

Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow

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Summary

Previous studies have provided evidence for the existence of adult human bone marrow stromal stem cells (BMSSCs) or mesenchymal stem cells. Using a combination of cell separation techniques, we have isolated an almost homogeneous population of BMSSCs from adult human bone marrow. Lacking phenotypic characteristics of leukocytes and mature stromal elements, BMSSCs are non-cycling and constitutively express telomerase activity *in vivo*. This mesenchymal stem cell population demonstrates extensive proliferation and retains the capacity for differentiation into bone, cartilage and adipose tissue *in*

vitro. In addition, clonal analysis demonstrated that individual BMSSC colonies exhibit a differential capacity to form new bone *in vivo*. These data are consistent with the existence of a second population of bone marrow stem cells in addition to those for the hematopoietic system. Our novel selection protocol provides a means to generate purified populations of BMSSCs for use in a range of different tissue engineering and gene therapy strategies.

Key words: Bone Marrow Stroma, Mesenchymal Stem Cells, STRO-1, Bone, Cartilage, Adipose, CFU-F

Introduction

Within the bone marrow (BM) hematopoiesis occurs in association with a complex stroma comprising a heterogeneous population of non-haemopoietic cells including fibroblasts, adipocytes, osteoblasts and other cellular elements of bone (Bianco, 1998; Lichtman, 1981; Weiss, 1976). Although the role played by BM stromal cells in the functional support of hematopoiesis has been extensively investigated (Allen et al., 1990; Dexter et al., 1977), other aspects of the biology of this tissue remain poorly understood. In particular, precise knowledge of the developmental relationship between the various stromal elements of the BM, both during ontogeny and in the postnatal organism, is currently lacking. Circumstantial evidence for the existence of precursor cells for marrow stromal tissue is provided by studies of the regeneration of BM following ablation of the tissue by various means (Knospe et al., 1972; Patt and Maloney, 1975) and by studies in rodents demonstrating the development of a BM organ following transplantation of BM to an ectopic site (Amsel and Dell, 1971; Tavassoli and Crosby, 1968).

A more direct demonstration of marrow stromal cell precursors was provided by the pioneering studies of Friedenstein and colleagues, who demonstrated the *in vitro* growth of adherent colonies of cells morphologically resembling fibroblasts (CFU-F) derived from explants of BM

(Castro-Malaspina et al., 1980; Friedenstein et al., 1970; Owen, 1988). A consistent feature of marrow CFU-F-derived colonies of virtually all species examined is their considerable heterogeneity in terms of size, morphology, enzyme histochemistry, proliferation and developmental potential (Friedenstein et al., 1987; Kuznetsov et al., 1997; Owen and Friedenstein, 1988). These observations are consistent with the hypothesised existence within marrow stromal tissue of a hierarchy of cellular differentiation supported at its apex by a small compartment of self-renewing, pluripotent stromal stem cells, known as bone marrow stromal cells, stromal precursor cells, bone marrow stromal stem cells and mesenchymal stem cells (Owen and Friedenstein, 1988).

The low incidence of clonogenic CFU-F in adult human BM (range 1-20×10⁵ mononuclear cells plated) (Gronthos and Simmons, 1996) was a major limitation to their study, a problem compounded until recently by the paucity of specific antibody reagents to facilitate CFU-F isolation and enrichment. Monoclonal antibody STRO-1 reacts with an as yet unidentified cell surface antigen expressed by a minor subpopulation of adult human BM (Simmons and Torok-Storb, 1991). Previous studies have shown that STRO-1 is non-reactive with haematopoietic progenitors, but included within the STRO-1⁺ population are essentially all detectable clonogenic CFU-Fs (Simmons and Torok-Storb, 1991).

Moreover, data from this laboratory demonstrate that within the STRO-1⁺ fraction in adult human BM are BMSSCs with the capacity to transfer a functional hematopoietic microenvironment *in vitro* and for differentiation into multiple stromal cell types including smooth muscle cells, adipocytes, osteoblasts and chondrocytes (Dennis et al., 2002; Gronthos et al., 1994; Simmons and Torok-Storb, 1991). However, use of the STRO-1 antibody is not sufficient to obtain the purity of BMSSCs required to properly study their properties, owing to the presence of contaminating populations of glycophorin-A-positive nucleated red cells and a small subset of B-lymphocytes.

Herein, we report the isolation of a highly enriched population of BMSSCs with clonogenic potential from adult human BM, on the basis of the use of STRO-1 in combination with an antibody directed to vascular cell adhesion molecule-1 (VCAM-1/CD106). In addition, we examine aspects of the molecular, cellular and developmental properties of this poorly characterized population of stromal stem cells. The isolation of BMSSCs is a prerequisite for the study of mechanisms that regulate their differentiation and is likely to be of therapeutic importance given the developmental potential of these cells.

Materials and Methods

Subjects and cell culture

BM aspirates were obtained from the posterior iliac crest of normal adult volunteers (20-35 years old) following informed consent, according to procedures approved by the ethics committee of the Royal Adelaide Hospital, South Australia. BM mononuclear cells (BMMNC) were prepared as previously described (Gronthos and Simmons, 1995). Primary BMSSC cultures were established in α -MEM supplemented with 20% fetal calf serum and 100 μ M L-ascorbate-2-phosphate as previously described (Gronthos and Simmons, 1995) for colony efficiency assays, RT-PCR, immunohistochemistry and developmental studies. BMSSC clonal cell lines were generated by limiting dilution from day 14 colonies derived from STRO-1^{BRIGHT}/VCAM-1⁺ sorted cells as described below, following subculture in serum-replete medium for proliferation, RT-PCR, immunohistochemistry and developmental studies.

Primary antibodies

Monoclonal antibodies (MAbs) STRO-1 (mouse IgM) (Simmons and Torok-Storb, 1991) and the anti-VCAM-1 antibody 6G10 (mouse IgG₁) (Simmons et al., 1992) were used as tissue culture supernatants diluted 1:2 and 1:4, respectively. Isotype-matched controls of irrelevant specificity, 1A6.12, (IgM), 3D3 (IgG₁) and 1D4.5 (IgG_{2a}) (1/4; kindly provided by L. K. Ashman, Medical Science Building, University of Newcastle, New South Wales, Australia). α -smooth muscle actin (5 μ g/ml, mouse IgG_{2a}; Immunotech), affinity-purified rabbit antiserum to collagen type I and rabbit immunoglobulin control (1/200; Chemicon International Inc., Temecula, CA). Anti-Ki-67-FITC or isotype-matched IgG₁-FITC antibody (1/10; Dakopatts A/S, Glostrup, Denmark).

Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) was performed as previously described (Gronthos, 1998; Gronthos and Simmons, 1995). In brief, approximately 1-3 \times 10⁸ BMMNCs were sequentially incubated with STRO-1 supernatant, anti-IgM-biotin, streptavidin microbeads and finally streptavidin-FITC (Caltag Laboratories, Burlingame, CA)

before being separated on a Mini MACS magnetic column (Miltenyi Biotec Inc., Auburn, CA).

Fluorescence-activated cell sorting and limiting dilution assays

The STRO-1⁺ (FITC labelled) isolated by MACS was incubated with purified anti-VCAM-1 antibody 6G10 or isotype control 1B5 for 30 minutes on ice, washed and incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (1/50; Southern Biotechnology Associates, Birmingham, AL) for an additional 20 minutes on ice. Cells were sorted using a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA). Limiting dilution assays were performed with STRO-1^{BRIGHT}/VCAM-1⁺ sorted cells seeded at plating densities of 1, 2, 3, 4, 5 and 10 cells per well (96-well plates) in replicates of 24 wells per plating density, using the automated cell deposition unit (ACDU) of the flow cytometer. The cells were cultured in serum-deprived medium in the presence of PDGF-BB and EGF (10 ng/ml) as previously described (Gronthos and Simmons, 1995). Colony efficiency assays were performed using Poisson distribution statistics by determining the number of wells with no clonogenic growth at day 14 of culture following staining of the cultures with 0.1% (w/v) toluidine blue in 1% paraformaldehyde. Aggregates of >50 cells were scored as CFU-F-derived colonies and aggregates of >10 and <50 cells were scored as clusters.

Analysis of cell cycle status

The STRO-1⁺ cells isolated by MACS were incubated with streptavidin-PE (Caltag; 1:50) for 15 minutes on ice. After washing with PBS, the cells were fixed for 10 minutes with 70% (v/v) ethanol on ice. Following washing, the cells were incubated with either anti-Ki-67-FITC or isotype-matched IgG₁-FITC antibody for 45 minutes on ice. The cells were then washed in PBS prior to flow cytometric analysis.

Immunostaining

STRO-1^{BRIGHT}/VCAM-1⁺ cells isolated by FACS were prepared as cytopins and fixed with cold acetone. After washing, the cells were blocked in 5% goat serum in PBS and then incubated with primary antibodies and the corresponding control immunoglobulins. The subsequent steps of immunoperoxidase staining were performed using Vectastain ABC immunoperoxidase kits for mouse and rabbit IgG, respectively (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Reverse transcriptase polymerase chain reaction analysis

Total cellular RNA was prepared from 2 \times 10⁴ STRO-1^{bright}/VCAM-1⁺ sorted cells collected as a bulk population and lysed using an RNazolB extraction method (Biotecx Lab. Inc., Houston, TX) according to the manufacturer's recommendations. RNA isolated from each subpopulation was then used as a template for cDNA synthesis, prepared using a First-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden). The expression of various transcripts was assessed by PCR amplification, using a standard protocol as described previously (Gronthos et al., 1999). Primers sets used in this study are shown in Table 1. Following amplification, each reaction mixture was analysed by using 1.5% agarose gel electrophoresis and visualised by ethidium bromide staining. RNA integrity was assessed by the expression of GAPDH.

Differentiation of CFU-F *in vitro*

We have previously reported the conditions needed for human BM stromal cells to develop a mineralized bone matrix *in vitro* as α MEM supplemented with 10% FCS, 100 μ M L-ascorbate-2-phosphate,

Table 1. PCR primer pairs used in this study

Gene	Primer sets	Accession number
GAPDH	Forward: 5'-cactgacacgttggcagtg-3' Reverse: 5'-catggagaaggctgggctc-3'	BC029618
Cbfa-1	Forward: 5'-gtggacgagcagagttca-3' Reverse: 5'-tggcaggtaggtgtgtagtg-3'	NM_004348
Osterix	Forward: 5'-ggcacaagaagcctactc-3' Reverse: 5'-cactggcagacagtcagaa-3'	AF477981
Collagen type I	Forward: 5'-aggcctccaacgagatcgatccg-3' Reverse: 5'-tacaggaagcagacagggccaactcg-3'	NM_000088
PTH-Receptor	Forward: 5'-aggaacagatcttctgctgca-3' Reverse: 5'-tgcattgtggatgtgtgctg-3'	X68596
Osteopontin	Forward: 5'-ttgctttgctcctagcga-3' Reverse: 5'-gtgaaaactcgggtgctg-3'	X13694
Osteocalcin	Forward: 5'-atgagagccctcacactcctc-3' Reverse: 5'-cgtagaagcgcgataggc-3'	X53698
Lipoprotein lipase	Forward: 5'-atggagagcaagccctgctc-3' Reverse: 5'-gttaggtccagctggatcgag-3'	NM_000237
PPAR γ 2	Forward: 5'-ctcctattgaccagaaagc-3' Reverse: 5'-gtagagctgagttctcag-3'	D83233
Leptin	Forward: 5'-atgcattgggaacctgtgc-3' Reverse: 5'-gcaccagggtgaggtcca-3'	NM_000230
Collagen type II	Forward: 5'-atgattcgctcggggctcc-3' Reverse: 5'-cattactccaactgggagc-3'	NM_033150
Collagen type X	Forward: 5'-agcagggttgcaggacca-3' Reverse: 5'-ttttccactccaggaggc-3'	NM_000493
Aggrecan	Forward: 5'-cactgttaccgccactccc-3' Reverse: 5'-accagcggaaagtccttcg-3'	NM_013227

dexamethasone 10^{-7} M and 3 mM inorganic phosphate (Gronthos et al., 1994). Mineral deposits were identified by positive von Kossa staining. Adipogenesis was induced in the presence of 0.5 mM methylisobutylmethylxanthine, 0.5 μ M hydrocortisone and 60 μ M indomethacin as previously described (Gimble, 1998). Oil Red O staining was used to identify lipid-laden fat cells. Chondrogenic differentiation was assessed in aggregate cultures treated with 10 ng/ml TGF- β 3 as described previously (Pittenger et al., 1999).

In vivo assay of bone formation

The adherent cells derived from STRO-1^{BRIGHT}/VCAM-1⁺ cells at passage two-three were trypsinised, mixed with 40 mg hydroxyapatite/tricalcium phosphate ceramic particles (Zimmer Corporation, Warsaw, IN) and then implanted into subcutaneous pockets on the dorsal surface of two-month-old SCID mice as described previously (Gronthos et al., 2000). These procedures were performed in accordance with an approved animal protocol (Adelaide University AEC# M/079/94). Implants were recovered after 6-8 weeks, fixed in 4% paraformaldehyde for 2 days, then decalcified for a further 10 days in 10% EDTA prior to embedding in paraffin. For histological analysis, 5 μ m sections of the implants were prepared and stained with haematoxylin and eosin. In situ hybridization for the human specific *alu* sequence was performed as previously described (Gronthos et al., 2000).

Telomerase repeat amplification protocol

Telomerase cell extracts prepared by the method of Kim et al. (Kim et al., 1994) were analysed for the presence of telomerase activity as previously described (Fong et al., 1997). To confirm the specificity of the telomerase products 2 μ l aliquots of each in CHAPS lysate was subjected to denaturation and resolved on a non-denaturing 12% polyacrylamide gel, then visualised by staining with SYBR green

Table 2. BM CFU-F express high levels of the STRO-1 antigen

Cell fraction	No. of CFU-F/ 10^5 cells	CFU-F recovery % total
BMMNC	9.4 \pm 2.3	100
STRO-1 ^{bright}	8,930 \pm 415	74
STRO-1 ^{intermediate}	48.0 \pm 7.5	3.2
STRO-1 ^{dull}	0.0	0.0

STRO-1⁺ BMMNC were first isolated by MACS and then sorted according to the level of STRO-1 expression, using FACS. The different FACS-sorted STRO-1 fractions were then cultured under standard clonogenic conditions. The data represent the mean number of day 14 CFU-F per 10^5 cells plated \pm s.e.m. ($n=5$ different BM samples).

fluorescent dye (FMC Bioproducts, OR) as recommended by the manufacturer. The telomerase repeat amplification protocol (TRAP) products were analysed using a FluorImager (Molecular Dynamics, Sunnyvale, CA).

Transmission electron microscopy

Approximately 2×10^4 STRO-1^{BRIGHT}/VCAM-1⁺ cells were fixed in 2.5% glutaraldehyde (EM Grade) in cacodylate buffer and then processed for embedding into resin as previously described (Gronthos et al., 1994). Ultrathin sections were examined using a JEOL 1200 EX II (Tokyo, Japan) transmission electron microscope.

Results

Purification of stromal precursor cells (CFU-F)

We elected to use the monoclonal antibody STRO-1 (Simmons and Torok-Storb, 1991) as the basis of a strategy to prospectively isolate clonogenic CFU-F. Immunoselection by MACS yielded a population with 75.3% STRO-1⁺ cells \pm 3.2 ($n=20$), whereas the incidence of STRO-1⁺ cells in the starting BMMNC samples was 6.5% \pm 0.73 ($n=20$). The STRO-1⁺ population exhibited a heterogeneous pattern of STRO-1 expression spanning four decades of fluorescence intensity, with the majority of cells exhibiting low (STRO-1^{DULL}) to intermediate (STRO-1^{INT}) levels of STRO-1 binding in addition to a small but incompletely resolved subpopulation characterised by very high level STRO-1 (STRO-1^{BRIGHT}) staining cells (Fig. 1A). STRO-1^{DULL}, STRO-1^{INT} and STRO-1^{BRIGHT} fractions were isolated by FACS and each population assayed for its CFU-F content. The majority (74%) of CFU-F were recovered in the minor STRO-1^{BRIGHT} subpopulation and correspondingly depleted or absent from the STRO-1^{DULL} and STRO-1^{INT} fractions (Table 2). Selection of the STRO-1^{BRIGHT} fraction resulted in a 950-fold enrichment of CFU-F relative to their incidence in unseparated BM mononuclear cells (BMMNC) samples prior to MACS/FACS separation (Table 2).

To further increase the quantity of CFU-F, we made use of previous studies that examined the expression of a broad range of cell surface molecules on CFU-F (Simmons et al., 1994). VCAM-1/CD106 was one of several cell surface molecules that fulfilled the criteria of minimal reactivity with BMMNC but produced a high yield of CFU-F following FACS. In accord with these data, dual-color FACS analysis of MACS-isolated STRO-1⁺ cells demonstrated VCAM-1 expression by a minor subpopulation of cells (1.4% \pm 0.3; $n=20$), which were

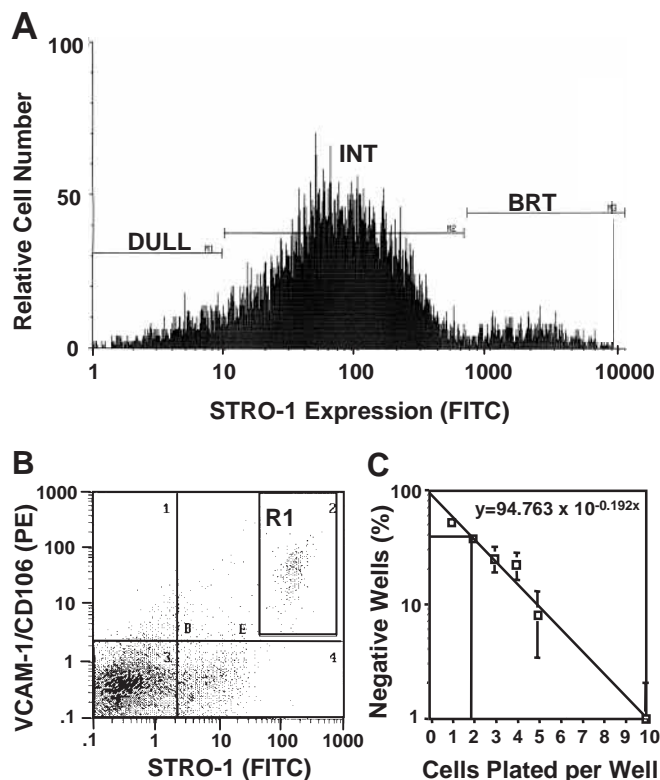


Fig. 1. Isolation and purification of BMSSCs. (A) Flow cytometric analysis of MACS-sorted STRO-1⁺ marrow cells demonstrated varying levels of STRO-1 expression. There were approximately three distinct populations: dull (DULL), intermediate (INT) and bright (BRT). (B) Dual-color flow cytometric analysis of VCAM-1 (PE) expression by STRO-1⁺ (FITC) marrow cells isolated by MACS depicting a discrete population of STRO-1^{BRIGHT}/VCAM-1⁺ cells (region: R1), approximately 0.02% of the total BMMNC population. (C) The incidence of clonogenic cell colonies (>50 cells) + clusters (>10<50 cells) based on STRO-1^{BRIGHT}/VCAM-1⁺ expression was determined by limiting dilution analysis of 24 replicates per cell concentration using Poisson distribution analysis (data derived from six independent experiments).

characterized by a high level of STRO-1 expression (STRO-1^{BRIGHT}) (Fig. 1B). By means of dual-colour cell sorting, CFU-F were found to be restricted to the small proportion of marrow cells co-expressing both antigens (STRO-1^{BRIGHT}/VCAM-1⁺) (data not shown). To determine the incidence of CFU-F in the STRO-1^{BRIGHT}/VCAM-1⁺ population, limiting dilution assays were performed. Analysis of the data from six different marrow samples yielded a mean frequency of approximately one CFU-F-derived colony (≥ 50 cells) per three STRO-1^{BRIGHT}/VCAM-1⁺ cells, whereas the incidence of all clonogenic CFU-F (measured as the sum of colonies plus clusters) was one per two STRO-1^{BRIGHT}/VCAM-1⁺ cells plated (Fig. 1C), using Poisson distribution statistics. Notably, of the 50% of wells deposited with single STRO-1^{BRIGHT}/VCAM-1⁺ cells that failed to produce clones (≥ 10 cells), a significant proportion (approximately 30%) contained either single or small groups of stromal cells that failed to produce colonies or clusters (<10 cells) in these assay conditions.

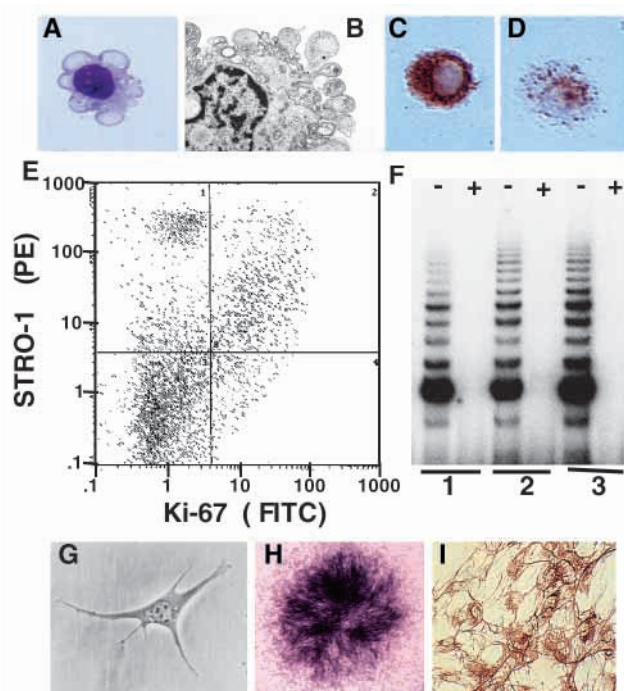
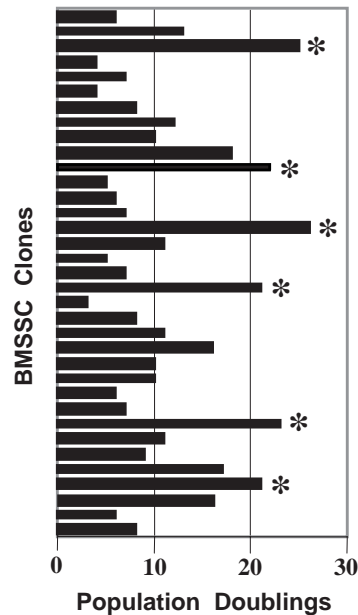


Fig. 2. Characterization of BMSSCs in vivo. (A) Light microscopic examination of cytopsin representing freshly sorted STRO-1^{BRIGHT}/VCAM-1⁺ marrow cells (1000 \times) counterstained with hematoxylin. (B) Transmission electron micrograph depicting the ultrastructure of freshly sorted STRO-1^{BRIGHT}/VCAM-1⁺ cells (15000 \times). Immunohistochemical staining of cytopsin preparations of the sorted STRO-1^{BRIGHT}/VCAM-1⁺ cells (1000 \times) with collagen type I (C) and α -SMA (D). (E) Dual-color flow cytometric analysis of Ki67 (FITC) expression by STRO-1⁺ (PE) marrow cells isolated by MACS. The majority of the STRO-1^{BRIGHT} marrow cells lack detectable binding of Ki67 relative to that of the isotype-matched control antibody, indicated by the vertical quad-stat marker. (F) Telomerase activity in different sorted cell populations was examined using a modified TRAP assay. TRAP products derived from CHAPS extracts of non-denatured (–) and denatured (+) total BM (lanes 1), STRO-1^{BRIGHT}/VCAM-1⁺ cells sorted fraction (lanes 2) and CD34⁺-sorted BM cells (lanes 3). TRAP products were resolved on a 12% polyacrylamide gel, stained with SYBR green fluorescent dye, and visualised using a fluorescence scanning system. (G) A typical light microscopic view of a single purified STRO-1^{BRIGHT}/VCAM-1⁺ cell, allowed to adhere to fibronectin-coated culture plates (400 \times). (H) A representative example of a day 14 CFU-F colony stained with toluidine blue is shown (200 \times). (I) Immunohistochemical staining showing all the cells that comprise the CFU-F colony express collagen type I (200 \times).

Characteristics of freshly isolated CFU-F

STRO-1^{BRIGHT}/VCAM-1⁺ cells sorted directly from fresh, marrow aspirates were large cells with heterochromatic nuclei and prominent nucleoli, agranular cytoplasm and numerous bleb-like projections of the cell membrane (Fig. 2A). Ultrastructural analysis demonstrated an extensive array of cytoplasmic microfilaments and a complex cell surface morphology dominated by the bleb-like membrane protrusions observed by light microscopy (Fig. 2B). Weibel-Palade bodies characteristic of endothelial cells were not detected in the enriched BMSSCs population. In addition,

Fig. 3. Proliferation potential of BMSSC clones. A total of 35 CFU-F colonies derived from STRO-1^{BRIGHT}/VCAM-1⁺ single sorted cells from two BM samples were analysed for their cumulative production of cells. A marked variation in proliferative capacity between individual BMSSCs is evident. The majority of clones (29/35, 83%) exhibited only moderate growth potential, which did not persist beyond 20 population doublings. 6/35 clones (17%) demonstrated continued growth extending beyond 20 population doublings.



immunohistochemical staining of cytospin preparations of the sorted STRO-1^{BRIGHT}/VCAM-1⁺ marrow cells showed strong staining in >90% of cells with anti-collagen type I antibody (Fig. 2C) but failed to show any reactivity with antibodies to factor-VIII-related antigen, a marker of vascular endothelial cells, or to CD45, the common leukocyte antigen (data not shown). A notable feature, however, was the expression of α -smooth muscle actin (α -SMA) in approximately 70% of STRO-1^{BRIGHT}/VCAM-1⁺ cells although the intensity of immunostaining varied somewhat between individual cells (Fig. 2D).

Dual-colour flow cytometric analysis demonstrated that the STRO-1^{BRIGHT} population lacked detectable expression of the Ki-67 antigen, demonstrating that these cells do not divide in vivo (Fig. 2E). In addition, telomerase activity commonly found in stem cell populations of other renewing tissues was also present in the total STRO-1⁺ population isolated by MACS and in the minor subpopulation of STRO-1^{BRIGHT}/VCAM-1⁺ FACS-sorted cells (Fig. 2F). Following transfer of the sorted cells in vitro, they rapidly attached and spread assuming a stellate morphology with long processes (Fig. 2G) capable of clonal expansion forming colonies consisting of collagen type I positive fibroblast-like cells (Fig. 2H,I).

An essential feature of stem cell populations in all renewing tissues is a capacity for extensive proliferation. Initial studies to examine the proliferative potential of BMSSCs were performed in which STRO-1^{BRIGHT}/VCAM-1⁺ cells were cultured in bulk and then serially passaged over the course of a number of weeks in culture. This analysis reproducibly demonstrated a cumulative expansion in the number of adherent stromal cells for over 40 population doublings prior to the onset of cellular senescence (data not shown). Since these data reflect the proliferative activity of a mixed population of BMSSCs, we chose to conduct a more rigorous examination of the growth potential of individual STRO-1^{BRIGHT}/VCAM-1⁺ BMSSCs obtained by means of the single cell deposition unit of the cell sorter. A total of 35 CFU-F colonies derived from two BM samples were expanded in

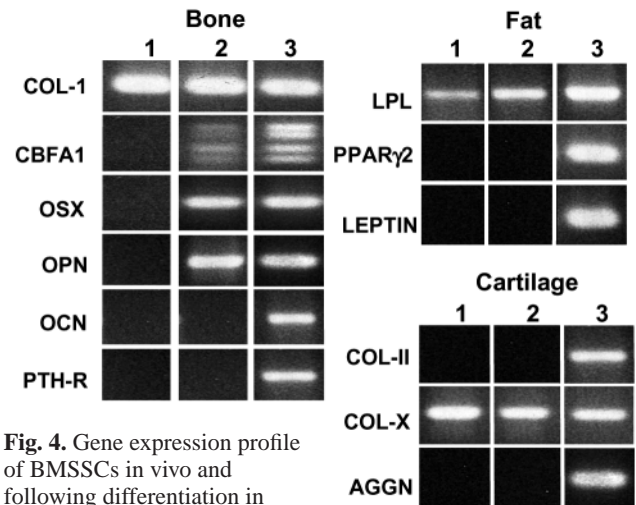


Fig. 4. Gene expression profile of BMSSCs in vivo and following differentiation in vitro. RT-PCR analysis of gene expression in STRO-1^{BRIGHT}/VCAM-1⁺-purified BMSSCs isolated directly from marrow aspirates (1), and when cultured in regular growth medium (2) or medium inductive for either for bone, fat or cartilage development (3) as described in Materials and Methods. The expression of a range of markers characteristic of each tissue is shown. Bone: transcription factors CBFA1 and osterix (OSX), collagen type I (COL-1), osteopontin (OPN), osteocalcin (OCN) and parathyroid hormone receptor (PTH-R). Fat: lipoprotein lipase (LPL), transcription factor PPAR γ 2 and leptin. Cartilage: collagen type II (COL-II), collagen type X (COL-X) and aggrecan (AGGN). Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. RNA integrity was assessed by GAPDH expression.

culture, as above, and analysed for their cumulative production of cells over a number of weeks in culture. There was marked variation in proliferative capacity between individual CFU-F (Fig. 3). The majority of clones (29/35; 83%) exhibited only moderate growth potential that did not persist beyond 20 population doublings. In contrast, a minor proportion of clones (6/35, 17%) demonstrated continued growth, extending beyond 20 population doublings, generating a mean of 3.0×10^8 cells per BMSSCs (range, $9.8 \times 10^6 - 5.2 \times 10^8$ cells). At this time, cells derived from highly proliferative clones (>20 population doublings) were analyzed for their expression of STRO-1 and VCAM-1 by flow cytometry. Although VCAM-1 expression was retained by the progeny of the initiating cells, the STRO-1 epitope was expressed by only a minor subpopulation in all clones analysed (range 3.2–15.8%; median 5.3%; $n=6$).

Gene expression profile of CFU-F in vivo and following proliferation and differentiation in vitro

We first surveyed the pattern of gene expression in freshly isolated STRO-1^{BRIGHT}/VCAM-1⁺ BMMNC by means of RT-PCR. There was no detectable expression of mature bone markers such as osteopontin, parathyroid hormone-receptor nor osteocalcin. Importantly, this population also lacked the expression of the essential early bone-cell-specific transcription factors CBFA1 and osterix (Fig. 4). A similar analysis of mRNA transcripts with restricted expression in adipose cells revealed constitutive expression of only lipoprotein lipase but not other adipocyte-related genes

including, leptin and the early adipocytic transcription factor, PPAR γ 2 (Fig. 4). Finally, expression analysis of genes restricted to the chondrocyte lineage demonstrated neither collagen type II nor aggrecan expression although collagen type X, a marker associated with hypertrophic chondrocytes, was consistently detected (Fig. 4).

Next, bulk cultures initiated with STRO-1^{BRIGHT}/VCAM-1⁺ cells were expanded by passing two to three times, at which point aliquots of cells were plated in conditions previously described to induce osteogenic (Gronthos et al., 1994), adipogenic (Gimble, 1998) and chondrogenic (Pittenger et al., 1999) differentiation of BMSSCs in vitro. Expression of CBFA-1, osterix and osteopontin were detected by RT-PCR in both induced and non-induced control cultures (Fig. 4), whereas osteocalcin and PTH-R transcripts were detected within 4-5 weeks of growth in osteoinduction media. Following 2-3 weeks of adipogenic induction, transcripts were detected for the fat-associated markers, PPAR γ 2 and leptin (Fig. 4). Similarly, in aggregate cultures, prolonged exposure to TGF β 3 induced the mRNA expression of the cartilage-associated matrix components collagen type II and aggrecan by RT-PCR (Fig. 4).

Developmental potential of BMSSC clones in vitro and in vivo

Comparison of the in vitro developmental potential of 64 highly proliferative BMSSC clones (20 population doublings), derived from single STRO-1^{BRIGHT}/VCAM-1⁺ cells ($n=3$ marrow samples), demonstrated the formation of von Kossa positive mineralized deposits by all clones in vitro after several weeks under osteogenic-induction conditions (Fig. 5A). Similarly, 95% of the same BMSSC clones formed clusters of Oil red O-positive lipid-laden adipocytes when cultured in adipogenic inductive media (Fig. 5B). A sample of 20 high proliferative clones were assayed for their chondrogenic potential using the well established aggregate culture system (Pittenger et al., 1999) in the presence of TGF β 3. Again, all clones exhibited synthesis of collagen type II by immunohistochemistry (Fig. 5C). However, with continuous subculture (greater than 25 population doublings) the BMSSCs clones showed either a reduced capacity or an inability to differentiate into all three stromal cell lineages (data not shown).

The capacity to develop a mineralised matrix in vitro, although consistent with osteogenic differentiation, may nevertheless not predict the capacity of the cells to produce an organised bone tissue in vivo. In view of this concern, all 64 highly proliferative BMSSC clones were assayed for their capacity to develop human bone tissue following ectopic transplantation in SCID mice, using hydroxyapatite/tricalcium phosphate (HA/TCP) particles as a carrier vehicle (Gronthos et al., 2000; Kuznetsov et al., 1997). Histological examination at week 8 showed that all of the implants contained an extensive network of blood vessels and fibrous tissue (Fig. 6A). Bone formation was evident in 35/64 clones (54%), whereas associated haematopoietic marrow/adipose elements were observed in 11/35 of the bone-producing clones (31%). Cartilage development, as assessed by immunohistochemical staining with an antibody to collagen type II, was not detected in any implant. The origin of the cellular material within the

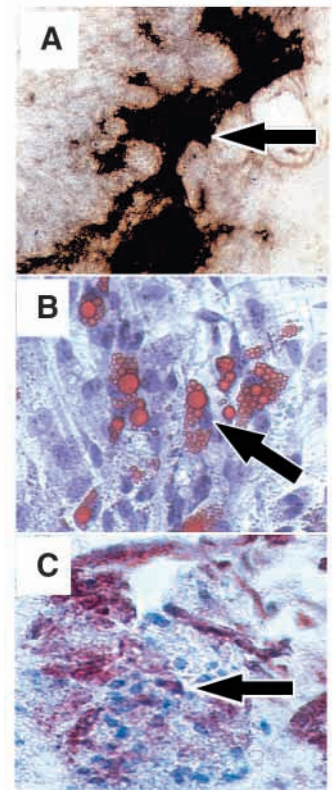


Fig. 5. Developmental potential of BMSSCs in vitro. Primary cultures of cells derived from STRO-1^{BRIGHT}/VCAM-1⁺ marrow cells were induced under osteogenic, adipocytic or chondrogenic conditions. (A) Mineralized deposits stained positively with the von Kossa reagent (arrow) formed within 4 weeks of culture under osteoinductive conditions (200 \times). (B) The presence of clusters of lipid containing adipocytes were also detected by Oil red-O staining (arrow) within 2 weeks of adipogenic induction (200 \times). (C) In aggregate cultures, collagen type II was present throughout the cellular mass following 3 weeks of chondrogenic induction (arrow) (200 \times).

recovered implants was assessed by in situ hybridization using a probe to the unique human repetitive *alu*. The interstitial tissue, bone lining cells and osteocytes within the newly formed bone were found to be positive for the *alu* sequence, confirming their human origin (Fig. 6B). Conversely, neither the endothelium-lining small blood vessels, haematopoietic cells nor the adipose and muscle tissue surrounding the HA/TCP carrier demonstrated hybridization with the *alu* probe and were therefore presumed to be of murine origin.

Discussion

A large body of evidence demonstrates that stromal tissue derived from adult BM of avian and mammalian species contains clonogenic progenitor cells (CFU-F), some of which are considered to be multi-potent BMSSCs with the capacity to differentiate into a range of mesenchymal cell lineages including adipose tissue, bone, cartilage, tendon and ligament (Bruder et al., 1994; Prockop, 1997). Despite considerable interest in the potential therapeutic applications of these cells, there is no well-defined protocol for the prospective isolation of human CFU-F in order to properly study their biological properties prior to cell culture. Current methodologies for the isolation of primitive BMSSCs are based upon those initially described by Friedenstein and colleagues, which rely upon the rapid adhesion of the stromal progenitor populations to tissue culture plastic and their subsequent rapid proliferation in vitro (Castro-Malaspina et al., 1980; Friedenstein et al., 1970; Kuznetsov et al., 1997; Pittenger et al., 1999). Such protocols result in a heterogeneous starting population of adherent BM cells, of which only a minor proportion represent multipotent BMSSCs. Moreover these protocols

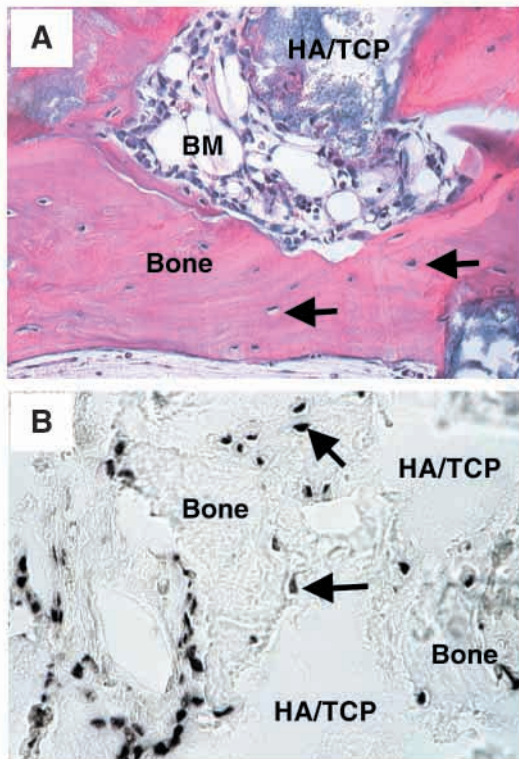


Fig. 6. Developmental potential of BMSSCs clones in vivo. Immunoselected STRO-1^{BRIGHT}/VCAM-1⁺ marrow-derived clones were expanded in vitro, then implanted subcutaneously into SCID mice using HA/TCP carrier. Implants were harvested 8 weeks after the transplant. (A) New bone formation containing osteocytes (arrow) was observed for a proportion of clones developing at the HA/TCP particle surfaces together with surrounding fibrous and hematopoietic tissue (BM). The sections were counter stained with haematoxylin and eosin (200 \times). (B) The osteocytes and bone lining cells (arrow) were found to be of human origin, as demonstrated by in situ hybridization using a DNA probe specific to the human *alu* repeat sequence (200 \times).

select for the progeny of CFU-F and not for the clonogenic progenitors themselves.

In this report, we describe the isolation of a minor subpopulation of adult human BMMNC that represent a near homogeneous population of CFU-F. This was achieved using the CFU-F reactive antibody STRO-1 (Simmons and Torok-Storb, 1991) in combination with an antibody to VCAM-1, a cell adhesion molecule constitutively expressed by marrow stromal tissue in vitro and in vivo (Simmons et al., 1992) and also by CFU-F (Simmons et al., 1994). This approach enabled the resolution of a discrete subpopulation of STRO-1^{BRIGHT}/VCAM-1⁺ marrow cells with a cloning efficiency for CFU-F approaching 50% using Poisson distribution statistics. This degree of enrichment for CFU-F in the STRO-1^{BRIGHT}/VCAM-1⁺ cell fraction (approximately 5,000-fold relative to unfractionated BM) substantially exceeds that previously reported for any mammalian species including mice and humans (Castro-Malaspina et al., 1980; Simmons and Torok-Storb, 1991; Van Vlasselaer et al., 1994; Waller et al., 1995). A significant factor contributing to the success of this enrichment strategy is the use of our previously defined serum-deprived culture conditions for the assay of CFU-F, in which

colony growth at low plating densities is stimulated by the addition of a combination of EGF and PDGF-BB (Gronthos and Simmons, 1995). CFU-F are generally assayed in medium supplemented with FCS as a growth stimulus. Although this is adequate for CFU-F growth from unfractionated bone marrow at high plating densities, when more enriched cell populations are assayed in FCS-containing medium, growth of CFU-F becomes suboptimal, particularly at limiting cell concentrations (Gronthos and Simmons, 1995). Indeed, in the current study, STRO-1^{BRIGHT}/VCAM-1⁺ CFU-F failed to grow in foetal bovine serum supplemented medium at plating densities <10 cells per well (data not shown), whereas in the serum-deprived assay, a significant proportion of cells exhibited clonogenic growth even when plated at 1 cell per well. It is important to note, however, that even under these highly efficient culture conditions a significant proportion of single STRO-1^{BRIGHT}/VCAM-1⁺ cells, although failing to proliferate sufficiently to qualify as either clusters or colonies, nevertheless contained stromal cells that either remained as single cells or underwent one or two divisions only. The nature of the STRO-1^{BRIGHT}/VCAM-1⁺ stromal cells that fail to either proliferate or do so only poorly remains to be determined. One possibility is that expression of the antigen identified by STRO-1 may encompass not only stromal progenitors but also partially differentiated stromal cells with correspondingly reduced growth potential in vitro. Alternatively, these cells may represent a subpopulation of stromal progenitors that fail to proliferate in these assay conditions. Nevertheless, the STRO-1^{BRIGHT}/VCAM-1⁺ BMMNC represent a virtually pure population of collagen type I positive, stromal progenitor cells with varying clonogenic efficiencies.

As a population, STRO-1^{BRIGHT}/VCAM-1⁺ cells have several important phenotypic characteristics previously attributed to stem cells in other renewing tissues (Fuchs and Segre, 2000). Firstly, freshly isolated BMNC-derived STRO-1^{BRIGHT} cells lacked expression of the Ki-67 antigen and thus appear to be a non-cycling population in vivo. This is in accord with previous studies demonstrating the quiescent nature of CFU-F in unfractionated rodent and human BM (Castro-Malaspina et al., 1981; Falla et al., 1993). Secondly, STRO-1^{BRIGHT}/VCAM-1⁺ cells and their progeny constitutively exhibit telomerase activity, a well documented feature of stem cell populations in renewing tissues that is lost during normal somatic cell proliferation and differentiation (Fuchs and Segre, 2000; Harle-Bachor and Boukamp, 1996). Recent studies have shown that human BMSSCs lose telomerase activity during ex vivo expansion, whereas enforced telomerase activity greatly enhanced the proliferative life-span and osteogenic potential of cultured BMSSCs (Shi et al., 2002; Simonsen et al., 2002). Thirdly, freshly sorted STRO-1^{BRIGHT}/VCAM-1⁺ cells exhibit an undifferentiated phenotype as demonstrated by the absence of gene products characteristic of endothelial cells and mature stromal elements such as osteogenic cells, adipocytes and chondrocytes within the BM. Most significantly in this regard, this population lacked detectable expression of transcription factors with pivotal roles in the early differentiation of bone (CBFA-1, osterix) and adipose (PPAR γ 2) tissues and the signature marker of chondrocytes, collagen type II (Ducy et al., 1997; Nakashima et al., 2002; Tontonoz et al., 1994).

A notable feature of the BM CFU-F population is the considerable heterogeneity in their potential for differentiation

and proliferation. In accord with the putative stromal stem cell hierarchy of cellular differentiation, we found that only a proportion of the STRO-1^{BRIGHT}/VCAM-1⁺ cell population was capable both of extensive proliferation in vitro beyond 20 population doublings and of differentiation into at least five of the cellular components of BM stromal tissue, namely myelosupportive stroma, smooth muscle cells, osteoblasts, adipocytes and chondroblasts. Similar observations were previously reported (Pittenger et al., 1999) for a population of plastic adherent fibroblast-like cells comprising 0.01-0.001% of nucleated cells in human BM.

We have previously demonstrated that all CFU-F clones derived from STRO-1⁺ human marrow demonstrated the capacity to synthesise a mineralized bone matrix in vitro, whereas only 48% of clones showed the capacity to form adipocytes in vitro (Gronthos et al., 1994). In the present study, highly proliferative clones, derived from single STRO-1^{BRIGHT}/VCAM-1⁺ cells, exhibited osteogenic, chondrogenic and adipogenic cell differentiation in vitro. However, when assayed in SCID mice for their capacity to generate human bone tissue following xenogeneic transplantation (Gronthos et al., 2000; Kuznetsov et al., 1997) only a little over half of the clones exhibited the potential to form bone in vivo. Thus the in vitro culture assay, despite evidence of many of the phenotypic characteristics of differentiated bone cells including expression of the master control gene CBFA1, does not accurately predict the osteogenic potential of BMSSCs clones in vivo, in accord with studies on murine BM stromal cell lines (Satomura et al., 2000). Use of the more stringent in vivo assay demonstrated that osteogenic differentiation is only exhibited by a subpopulation of clones and is consistent with the findings of Kuznetsov and colleagues (Kuznetsov et al., 1997).

In the present study, the data indicate that CFU-F in adult human BM represent a mixed population of multi-, bi- and uni-potential progenitors at different stages of differentiation, as initially proposed by Owen and Friedenstein (Owen and Friedenstein, 1988) and subsequently supported by others (Kuznetsov et al., 1997; Pittenger et al., 1999). Future studies designed to subset the STRO-1^{BRIGHT}/VCAM-1⁺ population may in time reveal a minor population with properties attributed to pluripotent stem cells. Efforts to identify BMSSCs in vivo have been hampered by the lack of precise knowledge regarding their anatomical distribution within the marrow. It has been suggested that that osteoprogenitors may be associated with the outer surfaces of the marrow vasculature (Bianco et al., 2001). In the present study, STRO-1^{BRIGHT}/VCAM-1⁺ cells proved to be a homogeneous population of large collagen type I positive cells lacking phenotypic characteristics of leukocytes or vascular endothelial cells. Of particular note is the expression of α -SMA by this population. In adult human BM in vivo, α -SMA is limited to vascular smooth muscle cells in the media of arteries, cells lining the abluminal surface of sinuses, pericytes lining capillaries and occasional flattened cells on the endosteal surface of bone. Expression of α -SMA is not detected in other marrow stromal elements such as reticular cells within haemopoietic cords, adipocytes or vascular endothelial cells (Galmiche et al., 1993). Collectively these observations suggest two possibilities for the identity and anatomical location of stromal progenitors in the BM: vascular smooth muscle cells/pericytes and endosteal cells. Furthermore, accumulating

data suggest that vascular pericytes may also fulfil the role of multipotential mesenchymal progenitors (Doherty et al., 1998; Schor et al., 1995).

The biological properties of the BMSSCs described herein should be viewed in the context of recent reports that demonstrate that the inherent developmental potential of stem cells derived from various mammalian tissues may be more similar than previously suspected (Azizi et al., 1998; Bjornson et al., 1999; Gussoni et al., 1999). This suggests that the developmental plasticity of stem cells is dictated by the local tissue microenvironment in which they lodge, and it would therefore be of great interest to examine whether the population of candidate stem cells described herein can regenerate tissues other than the marrow stroma and associated skeletal tissues (Prockop, 1997). It is conceivable therefore that the properties exhibited by the STRO-1^{BRIGHT}/VCAM-1⁺ population may, in time, be useful for a range of novel cellular therapies that extends beyond their more obvious use in the treatment of disorders of the haemopoietic and skeletal systems.

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