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Differential Cell Surface Expression of the STRO-1 and Alkaline Phosphatase Antigens on Discrete Developmental Stages in Primary Cultures of Human Bone Cells

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ABSTRACT

Human osteoblast-like cells can be readily cultured from explants of trabecular bone, reproducibly expressing the characteristics of cells belonging to the osteoblastic lineage. Dual-color fluorescence-activated cell sorting was employed to develop a model of bone cell development in primary cultures of normal human bone cells (NHBCs) based on the cell surface expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker alkaline phosphatase (ALP). Cells expressing the STRO-1 antigen exclusively (STRO-1^+/ALP^-), were found to exhibit qualities preosteoblastic in nature both functionally by their reduced ability to form a mineralized bone matrix over time, as measured by calcium release assay, and in the lack of their expression of various bone-related markers including bone sialoprotein, osteopontin, and parathyroid hormone receptor based on reverse trancriptase polymerase chain reaction (PCR) analysis. The majority of the NHBCs which expressed the STRO-1^-/ALP^+ and STRO-1^-/ALP^- phenotypes appeared to represent fully differentiated osteoblasts, while the STRO-1^+/ALP^+ subset represented an intermediate preosteoblastic stage of development. All STRO-1/ALP NHBC subsets were also found to express the DNA-binding transcription factor CBFA-1, confirming that these cultures represent committed osteogenic cells. In addition, our primer sets yielded four distinct alternative splice variants of the expected PCR product for CBFA-1 in each of the STRO-1/ALP subsets, with the exception of the proposed preosteoblastic STRO-1^+/ALP^- subpopulation. Furthermore, upon re-culture of the four different STRO-1/ALP subsets only the STRO-1^+/ALP^- subpopulation was able to give rise to all of the four subsets yielding the same proportions of STRO-1/ALP expression as in the original primary cultures. The data presented in this study demonstrate a hierarchy of bone cell development in vitro and facilitate the study of bone cell differentiation and function. (J Bone Miner Res 1999;14:47–56)

INTRODUCTION

BONE IS A HIGHLY ORGANIZED structure comprising a calcified connective tissue matrix formed by the proliferation and differentiation of osteoprogenitors into mature osteoblasts (reviewed in Refs. 1 and 2). Evidence exists that the osteogenic cell lineage belongs to the stromal fibroblastic system of the bone marrow, which includes other stromal tissues, such as cartilage, smooth muscle, and fat.[3–5] The osteogenic cell lineage is thought to arise from a population of uncommitted multipotential stromal precursor cells which reside in the bone marrow spaces and the surrounding connective tissue. Due to the lack of well defined markers, little is known of the precise developmentally regulated changes in phenotype and patterns of gene expression which occur during the differentiation and maturation of human osteoprogenitor cells into functional osteoblasts and, ultimately, terminally differentiated osteocytes.

To date, the best characterized cells of the osteogenic lineage are the osteoblasts, which are responsible for the

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production of the bone matrix constituents.[6] Osteoblasts are identified morphologically by their cuboidal appearance and by their association with newly synthesized bone matrix at sites of active bone formation. These committed osteogenic cells are also characterized histologically by their expression of high levels of alkaline phosphatase (ALP) activity and biochemically by the synthesis of certain bone-associated matrix proteins, such as collagen type I (COLI), osteocalcin (OC), bone sialoprotein (BSP), osteonectin (ON), and osteopontin (OP).[6–9] However, there is heterogeneity of the osteoblast phenotype according to the proliferative and functional state of the cell population.[6,10] Together these markers are useful in determining osteogenic commitment but cannot yet be associated unambiguously with cells at discrete stages of osteogenic differentiation. Moreover, the less differentiated precursors which maintain the osteogenic cell population remain unidentified in the absence of reliable markers for these cells.

We have previously described a monoclonal antibody (MAb), STRO-1, that binds to an as yet unidentified cell surface antigen expressed by a subset of human bone marrow cells.[11] Included in this subset are all assayable colony-forming units-fibroblast, which under different culture conditions are capable of giving rise to an array of stromal cell types, including smooth muscle cells, fibroblasts, lipid-laden adipocytes, and functional osteoblasts.[11,12]

The isolation and culture of osteoblasts derived from rodent bone tissue has been possible through the use of progressive enzymatic digestion[13,14] or by cell migration from the outgrowth of bone explants.[15,16] Similar culture systems have been established for assessing the biology of normal human bone cells (NHBCs).[17–22] Human osteoblast-like cells can be readily cultured from explants of trabecular bone, reproducibly expressing the characteristics of cells belonging to the osteoblastic lineage, including the ability to form a mineralized bone matrix (hydroxyapatite), the presence of high levels of ALP activity, and the synthesis of OC.[18,20,22–25] However, it is not known what proportion of the cell populations in these cultures are representative of osteoprogenitor cells or indeed other stages of bone cell development such as preosteoblasts, osteoblasts, and osteocytes.

The intention of this study was, therefore, to characterize better this culture system by defining objective criteria to identify and isolate the various cellular components that comprise this heterogeneous population of bone cells. The dissection of this population of NHBCs was based on the use of antibodies to two independent cell surface markers. The bone/liver/kidney isoform of ALP, although not specific to the osteogenic cell lineage, was used as a general marker of committed osteoblasts. The STRO-1 antigen, which is expressed on multipotential bone marrow stromal precursor cells with osteogenic potential, was used as a marker representative of a preosteoprogenitor phenotype. We employed dual-color fluorescence-activated cell sorting (FACS) to subdivide the NHBC population according to the cell surface expression of STRO-1 and ALP. Phenotypically distinct subpopulations were identified, and each was subjected to an analysis of their osteogenic commitment, based both on their expression of bone-associated matrix proteins and the osteogenic transcription factor CBFA1, and on the ability of each subpopulation to form a mineralized bone matrix in vitro.

MATERIALS AND METHODS

Normal human bone cells

Trabecular bone specimens were obtained from normal patients (58–80 years old) during routine knee and hip replacements from the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital. Bone explants were cultured as previously described.[26] Briefly, bone chips were grown in T-75 tissue-culture flasks in α-modified Eagle's medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 μM L-ascorbate-2-phosphate (Novachem, Melbourne, Australia). Cultures were fed three times per week for 5–6 weeks and incubated at 37°C in 5% CO_2 until confluent.

Enzymatic digestion of NHBC cultures

Single-cell suspensions were obtained from confluent primary NHBC cultures by enzymatic digestion.[26] The cultures were washed twice in phosphate-buffered saline, pH 7.4, and then digested in a solution of COLI (3 mg/ml; Worthington Biochemical Co., Freehold, NJ, U.S.A.) and dispase (4 mg/ml) (Neutral Protease Grade II; Boehringer Mannheim, GMBH, Mannheim, Germany) for 90 minutes at 37°C. Cell suspensions were then washed with growth medium before being passed through a Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) to obtain a single cell suspension.

Flow cytometric analysis

Following enzymatic digestion, NHBCs were resuspended in blocking buffer (Hank's balanced salt solution [HBSS] + 20 mM HEPES, 1% normal human serum, 1% bovine serum albumin (Cohn fraction V; Sigma Aldrich Pty. Ltd., New South Wales, Australia), and 5% FCS for 20 minutes on ice. Approximately $1–3 \times 10^7$ cells were pelleted in 14-ml polypropylene tubes (Falcon Labware, Lincoln Park, NJ, U.S.A.) and resuspended in 200 μl of saturating concentrations of B4–78[27] (mouse immunoglobulin G [IgG_1] anti-human bone/liver/kidney ALP; Developmental Studies Hybridoma Bank, Iowa University, Iowa City, IA, U.S.A.) and STRO-1 for 45 minutes on ice. The isotype matched negative control antibodies, IgG_1 (3D3) and IgM (1 A6.12) (kindly provided by Dr. L.K. Ashman, Hanson Center for Cancer Research, Adelaide, South Australia), were used under identical conditions. The cells were then washed in HBSS with 5% FCS and incubated with a goat anti-mouse IgG (γ-chain specific) phycoerythrin (PE) (1:50) and a goat anti-mouse IgM (μ-chain–specific) fluorescein isothiocyanate (FITC, 1:30) (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) for 45 minutes on ice. The cells were then washed twice more and resuspended to

TABLE 1. PCR PRIMER SEQUENCES AND EXPECTED PRODUCT SIZE

Transcript name	*RT-PCR primer set (5′–3′) Upper primer (UP) Lower primer (LP)*	*Expected product size (bp)*
GAPDH[29]	UP: TTGACCTCAACTACATGG LP: TCATACCAGGAAATGAGC	992
Human OP[30]	UP: TTGCTTTTGCCTCCTAGGCA LP: GTGAAAACTTCGGTTGCTGG	430
Human CBFA1[31]	UP: GTGGACGAGGCAAGAGTTTCA LP: TGGCAGGTAGGTGTGGTAGTG	632
Human COLI[32]	UP: AGGGCTCCAACGAGATCGAGATCCG LP: TACAGGAAGCAGACAGGGCCAACGTCG	222
Human PTHR[33]	UP: AGGAACAGATCTTCCTGCTGCA LP: TGCATGTGGATGTAGTTGCGCGT	571
Human BSP[34]	UP: TCAGCATTTTGGGAATGGCC LP: GAGGTTGTTGTCTTCGAGGT	657
Human ON[35]	UP: ATGAGGGCCTGGATCTTCTT LP: CTGCTTCTCAGTCAGAAGGT	576
Human OCN[36]	UP: ATGAGAGCCCTCACACTCCTC LP: CGTAGAAGCGCCGATAGGC	289

~10^6 cells/ml prior to being sorted using a FACStar^{PLUS} (Becton Dickinson, Sunnyvale, CA, U.S.A.) flow cytometer. Positivity for each antibody was defined as the level of fluorescence > 99% of the isotype-matched control antibodies. Following the initial sort, each STRO-1/ALP subpopulation was resorted and analyzed to ensure a purity of > 99%.

RNA isolation and first-strand cDNA synthesis

Total cellular RNA was routinely prepared from 2×10^4 FACS double-sorted NHBC subpopulations using the RNAzolB extraction method (Biotecx Laboratories Inc., Houston, TX, U.S.A.), per the manufacturer's recommendations. RNA isolated from each subpopulation was then used as a template for cDNA synthesis. cDNA was prepared using a first-strand cDNA synthesis kit from Pharmacia Biotech (Uppsala, Sweden) according to the manufacturer's instructions. Briefly, total RNA was resuspended in 8 μl of diethylpyrocarbonate-treated water and subsequently heated to 65°C for 10 minutes. Following snap cooling on ice, the RNA was added to 7 μl of premix containing reaction buffer, oligo-dT as primer, and Superscript Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (RT). Following incubation at 42°C for 60 minutes, the volume of the reaction was adjusted to 50 μl with the addition of 35 μl of sterile water.

Polymerase chain reaction

Due to limited cell numbers, the expression of various bone-related transcripts (Table 1) was assessed by polymerase chain reaction (PCR) amplification, using a standard protocol.[28] Two microliters of the resultant first-strand cDNA mixture from each subpopulation was diluted in a 50-μl PCR reaction (1× buffer = 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)2SO_4$, 0.45% Triton X-100, 200 μg/ml gelatin, 2 mM $MgCl_2$, 200 μM each dNTP) containing 100 ng of each primer detailed in Table 1. This mixture was heated to 96°C for 3 minutes, snap chilled, and 1 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) was added. Reaction mixes were overlayed with mineral oil, and amplification was achieved by incubation in a Perkin-Elmer Cetus thermal cycler. Primer design enabled typical cycling conditions of 94°C/2 minutes, 60°C/30 s, 72°C/1 minute for 40 cycles, with a final 10 minute incubation at 72°C. To control for the integrity of the various RNA preparations, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and/or β_2-microglobulin was also assessed. Following amplification, 10 μl of each reaction mixture was analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Mineralization in vitro

We have previously reported the conditions for the induction of human bone marrow stromal cells to develop a mineralized bone matrix in vitro.[12] Briefly, primary sorted NHBCs were cultured (5×10^3 cells/well 96-well microtiter plate) in α-MEM (Flow Laboratories) supplemented with 20% FCS, L-glutamine (2 mM), β-mercaptoethanol (5×10^{-5} M), L-ascorbic acid 2-phosphate (100 μM) (ASC-2P; Novachem, Melbourne, Australia), dexamethasone sodium phosphate (10^{-8} M) (DEX; David Bull Laboratories, Sydney, Australia), KH_2PO_4 (1.8 mM) (BDH Chemicals Ltd. Poole, Dorset, U.K.), and HEPES (10 mM) at 37°C, 5% CO_2. The media was changed twice a week for a period of 7 weeks.

Measurement of calcium levels

Single-cell suspensions of primary sorted NHBCs were seeded at a density of 5×10^3 cells/well in a 96-well microtiter plate (NUNCs). Calcium levels were determined as described elsewhere[37] from triplicate cultures at weekly intervals for 7 weeks. The cultures were washed four times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and incubated overnight with 0.6 N HCl. Samples of the solu-

bilized matrix were then reacted with o-cresol-phthalein-complexon (Test Combination Calcium; Boehringer Mannheim), and the colormetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium according to the manufacturer's recommendations.

Transmission electron microscopy

Seven-week-old mineralized NHBC cultures were washed in 0.05 M sodium cacodylate buffer and then fixed in 2.5% glutaraldehyde (EM grade) in 0.05 M sodium cacodylate buffer for 2 h at room temperature. After washing with 0.05 M cacodylate buffer, the cultures were postfixed with 2% osmium tetroxide (VIII; BDH Chemicals) in cacodylate buffer for 1 h at room temperature and then washed in cacodylate buffer. Following this, the cultures were dehydrated with graded alcohol (70, 90, and 100% ethanol solutions) for two 15-minute changes for each alcohol grade. Epoxy resin (TAAB Laboratories; Berkshire, U.K.) was then used to infiltrate the cultures overnight at 37°C. Polymerization of the resin was carried out at 60°C for 24 h under vacuum. Ultrathin sections were cut on an 8800 Ultrotome II (LKB, Broma, U.K.) and mounted onto copper grids. Sections were then examined using a 1200 EX II transmission electron microscope (JEOL, Tokyo, Japan). Photographs were taken using EM Technical film (Ilford, Mobberly, Cheshire, U.K.). Mineral deposits were analyzed using a Tracor-Northern Series II EDX System (EDX, energy dispersive X-ray microanalyzer; Tracor Europa B.V., Bilthoven, The Netherlands). Electron diffraction analysis was performed using a camera length of 100 cm on the mineralized bone cultures and on a commercially available hydroxyapatite standard (hydroxyapatite type 1; Sigma).

Statistical analysis

One-way analysis of variance was used to determine any differences in the calcium concentrations between the different STRO-1/ALP NHBC subsets. Statistical significance ($p^2 = 0.05$) in the level of calcium detected between the different STRO-1/ALP subgroups at each time point was determined using Fisher's projected least significant difference (PLSD) test.

RESULTS

Cell surface expression of STRO-1 and ALP on primary NHBCs

Dual-color FACS was employed to determine the pattern of expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker ALP on enzymatically treated single-cell suspensions of 5- to 6-week-old primary NHBC cultures derived from 12 different donors. The NHBC population demonstrated a heterogeneous but highly reproducible pattern of expression for both the STRO-1 and ALP antigens (Fig. 1). The majority of the NHBCs (62.2 ± 2.7% SEM, $n = 12$) demonstrated no detectable levels of STRO-1 or ALP at the cell surface ($STRO\text{-}1^-/ALP^-$). A high proportion of the remaining

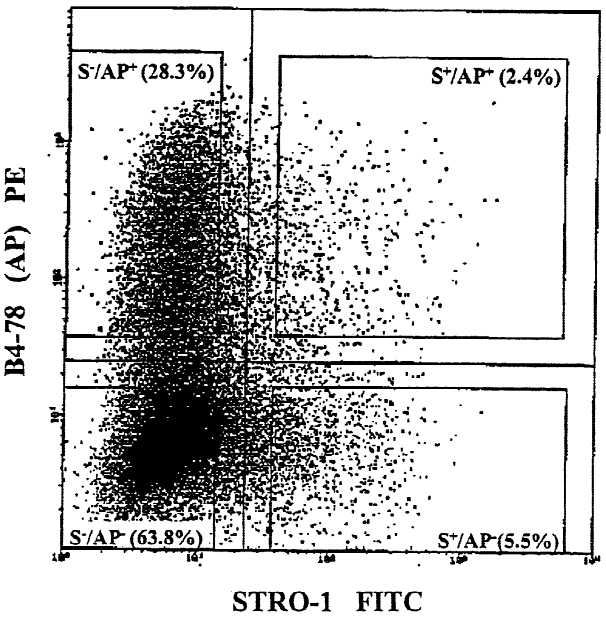

FIG. 1. A representative histogram demonstrating the immunoreactivity of the MAbs B4-78 and STRO-1 with NHBCs. Dual-color flow cytometric analysis depicting the cell surface expression of the STRO-1 and ALP antigens on single-cell suspensions of NHBCs. A typical dot plot histogram is shown from a representative experiment where 5×10^4 events were collected as list mode data. The vertical and horizontal lines were set to the reactivity levels obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC), respectively.

NHBC population (30.8 ± 2.5 SEM, $n = 12$) expressed the osteoblastic marker ALP exclusively ($STRO\text{-}1^-/ALP^+$), while only a minor population (3.6 ± 0.6 SEM, $n = 12$) of cells expressed the STRO-1 antigen alone ($STRO\text{-}1^+/ALP^-$). In addition, a small proportion of cells (3.4 ± 0.7 SEM, $n = 12$) were also found to coexpress the ALP and STRO-1 antigens ($STRO\text{-}1^+/ALP^+$). The number of cells expressing B4-78 in a given sample correlated to the number of cells expressing ALP activity histochemically (data not shown). Following FACS of each of the four different STRO-1/ALP subpopulations, each was resorted and analyzed to ensure a purity of > 99% (Fig. 2) for all subsequent experiments.

In vitro mineralization potential of the different STRO-1/ALP subpopulations

The ability of the different STRO-1/ALP sorted subpopulations to form a mineralized bone matrix was determined by culturing the cells in osteoinductive conditions in the presence of ASC-2P, DEX and PO_{4i} as previously described.[12] A mineralized matrix formed in all the STRO-1/ALP NHBC subsets (Fig. 3) and was identified as hydroxyapatite by X-ray diffraction and EDX analysis (Fig. 4). The most rapid onset of mineral deposition was observed in the $STRO\text{-}1^-/ALP^+$ subpopulation. The level of

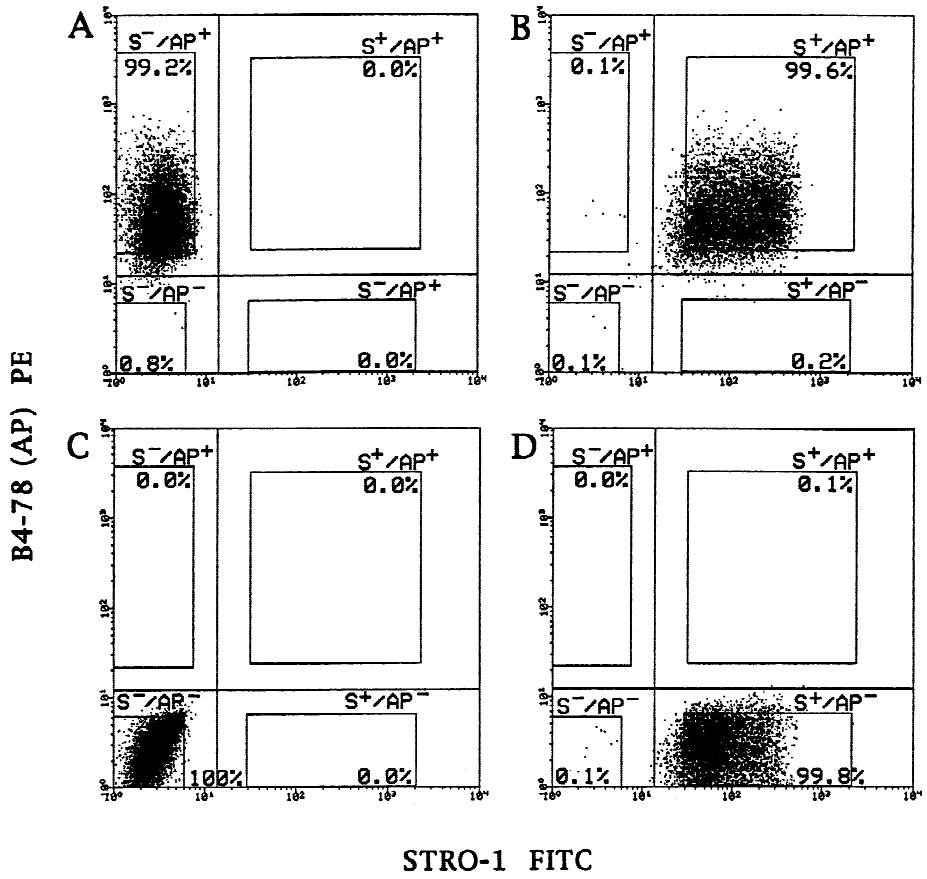

FIG. 2. Double sorting experiments were conducted on each of the STRO-1/ALP NHBC subsets sorted initially from primary cultures to ensure a purity of 99% for each phenotype. (A) STRO-1$^-$/AP$^+$; (B) STRO-1$^+$/AP$^+$; (C) STRO-1$^-$/AP$^-$; (D) STRO-1$^+$/AP$^-$. A typical dot plot histogram is shown from a representative experiment where 2×10^4 events were collected as list mode data. The vertical and horizontal lines were set to the reactivity levels obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC), respectively.

mineral formation by the STRO-1$^-$/ALP$^+$ cells between 14 and 35 days of osteoinduction was significantly greater (Fisher's PLSD test; $p^2 = 0.05$) than that demonstrated by the STRO-1$^+$/ALP$^-$ and STRO-1$^-$/ALP$^-$ subgroups and significantly greater ($p^2 = 0.05$) than the level of mineral formation by the STRO-1$^+$/ALP$^+$ subpopulation between 14 and 28 days. The STRO-1$^+$/ALP$^+$ subpopulation demonstrated a potential for mineral formation intermediate between that of the STRO-1$^+$/ALP$^-$ and STRO-1$^-$/ALP$^+$ populations. Differences in mineral deposition between the STRO-1$^+$/ALP$^+$ and both the STRO-1$^+$/ALP$^-$ and STRO-1$^-$/ALP$^-$ subpopulations were significant ($p^2 = 0.05$) at 14–35 days and at 14 and 35 days, respectively. The lowest potential for mineral formation was exhibited by the STRO-1$^+$/ALP$^-$ and STRO-1$^-$/ALP$^-$ NHBC fractions, which also demonstrated a delay in the onset of mineralization relative to the STRO-1$^-$/ALP$^+$ subpopulation and at some of the time points assayed for the STRO-1$^+$/ALP$^+$ subgroup. Approximately equivalent levels of mineral deposition were evident at all the time points measured, with the exception at day 35 when a significantly ($p^2 = 0.05$) higher level of mineral formation was observed in the STRO-1$^-$/ALP$^-$ subgroup relative to the STRO-1$^+$/ALP$^-$ subpopulation. Based upon these studies, the overall ability of each STRO-1/ALP NHBC subset to form a mineralized matrix at the end of the osteoinduction period was ranked as follows: STRO-1$^-$/ALP$^+$ > STRO-1$^+$/ALP$^+$ > STRO-1$^-$/ALP$^-$ > STRO-1$^+$/ALP$^-$.

Differential expression of bone-related markers between the different STRO-1/ALP subpopulations

The expression of various genes associated with osteogenic development was determined for all the different NHBC STRO-1/ALP subsets. RT-PCR amplification was employed due to the small numbers of cells (2×10^4) available following double sorting. The mRNA integrity in each of the sorted subpopulations was confirmed by reproducible and consistent amplification of the ''house-keeping'' gene GAPDH. As anticipated, RT-PCR analysis of mRNA isolated from the unfractionated (bulk) NHBCs demonstrated expression of all the bone-related genes examined including OC, COLI, ON, OP, BSP, and the parathyroid hormone receptor (PTHR). The four STRO-1/ALP NHBC subpopulations were characterized by distinct patterns of expression of these bone markers (Fig. 5). Although all

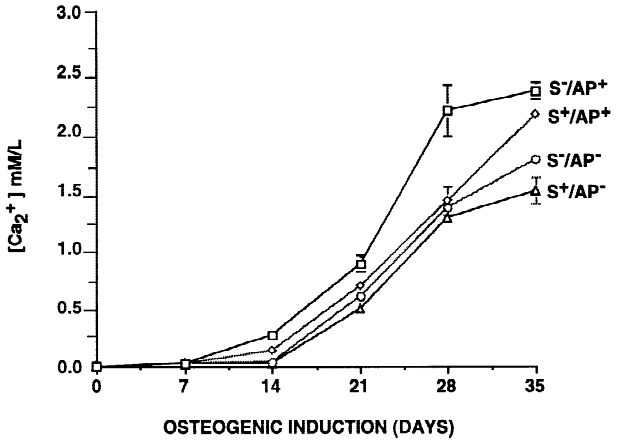

FIG. 3. The mean calcium levels were determined from cultures established from three different donors under osteoinductive conditions (ASC-2P, DEX, and PO_{4i}) at weekly intervals for 35 days. The release of free calcium was achieved by treating the adherent cell layers of NHBC cultures under acidic conditions. Samples were then reacted with o-cresol-phthalein-complexon and the colormetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium for each STRO-1/ALP NHBC subset over the time interval.

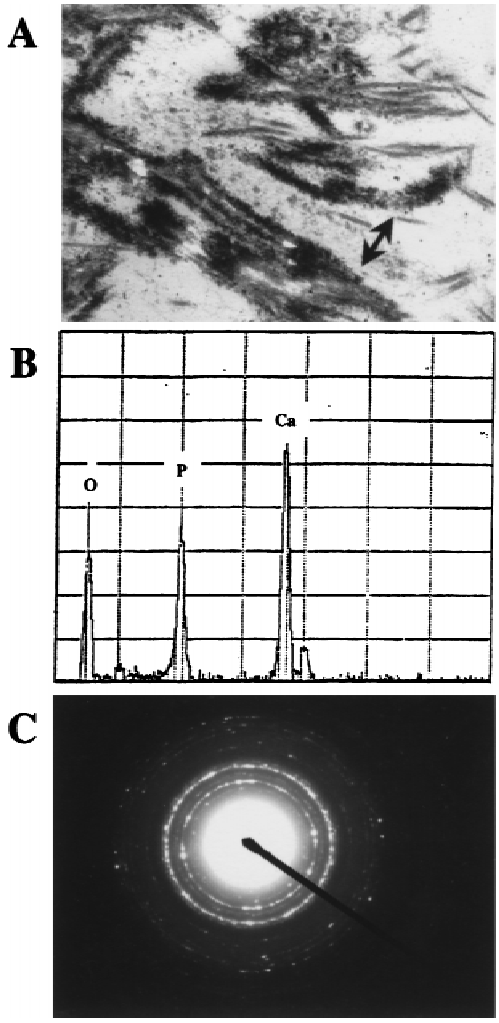

FIG. 4. A representative transmission electronmicrograh of the adherent cell layers of NHBC cultured for 5 weeks in the presence of ASC-2P, DEX, and PO_{4i}. (A) Examination of the extracellular matrix of the adherent layers from a typical bulk culture, demonstrating the presence of deposits of hydroxyapatite-like crystals (double arrow) in association with a network of collagen fibrils (×20,000). (B) EDX analysis of similar mineral deposits demonstrating major peaks for calcium (Ca) and phosphorous (P) with a mean Ca/P ratio consistant with hydroxyapatite. (C) The X-ray diffraction pattern of the mineral deposits in the same cultures was found to be identical to that of the hydroxyapatite standard.

subpopulations expressed transcripts for COLI, ON, and OC, only the STRO-1^-/ALP^+ and STRO-1^-/ALP^- subgroups exhibited OP and BSP. The STRO-1^-/ALP^+ fraction was unique among the four NHBC subpopulations in its expression of PTHR and in demonstrating a pattern of gene expression identical to that of the unfractionated bulk NHBC population. The STRO-1^+/ALP^- and STRO-1^+/ALP^+ subgroups demonstrated a similar pattern of expression characterized by the absence of transcripts for OP, BSP, and PTHR. Given recent reports identifying the key role of the runt-containing transcription factor CBFA1 in the regulation of bone cell differentiation, we also examined CBFA1 expression in the various fractions. Primers were designed to amplify a 635 base pair fragment, and, in accord with this, a band of the expected size was identified in all four STRO-1/ALP subpopulations. Unexpectedly, all the STRO-1/ALP subgroups with the exception of the STRO-1^+/ALP^- subpopulation yielded three additional smaller PCR products. DNA sequence analysis of these products indicated that the smaller amplified bands represented spliced variants of the full-length transcript (data not shown). Although all the sorted groups of NHBC expressed CBFA1 transcripts, only the full-length transcript was observed in the STRO-1^+/ALP^- subgroup.

We next examined whether the discordant pattern of gene expression among the various STRO-1/ALP subpopulations was a result of possible differences in the cycling status among the different fractions. Three-color FACS analysis of primary NHBC based on their expression of STRO-1, ALP, and the cell cycling–specific antigen Ki-67[38,39] revealed that the majority of the cells (> 80%) in each of the four STRO-1/ALP subsets were actively dividing, as indicated by their expression of the Ki-67 antigen (data not shown).

STRO-1 and ALP expression following reculture of NHBC subpopulations

A series of reculture experiments was performed in order to examine the fidelity of the cell surface phenotype of each of the four NHBC subpopulations initially isolated based on their expression of the STRO-1 and ALP antigens. Each subpopulation obtained was resorted to ensure phenotypic purity and then recultured for a period of 2 weeks under

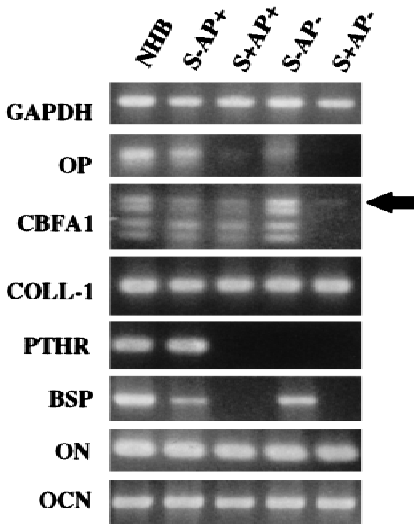

FIG. 5. Expression of numerous bone-related transcripts (ON, osteonectin; CBFA1; COLI, collagen type I; OC, osteocalcin; BSP, bone sialoprotein; OP, osteopontin) was assessed in the various STRO-1/ALP–sorted NHBC subpopulations by RT-PCR from a representative culture. Expression of GAPDH was assessed as an indicator of mRNA integrity. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

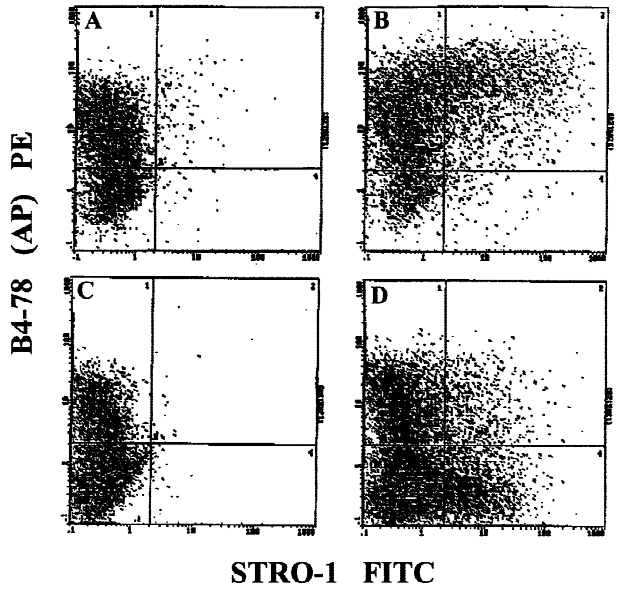

FIG. 6. A representative flow cytometric analysis of the different STRO-1/ALP NHBC subpoulations: (A) STRO-1^-/ALP^+; (B) STRO-1^+/ALP^+; (C) STRO-1^-/ALP^-; (D) STRO-1^+/ALP^- following reculture for 2 weeks under standard culture conditions supplemented with ASC-2P. A typical dot plot histogram is shown from a representative experiment where 2×10^4 events were collected as list mode data. The vertical and horizontal lines were set to the reactivity levels obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC), respectively.

standard growth conditions. Reculture experiments were performed on primary NHBCs from three different donors ($n = 3$). Following this, the cultured cells were subjected to flow cytometric analysis using STRO-1 and the B4-78 MAbs. Data from a representative experiment are shown in Fig. 6. Over the 2 weeks of culture following their initial isolation, none of the four STRO-1/ALP subpopulations maintained the phenotype for which they were initially selected. The NHBC fraction which originally expressed the STRO-1^+/ALP^- phenotype demonstrated the same STRO-1/ALP pattern of expression as observed in the initial primary NHBC cultures (Fig. 6D). This was in contrast to the other three STRO-1/ALP subgroups, which demonstrated alternate STRO-1/ALP distribution patterns quite distinct from that exhibited by unfractionated NHBCs. The majority of the NHBC (73.0 ± 0.1% SEM, $n = 3$) sorted on the basis of the STRO-1^+/ALP^+ phenotype were found to have lost cell surface expression of the STRO-1 antigen but retained their expression of ALP after reculture (Fig. 6B). Similarly, the majority (76.5 ± 7.2% SEM, $n = 3$) of the NHBCs sorted initially on the basis of the STRO-1^-/ALP^+ phenotype retained their expression of ALP after 2 weeks in culture (Fig. 6A). A proportion (29.7 ± 5.7% SEM, $n = 3$) of those NHBCs which initially lacked any cell surface expression of STRO-1 or ALP (STRO-1^-/ALP^-) were now found to express the ALP antigen following reculture (Fig. 6C).

DISCUSSION

The development of osteoblasts from undifferentiated precursor cells occurs through a series of cellular transitional stages which can be identified based on morphological, biochemical, and molecular criteria.[6] Current evidence suggests that COLI, the major constituent of the extracellular matrix in bone is expressed in all of the developmental stages within the bone cell lineage. Other markers, such as ALP, ON, OP, and PTHR/parathyroid hormone–related protein receptor (PTHrpR) are also thought to be expressed in early preosteoblasts and functionally active mature osteoblasts but are absent or only weakly present in differentiated osteocytes. In contrast, the noncollagenous proteins OC and BSP appear to be restricted in their expression to the mature osteoblasts and osteocytes. In the present study, we utilized primary cultures of NHBCs as an in vitro model of osteogenesis. Under appropriate culture conditions, NHBCs exhibit characteristics attributed to functionally active osteoblasts, including the ability to form a mineralized bone matrix composed of hydroxyapatite in vitro[24,40,41] and to express all the above bone-related markers.[18,20,22–25,42,43] Despite the utility of cultured bone cells as an in vitro model of human bone cell development, such culture systems are inherently complex and contain a range of bone cell types at various stages of differentiation. The present study was, therefore, performed in order to develop a means of identifying and isolating NHBCs at various stages of development.

Several studies have examined the expression of ALP as an early marker of osteogenic differentiation to identify committed bone cells in cultures comprised of heteroge-

neous cell populations. Flow cytometric analysis has been previously used to quantitate the number of ALP expressing fixed stromal cells derived from rat bone marrow.[44] Subsequent studies by Aubin and colleages[45,46] utilized MAbs reactive to ALP in order to isolate living cells with osteogenic potential from cultures derived from adult rat bone marrow and from fetal rat calvaria. In the present study, we employed dual-color FACS to identify and isolate four distinct subsets within the NHBC population based on the cell surface expression of the stromal precursor cell marker STRO-1 and the osteoblastic marker ALP. We hypothesized that NHBCs expressing the ALP antigen represented a more differentiated bone cell phenotype compared with those cells expressing the STRO-1 antigen alone. To test our hypothesis, the different STRO-1/ALP subsets were first placed under osteoinductive conditions to assess the ability of each phenotype to form a mineralized bone matrix, a function that is characteristically attributed to mature osteoblasts.

Compared with the other NHBC subpopulations, cells expressing the STRO-1 antigen alone demonstrated both a delayed onset in mineral formation and a reduced capacity for the formation of a mineralized bone matrix over time. In addition, this subpopulation lacked expression of markers characteristic of more mature bone cells, such as BSP and OP, and of the four subpopulations examined this is the only one that upon sorting and reculture has the capacity to generate all the four STRO-1/ALP fractions de novo. Collectively, these data are consistent with the hypothesis that the STRO-1^+/ALP^- subpopulation in NHBC cultures represents an early osteoprogenitor population. In contrast, the STRO-1^-/ALP^+ fraction exhibits both rapid mineralization kinetics in vitro and expression of all the bone markers examined and thus appears representative of a population of fully differentiated osteoblasts. Intermediate between the two both phenotypically and functionally is the STRO-1^+/ALP^+, which we propose represents a preosteoblastic cell population. A clear parent–progeny relationship among these three populations is established by the resorting experiments, which, together with the phenotypic and functional data, constitute the basis for the hierarchical scheme presented in Fig. 7.

In proposing this scheme, several points are of note. First, although widely regarded as a specific biosynthetic product of mature osteoblasts, OC expression was nevertheless detected in all the STRO-1/ALP subpopulations. The reasons for this are unclear but may, for example, reflect lack of fidelity of expression under the in vitro culture conditions utilized or the presence of contaminating OC^+ cells in each of the sorted fractions. It should be noted that OC expression in these studies was demonstrated by RT-PCR only and it will therefore be important in future studies to examine OC expression at the protein level in each of the STRO-1/ALP subpopulations. An alternative possibility is that OC expression may not be tightly restricted to mature osteoblasts, as is currently believed, and may be expressed at earlier developmental stages. In support of this notion, OC has been previously detected in preosteoblasts in vivo.[47]

Second, the physiological counterpart of the NHBC population which lacks both STRO-1 and ALP remains to be determined. Given that the STRO-1^-/ALP^- subgroup constitutes the majority of the NHBC population, this cell fraction may be representative of other differentiated bone cells, such as osteocytes and/or bone lining cells due to the reduced functional properties of these cells to form a mineralized bone matrix compared with the STRO-1^-/ALP^+ subgroup. In support of this view, osteocytes and bone lining cells have been shown to have little or no ALP activity and lack the expression of the PTHR in vivo,[48–50] consistent with the pattern of gene expression exhibited by the STRO-1^-/ALP^- subpopulation in vitro. In addition, following reculture of the STRO-1^-/ALP^-–selected subgroup, a minor proportion of the cells were found to develop a STRO-1^-/ALP^+ phenotype. This may imply a plasticity during bone cell development where for example bone lining cells may be capable of differentiating into osteoblasts and visa versa.

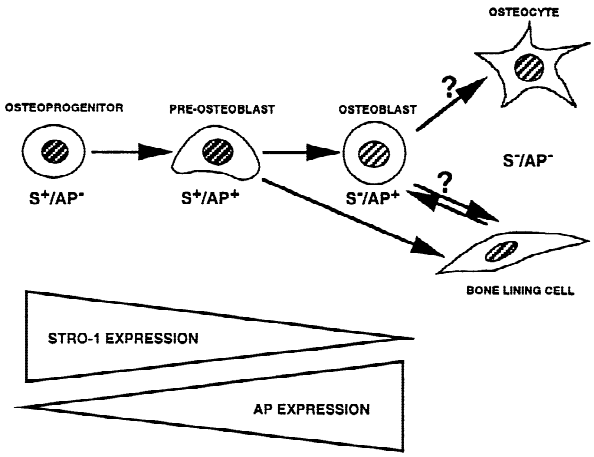

FIG. 7. Proposed hierarchy of bone cell development based on the cell surface expression of the STRO-1 (S) and ALP antigens in primary cultures of NHBCs.

The discovery of the DNA-binding protein CBFA1 has provided a marker of early osteogenic commitment.[51–53] CBFA1 is a homolog to the runt family of transcriptional factors and has been specifically associated with osteogenic tissue in rodents.[51–53] The precise function of CBFA1 is unknown but it is thought to regulate the osteoblast-specific gene, OC, by complexing with the OC promoter. Although an important marker of early osteogenesis, CBFA1 cannot be used to identify, isolate, and manipulate living osteogenic cells within a heterogeneous stromal cell population. In the present study, all STRO-1/ALP NHBC subsets were found to express CBFA1, confirming that primary NHBC cultures are representative of committed osteogenic cells. Furthermore, our primer sets yielded three distinct splice variants in addition to the expected PCR product for CBFA1 in each of the STRO-1/ALP subsets, with the exception of the STRO-1^+/ALP^- subpopulation. The low levels of CBFA1 expression observed in the STRO-1^+/ALP^- fraction may be due to the presence of only a minor population of $CBFA1^+$ cells within this subset. The availability of antibodies reactive to the CBFA1 gene product may resolve this issue. Recent reports have identified two alterna-

tive spliced variants at the 3′ end of the human CBFA1 gene, which have been shown to affect protein function.[54] We are currently pursuing the possibility that the expression of the alternative splice variants in this study are associated with the switch from a preosteoblastic phenotype to a functional osteoblast and a mature osteocyte.

Full characterization of the STRO-1/ALP NHBC subpopulations will depend on the further development of MAbs with restricted reactivity to specific developmental stages during osteogenesis. Osteocyte-specific MAbs, OB7.3,[55] OB37.11,[56] and SB-5,[57] and osteoblast-specific MAbs SB-2 and SB-3[57] have previously been developed reactive to avian bone cells which recognize as yet unidentified antigens. Unfortunately, these reagents do not cross-react with mammalian bone cells in vivo or in vitro and therefore cannot be used to dissect our model of bone cell development. In humans, the MAb OB/M[58] reacts with a cytoplasmic antigen in osteocytes and may be useful in determining whether the STRO-1^-/ALP^- NHBC subpopulation represents an osteocyte-like phenotype. To complicate matters further, other markers such as CD44, which appear to react specifically to osteocytes in vivo,[59] have been found to be constitutively expressed by all cells in primary cultures of NHBC (Gronthos S, unpublished observation). Similarly, other MAbs known to react with human stromal precursors/osteoprogenitors (SH-2, SH-3, and SH-4,[60] and SB-10[61]) and osteoblasts (SB-20 and SB-21)[61] in vivo demonstrate ubiquitous expression in stromal cultures derived from human bone and bone marrow. This typifies the limitations of any in vitro culture system where antigen expression can vary from that observed in vivo for cells of similar ontogeny. The continuing development of novel lineage and stage-specific markers will allow us to use this system to subset further the NHBC population and identify more accurately all the different bone cell types within, therefore facilitating the study of osteoblast function, differentiation, and pathogenesis of various bone diseases.

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