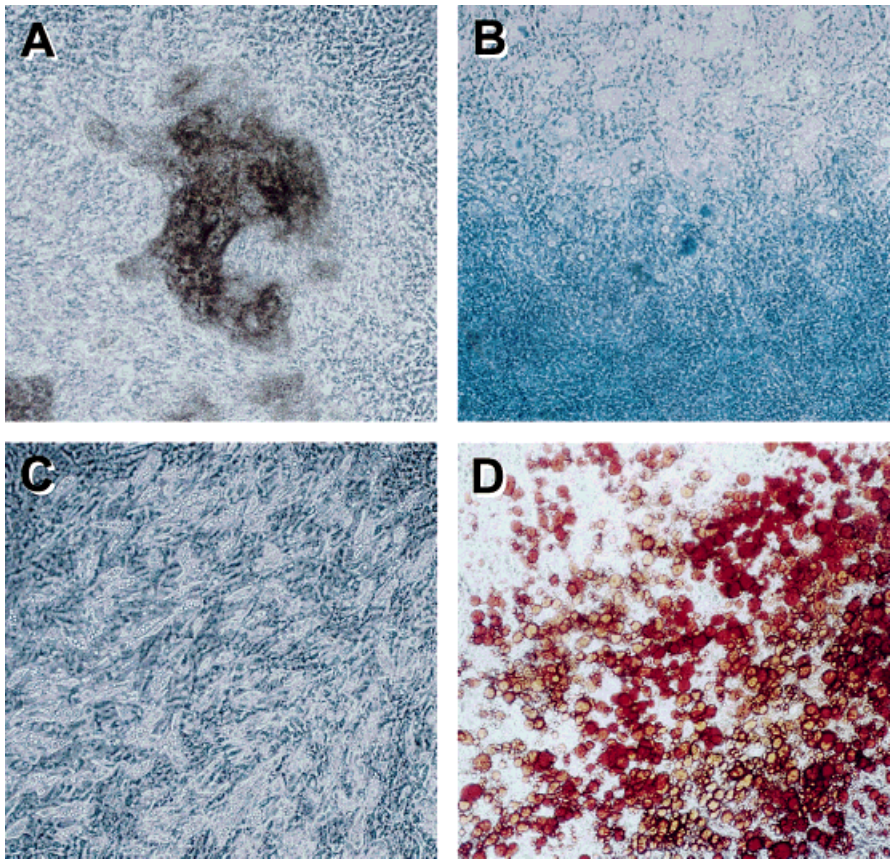

Fig. 4. Phase-contrast micrographs of C-8. C-8 formed nodules at 4 weeks after confluency (**A**), and the characteristics were similar to those of nodules formed in C-3 cultures. Interestingly, this cell line also formed Alcian blue-positive clusters indicative of accumulation of a cartilaginous matrix (**B**). Other areas of the culture showed cells with globular inclusions (**C**), which were positively stained with Oil Red O, indicative of adipogenesis (**D**).

in all lines (Fig. 5), irrespective of the phenotypic expression pattern of each individual clonal line (Fig. 2) and of the simultaneous fluctuations in the levels of phenotype-characteristic genes.

Osteogenesis by Murine Clonal Stromal Cell Lines In Vivo

In vivo transplantation provides the opportunity to determine true osteogenic capacity as identified by the formation of histologically identifiable bone [Bianco and Robey, 1999; Krebsbach et al., 1997, 1998]. The clonal lines were transplanted into critical size calvarial defects in combination with collagen sponges in order to determine their actual osteogenic capacity. C-3, C-4, and C-8 formed bone with clearly identifiable osteocytes and osteoblasts within or in contact with the collagen sponge. Fibroblastic cells were found to be scattered within the remaining portion of the vehicle. By contrast, no bone was observed in the transplants of C-10 or C-11, and only fibroblastic cells were noted within the vehicle (Fig. 6). No

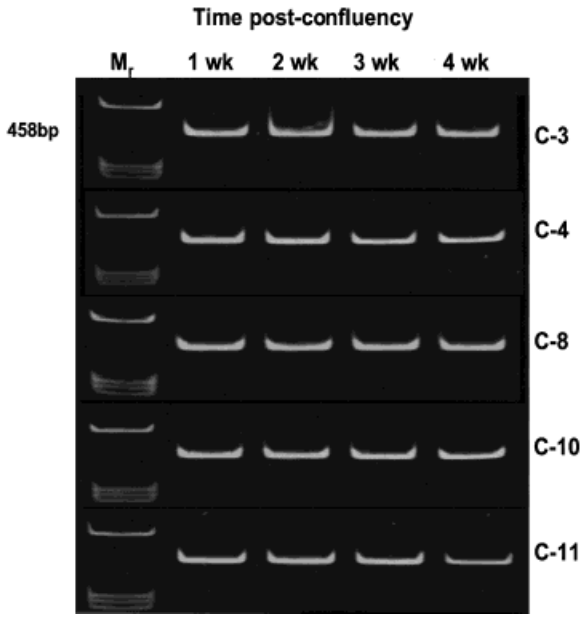

Fig. 5. Expression of *cbfa1* by reverse transcription-polymerase chain reaction (RT-PCR). After reaching confluency, mRNA extracted from the murine clonal BMSC lines at 1-week intervals (see Fig. 2) were used to examine the levels of *cbfa1*. All the murine clonal lines expressed *cbfa1* and maintained similar levels throughout the course of the experiment.

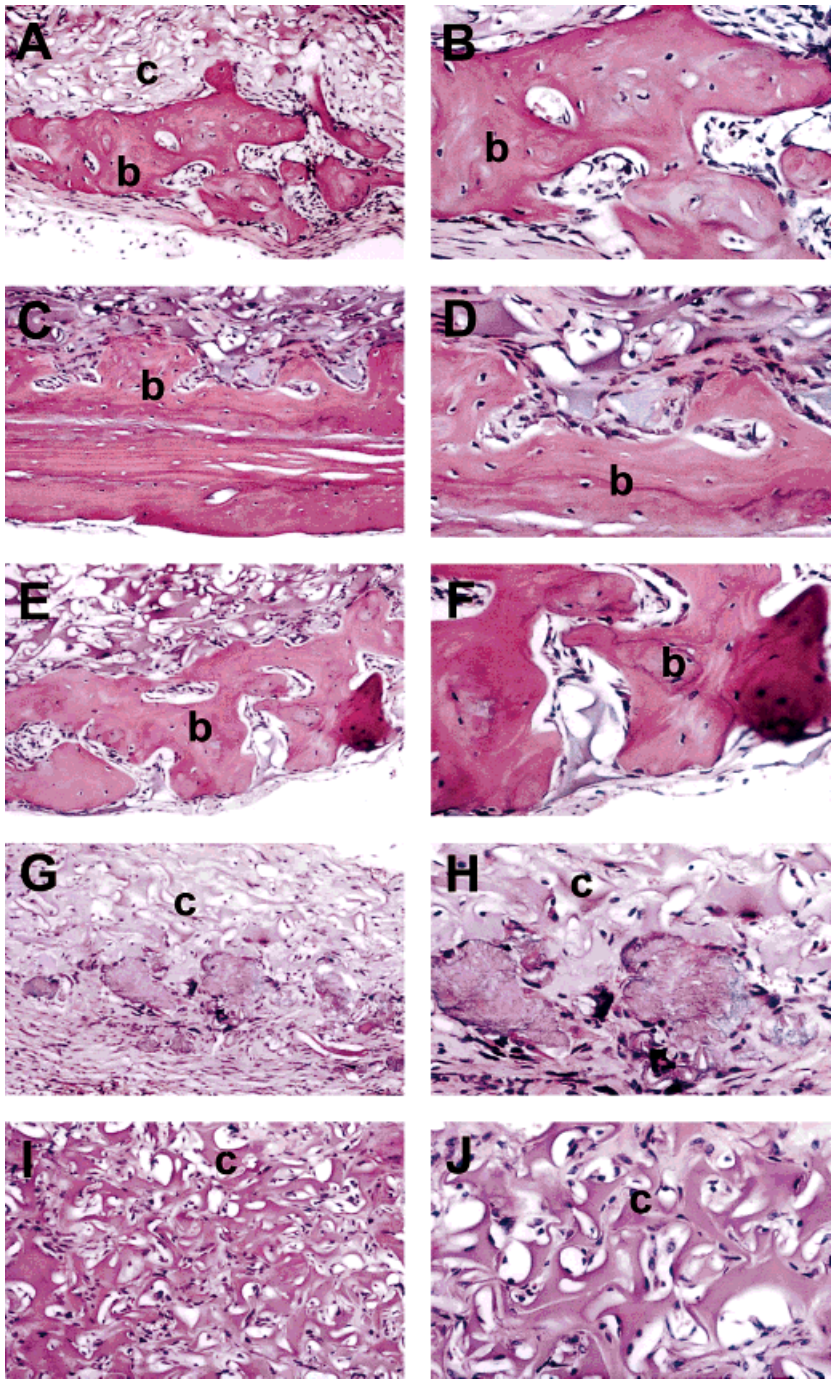

Fig. 6. Photomicrographs of transplants of five murine clonal stromal cell lines. Cells were absorbed to Gelfoam sponge and transplanted into critical sized bone defects in the calvariae of immunocompromised mice. The transplants were harvested at 4 weeks after transplantation (H&E staining). **A,B:** Bone formation by C-3. **C, D:** Bone formation by C-4. **E,F:** Bone formation by C-8. C-10 (**G,H**) and C-11 (**I, J**) failed to form bone. b, newly formed bone; c, collagen sponge carrier.

bone formation was observed in the transplants of mouse skin fibroblasts used as a negative control (data not shown).

To confirm the donor origin of newly formed bone in the murine clonal cell line transplants, PCR for mouse Y chromosome-specific repetitive sequence was performed. A 617-bp PCR product was identified in the reaction of newly formed bone and fibroblastic cells in Gelfoam surrounding newly formed bone, while host bone from the female recipient lacked amplified DNA of mouse Y chromosome-specific repetitive sequence (Fig. 7).

DISCUSSION

Multipotent Versus Restricted Progenitors in the Stromal System

In this study, we isolated and characterized five spontaneously immortalized murine clonal

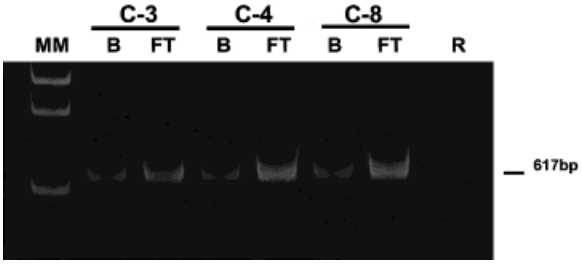

Fig. 7. Polymerase chain reaction (PCR) analysis for a murine Y chromosome-specific repetitive sequence. Newly formed bone in the transplant (B), fibrous tissue (FT) around new bone and recipient female bone (R) were retrieved from eosin-stained sections by microexcision under the microscope. DNA was extracted with 50 mM NaOH treatment, followed by neutralization with 1 M Tris-HCl (pH 7.5). PCR were performed using specific primers for a murine Y chromosome-specific repetitive sequence. The amplified DNA fragment was visualized by ethidium bromide staining. MM, molecular-weight markers.

stromal cell lines. Characterization of the cell lines using three different approaches (expression of phenotypically characteristic mRNAs, in vitro differentiation, in vivo osteogenesis) revealed marked differences in the growth rate, and in differentiation potential both in vitro and in vivo. Three clones were found to proliferate rapidly and were osteogenic in vivo (C-3, C-4, and C-8), while the remaining two proliferated more slowly and were nonosteogenic (C-10, C-11), suggesting that capacity for bone formation may be associated with high rates of cell growth. One clone was found to be multipotential (C-8), while the others exhibited a more restricted pattern of phenotypic expression.

These data demonstrate that although the entire (nonclonal) population of marrow stromal cells behaves as a source of progenitors for multiple skeletal connective tissues (bone, cartilage, hematopoiesis-supporting stroma, adipocytes, fibroblasts), as first demonstrated by Friedenstein and Owen and coworkers [Friedenstein et al., 1970; Owen and Friedenstein, 1988], only a subset of stromal cells (reflected in at least one of our clonal lines) is indeed endowed with multiple differentiation potential. Because the murine clonal lines that were isolated in this study have been passaged more than 30 times, the propensity for spontaneous immortalization of murine cells precludes assessment of their ability to continuously self-replicate. Therefore, we regard our data as only providing evidence for multipotential progenitors within the marrow stromal cell population, with no inference on their identity as true "stem" cells, for which ability to continuously self-renew, independent of immortalization, must be demonstrated [Morrison et al., 1997].

Stromal Cell Differentiation

Analysis of the differentiation potential of stromal cell lines can rely on different approaches, including assessment of gene expression profiles characteristic of a particular phenotype, morphological/biochemical characterization of the cultured cells in vitro, and histology of tissue formed upon in vivo transplantation of ex vivo expanded cells. It is important to note that the three methods may yield significantly divergent information (Table II). Interestingly, all the clones expressed many of the markers characteristic of osteoblastic, chondrogenic and adipocytic phenotypes at some point during the extended culture period, albeit with varying levels. These data suggest that individual members of the stromal cell system are inherently flexible with respect to phenotype and well demonstrate the potential for plasticity in the stromal cell system. However, clearly some of clonal lines had lost some of their flexibility and exhibited a limited profile of phenotypic markers, which may also relate to the decrease in growth rate exhibited by these particular lines.

In our study, expression of osteoblastic, chondrocytic, and adipocytic marker genes did not predict in all cases actual generation of differentiated osteoblasts, chondrocytes and/or adipocytes in culture, or in vivo. For the full development of a phenotype beyond the expression of particular markers, additional environmental cues provided by paracrine and endocrine factors, as well as by three-dimensional structure, may be required. For example, low oxygen tensions conducive for chondrogenesis, are mimicked in "mass" cultures, but not necessarily in cultures of adherent monolayers [Johnstone et al., 1998]. In addition, the in vivo transplantation model system used in this study is clearly not conducive for cartilage formation. Furthermore, in our study, the formation of so-called "bone nodules" in vitro did not predict actual osteogenic capacity in vivo. The clonal line C-4 did not form bone nodules in culture but was able to form bone in vivo. Previous reports have suggested that "bone nodule" formation in vitro is a good marker of osteoblastic cells [Bellows et al., 1986; Nefussi

TABLE II. Phenotypic Characterization of Murine Bone Marrow Stromal Cell Lines

Cell line	Markers (RT-PCR)	In vitro differentiation	In vivo differentiation
C-3	Tri-potential (OB, C, A)	OB	Bone
C-4	Uni-potential (OB)	—	Bone
C-8	Tri-potential (OB, C, A)	Tripotential (OB, C, A)	Bone
C-10	Bi-potential (?) (OB, A)	—	—
C-11	Bi-potential (?) (OB, A)	—	—

OB, Osteoblastic.
C, Chondrogenic.
A, Adipogenic.

et al., 1985]. However, mineral deposited in many cell culture model systems is often spurious, and not characteristic of mineral formed in vivo (E.D. Eanes, NIDCR, personal communication). In this study, the most consistent predictor of osteogenic capacity was production of BSP, whose expression is highly restricted to osteoblasts in vivo.

cbfa1 Expression and Its Significance During Development and Postnatal Bone Formation

A rational layout of the hierarchy of the stromal progenitor cells with different differentiation potential has long been missing, and the need for appropriate markers suited to address the issue repeatedly emphasized. The identification of *cbfa1* as a pivotal transcription factor regulating osteogenic differentiation during embryonic development [Ducy et al., 1997] has provided a new paradigm for investigating the lineage hierarchy within the stromal cell system. Inactivation of the *cbfa1* gene in mice prevents the development of a marrow cavity and therefore the appearance of the myelosupportive and adipocytic stromal cells that are found in the intact marrow [Komori et al., 1997]. Developmentally, *cbfa1* expression (and its histological correlate, osteogenic cells) lies upstream of lineage divergence and restriction in the stromal system. Likewise, when clonal stromal cells are transplanted in vivo in immunocompromised mice, osteogenesis precedes the development of a complete hematopoietic marrow within the ectopic ossicle [Kuznetsov et al., 1997]. Hematopoiesis (and the cognate supporting stromal cells) and marrow adipocytes develop in such systems in temporal succession with, and in tight dependence on, the formation of bone by competent osteoblasts. Both natural developmental patterns and experimental models of bone and marrow ontogeny thus indicate a temporal and possibly mechanistic priority of bone formation over the formation of a marrow stromal tissue.

Thus, *cbfa1* drives the osteogenic differentiation of embryonic mesenchymal cells by regulating the expression of a set of tissue-specific genes, and is indispensable for generation of competent osteoblasts in vivo. Function and tissue specificity of *cbfa1* make it match, in the context of osteogenic commitment, the generic sketch profile of an "ideal" marker which would be (1) tissue specific, (2) of known molecular identity, and (3) relevant to the desired tissue specific functions/differentiation pathway. Surprisingly, the significance of *cbfa1* within this context has been somewhat overlooked.

We have shown in this report that *cbfa1* expression is a common denominator of different murine stromal cell lines characterized by heterogeneous differentiation potential. Interestingly, collagen type I and AP were also commonly expressed in our cell lines. Consequently, it can be surmised that expression of *cbfa1* and of other less stringent markers of the osteogenic phenotype, are early events during stromal cell differentiation, which would explain their recurrence across the clonal cell lines with broad or restricted differentiation potential and divergent growth capacity. However, unlike the other early markers (collagen type I, AP) and late markers of phenotypic differentiation (BSP, collagen type II, LPL), *cbfa1* transcripts remained stable over time in culture, even in the face of shifting profiles of gene expression within an individual clonal strain. Expression of what can be regarded as the most stringent marker of osteogenic commitment available to date thus appears to be a very basic property of functionally different marrow stromal cells.

Furthermore, expression of *cbfa1* transcripts does not prevent the ability of stromal cells to differentiate toward chondrogenic or adipocytic phenotypes, nor does it predict an osteogenic behavior of stromal cells either in vitro, or more significantly, upon in vivo transplantation. These observations suggest that while *cbfa1* is absolutely required for osteogenic differentiation, the initial *cbfa1*-driven osteogenic commitment may in fact be reversible and nonobligatory, and that additional differentiation pathways normally entered by stromal cells (chondrogenesis, adipogenesis) emanate from a common precursor provided by osteogenically competent cells.

In conclusion, five clonal, spontaneously immortalized murine bone marrow stromal cell lines were isolated and characterized. All the lines expressed *cbfa1* irrespective of their ability to differentiate into nonosteoblastic phenotypes. In addition, one line maintained the ability to form a chondrogenic nodule and adipocytes. These results support further the osteoblastic origin of bone marrow stromal cells and confirm the existence of a multipotential progenitor in the post-natal bone marrow. Consequently, bone marrow stromal cells that are committed to osteogenesis (expressing *cbfa1*) are also pluripotent. Further studies are required to determine the factors that are required to induce a particular phenotype, and to maintain it in the bone marrow microenvironment.

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