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## STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) as markers of primitive human marrow stromal cells and their more differentiated progeny: a comparative investigation in vitro

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**Abstract** There is widespread interest in the use of bone marrow stromal cells (BMSC) for tissue reconstruction and repair and for gene therapy. BMSC represent the differentiated progeny of CFU-F, which however comprise a developmentally heterogeneous population as is reflected in the cellular heterogeneity of the cell populations to which they give rise. We have compared the efficacy of monoclonal antibodies recognising a series of stromal antigens, viz. STRO-1, HOP-26, CD49a and SB-10/CD166, as tools for the enrichment of CFU-F prior to culture and as developmental markers for culture-expanded BMSC. In freshly isolated bone marrow mononuclear cells (BMMNC), the proportion of antigen-positive cells was 27%, 46%, 5% and 19% for STRO-1, HOP-26, CD49a and CD166, respectively. All CD49a<sup>+</sup> cells co-expressed STRO-1. The degree of CFU-F enrichment obtained with anti-CD49a (~18-fold) by a one-pass immunoselection strategy was significantly greater than that of all other antibodies tested. BMSC expressed higher levels of all antigens investigated (except for HOP-26) compared with BMMNC. Expression of STRO-1 and CD49a remained restricted to a subset of BMSC, whereas all BMSC were SB-10/CD166 positive. Treatment with dexamethasone (10 nM), which promotes the differentiation and further maturation of cells of the osteogenic

lineage in this cell culture system, increased the expression of CD49a and HOP-26. The CD49a<sup>+</sup> and HOP-26<sup>+</sup> fractions of BMSC were further subdivided by dual-labelling with anti-STRO-1 and B4-78 (an antibody recognising the B/L/K isoform of the enzyme alkaline phosphatase), respectively. By using a variety of criteria, the HOP-26 antigen was identified as CD63, a member of the tetraspanin family of proteins thought to modulate integrin compartmentalisation and signalling.

**Keywords** CFU-F · Bone marrow stromal cells · STRO-1 · HOP-26/CD63 · CD49a · Human

### Introduction

The pioneering studies of Friedenstein and colleagues (Friedenstein et al. 1966, 1970; Friedenstein 1980) revealed that adult bone marrow contains rare ( $1$  in  $10^4$ – $10^5$  bone marrow mononuclear cells; BMMNC) clonogenic precursors (CFU-F) associated with the soft fibrous tissue of the marrow stroma. They revealed further that, within the CFU-F population, are cells capable of forming bone and reconstituting the haematopoietic microenvironment (Friedenstein et al. 1987). This observation, which has subsequently been confirmed by others (Kuznetsov et al. 1997a, 1997bb), has been cited as evidence for the existence of marrow stromal stem cells. In the absence of definitive proof, however, it has been suggested by some workers that these cells should be considered as multipotential progenitors rather than true stem cells (Bianco and Cossu 1999; Bianco et al. 2001).

When explanted in vitro, CFU-F give rise to adherent cultures of bone marrow stromal cells (BMSC). The results of studies to date suggest that appropriately primed BMSC may have a clinical application in the reconstruction and repair of cartilage and bone, in the haematopoietic microenvironment (Bruder et al. 1994, 1998a, 1998b;

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Connolly 1998; Krebsbach et al. 1998; Yoo and Johnstone 1998; Oreffo and Triffitt 1999; Nilsson et al. 1999; Petite et al. 2000; Kon et al. 2000; Koc et al. 2000) and in the correction of inherited disorders of the skeleton (Horowitz et al. 1999, 2001; McMahon et al. 2001). In addition, evidence is accumulating that a subset of BMSC has the capacity to differentiate into cells of a multiplicity of tissue types; these may represent a viable, and ethically less controversial, alternative to the use of human embryonic stem cells (Ferrari et al. 1998; Kopen et al. 1999; Makino et al. 1999; Woodbury et al. 2000; Mezey et al. 2000; Lagasse et al. 2000; Jiang et al. 2002).

CFU-F comprise a heterogeneous population of cells at different stages of differentiation and lineage commitment. In addition, little is known of the factors that regulate their numbers and promote the proliferation and further differentiation of their immediate progeny. This lack of knowledge presents a major obstacle to the widespread clinical exploitation of their developmental potential and, to a significant extent, reflects the current lack of a stromal CD repertoire analogous to that developed for cells of the haematopoietic system.

Of the available antibodies that appear to recognise stage- and/or lineage-specific stromal antigens, the most extensively used has been STRO-1. This recognises a trypsin-resistant cell-surface antigen expressed by a subset of freshly isolated BMMNC that includes essentially all assayable CFU-F (Simmons and Torok-Storb 1991; Gronthos et al. 1994). Other antibodies reported to recognise antigens expressed by the CFU-F population of human bone and marrow include HOP-26 (Joyner et al. 1997), SB-10 (subsequently shown to recognise the activated leukocyte adhesion molecule CD166: ALCAM; Bruder et al. 1997, 1998c) and, more recently, those recognising CD49a, the  $\alpha 1$  integrin subunit (Deschaseaux and Charbord 2000). Each of these antibodies has been reported to facilitate the isolation of CFU-F from adult human bone marrow and to provide a means for assessing the developmental status of the cells to which they give rise, in particular those of the osteogenic (bone-forming) lineage.

In this investigation, the colony forming efficiency (CFE) of BMMNC subsets obtained by using monoclonal antibodies recognising STRO-1, CD166, HOP-26 and CD49a has been determined, as has the extent to which the expression of their cognate antigens remains developmentally regulated in culture. Our results show that, when used in isolation, the proportion of CFU-F recovered in the CD49a<sup>+</sup> fraction is consistently greater than that obtained by using any of the other antibodies tested. In vitro, the expression of all antigens is up-regulated, although that of STRO-1, CD49a and HOP-26, which we identify here as the lysosomal membrane glycoprotein CD63, remains restricted to a subset of BMSC. The relative merits of these antibodies as a means of identifying and isolating CFU-F from adult human bone marrow is considered, as is their potential utility as developmental markers of cells of the osteoblast lineage.

## Materials and methods

### Subjects

Femoral reamings were obtained during routine surgery from osteoarthritic patients. Patients with a history of corticosterone treatment were excluded from this study. Samples were obtained according to procedures approved by the local ethics committee.

### Cell preparation and culture

BMMNC were isolated by density gradient centrifugation over Ficoll (1.077 g/ml; Lymphoprep, Nycomed, Birmingham, UK), washed in serum-free Dulbecco's modified Eagles medium (DMEM; Life Technologies, Paisley, UK) and filtered through a 70- $\mu$ M pore-size cell strainer (Becton Dickinson Labware, Farnborough, Milton Keynes, UK) to obtain a single cell suspension.

To obtain BMSC cultures, BMMNC were seeded at a density of  $3 \times 10^4$  cells/cm<sup>2</sup> in DMEM supplemented with 15% (v/v) fetal bovine serum (FBS; Sigma, Poole, UK) and 100  $\mu$ M L-ascorbate-2-phosphate (Alpha Laboratories, Eastleigh, UK; Nomura et al. 1969). Non-adherent haematopoietic cells were removed after 5–7 days by washing twice with calcium/magnesium-free phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK) and the cultures were fed twice weekly thereafter. To increase cell yields, BMSC were sub-cultured at day 14 as follows. Cultures were rinsed twice with PBS then incubated in serum-free DMEM containing 25 U/ml collagenase VII (Sigma) and an additional 2 mM CaCl<sub>2</sub> for 90 min at 37°C. After being rinsed twice with PBS, cultures were incubated in 30  $\mu$ l/cm<sup>2</sup> trypsin-EDTA (Life Technologies) for 5–10 min at 37°C until the cells became detached. Medium was added and the cells were then grown until a confluent monolayer was formed (2–4 weeks; termed secondary cultures).

MG-63 and SaOS-2 osteosarcoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Md., USA) and were grown in DMEM supplemented with 10% FBS. Prior to characterisation studies, cells were cultured until a confluent monolayer had formed and were then harvested following treatment with trypsin-EDTA. Rat basophilic leukaemia cells (RBL-2H3) transfected with human CD63 (RBL-CD63<sup>+</sup>; Smith et al. 1995) or CD9 (RBL-CD9<sup>+</sup>; Higginbottom et al. 2000) were grown in DMEM supplemented with 10% FBS and 400  $\mu$ g/ml G418 (Sigma).

### Antibodies

The antibody-producing hybridoma cell line STRO-1 (Simmons and Torok-Storb 1991) was obtained from Dr. P.J. Simmons, Peter MacCallum Cancer Institute, Melbourne, Australia. The B4-78 hybridoma (Lawson et al. 1985) was obtained from the Developmental Studies Hybridoma Bank, Iowa, USA. STRO-1 (IgM subclass) was used in the form of undiluted tissue culture supernatant and was routinely tested for giving more than 95% reactivity with the human osteosarcoma cell line MG-63, a cell line known to express high levels of the STRO-1 antigen (Stewart et al. 1999). Purified anti-alkaline phosphatase (AP) antibody from the B4-78 hybridoma (IgG<sub>1</sub> subclass) was generated by using a protein G column (Pharmacia Biotech, St. Albans, UK). HOP-26 antibody (Joyner et al. 1997; IgM subclass) was obtained from Dr. J.T. Triffitt, Nuffield Orthopaedic Centre, Headington, Oxford, UK and used as undiluted tissue culture supernatant. An antibody raised against activated lymphocyte cellular adhesion molecule (ALCAM), CD166 (clone 18; IgG<sub>1</sub> subclass) was obtained from Antigenix America, New York, USA. An antibody raised against the  $\alpha 1$  integrin subunit, CD49a (clone TS2/7; IgG<sub>1</sub> subclass), was obtained from Serotec, Oxford, UK and an antibody raised against CD63 (clone H5C6; IgG<sub>1</sub> subclass) was obtained from PharMingen, USA. Culture supernatant from an IgM-secreting hybridoma reactive with an irrelevant antigen (in-house) and non-immune mouse IgG<sub>1</sub> obtained from Sigma (Poole, UK) were used

as negative controls. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM, FITC-conjugated anti-mouse IgG<sub>1</sub> and R-phycoerythrin (RPE)-conjugated anti-mouse IgG<sub>1</sub> antibodies were supplied by Cambridge Bioscience, Cambridge, UK.

#### Magnetic-activated cell sorting

Freshly isolated BMMNC were resuspended in Hanks' buffered saline solution (HBSS; Life Technologies) containing 10 mM HEPES (Sigma), 10% normal human AB serum (Blood Transfusion Service, Bristol, UK), 1% bovine serum albumin (BSA; Sigma) and 5% FBS (termed blocking buffer) at a concentration of 10<sup>7</sup>/ml and incubated on ice for 20–30 min in order to block non-specific binding sites. Cells (10<sup>8</sup>) were then pelleted and resuspended in 5 ml of either STRO-1 or HOP-26 supernatant or in 1 ml DMEM supplemented with 10% FBS containing 10 µg/ml CD166 or CD63 antibodies or 2 µg CD49a antibody. Cells were incubated on ice for 60 min, with regular mixing, washed in PBS supplemented with 2 mM EDTA (Sigma) and 0.5% (w/v) BSA (Sigma; MACS buffer, degassed according to instructions) and resuspended in 1 ml blocking buffer containing 200 µl rat anti-mouse IgM or IgG<sub>1</sub> MicroBeads (Miltenyi Biotec, Surrey, UK). After 30 min on ice, FITC-conjugated anti-mouse IgM or IgG<sub>1</sub> antibody was added (1/25 dilution) and incubation continued for a further 15 min. Cells were then washed and resuspended in 1 ml MACS buffer. Finally, cells were applied to MS separation columns (Miltenyi Biotec) and separated into positively- and negatively-labelled fractions by using a MiniMACS separation unit (Miltenyi Biotec) as outlined in the protocols supplied. The purity of recovered cell fractions was assessed by flow cytometry (range: 55%–95%). Remaining cells were seeded into 25-cm<sup>2</sup> flasks at a density of 7×10<sup>3</sup>/cm<sup>2</sup> and cultured as described for BMSC, in triplicate or quadruplicate. Unseparated BMMNC were seeded at an equivalent density and grown under identical conditions for comparison.

#### Determination of the colony forming efficiency and number of colonies expressing AP

After 14–17 days of BMSC culture, the colonies formed were washed twice with PBS, fixed in methanol for 5 min at room temperature and allowed to air-dry. The number of colonies expressing AP was determined as previously described (Scutt and Bertram 1999). The total number, diameter and morphological characteristics of colonies present was assessed after staining with methylene blue (Scutt and Bertram 1999) by using an Olympus SZ-CTV microscope (×15 objective) and a measuring graticule.

#### Flow cytometry

Flow cytometric analysis was carried out as previously described (Stewart et al. 1999). The procedure for the single labelling of cells was as described below for dual labelling, with the exception that cells were incubated with either STRO-1, HOP-26, anti-CD166, anti-CD49a, B4–78 or isotype-matched control antibody alone and binding was detected by using either FITC-labelled goat anti-mouse IgM or goat anti-mouse IgG<sub>1</sub> antibodies (1/25 dilution). Positivity was recorded as the percentage of labelled cells with fluorescence ≥95% control cells.

For competition binding experiments, cells were preincubated with either HOP-26, CD63 or equivalent concentrations of isotype-matched control antibodies for 2 h at 4°C and then washed once in HBSS supplemented with 10 mM HEPES and 5% FBS, pH 7.35 (HBSS/FBS) prior to labelling for either CD63 or HOP-26 expression, respectively, as described below for dual staining. Binding was detected by using either FITC-labelled goat anti-mouse IgM or goat anti-mouse IgG<sub>1</sub> antibodies (1/25 dilution). Positivity was recorded as the percentage of labelled cells with fluorescence ≥95% control cells.

For dual staining, cells were resuspended in blocking buffer at a concentration of 10<sup>6</sup>/ml and incubated for 20–30 min on ice. Cells (10<sup>5</sup>) were pelleted and resuspended in 500 µl STRO-1 or HOP-26 supernatant, representing a saturating concentration of the antibodies and containing an optimised dilution of mouse IgG<sub>1</sub> antibody directed against either AP or CD49a. Negative control mouse IgM and IgG<sub>1</sub> antibodies were used under identical conditions. Cells were incubated for 60 min on ice and then washed in HBSS/FCS. Antibody binding was detected by resuspending cells in 100 µl blocking buffer containing FITC-labelled goat anti-mouse IgM (1/25 dilution) and RPE-labelled goat anti-mouse IgG<sub>1</sub> (1/200 dilution) antibodies and incubating them for 45 min on ice. Finally, cells were washed once and resuspended in HBSS/FBS for analysis on a FACStar<sup>PLUS</sup> flow cytometer (Becton Dickinson, Oxford, UK). Control samples were used to align the cytometer and to set compensation for the interference between fluorescence signals; 10,000 events were collected for each sample and data was analysed by using the Cell Quest software package (Becton Dickinson).

#### Statistical analysis

Statistical analysis (ANOVA; Mann-Whitney U-test) was carried out by using version 4.5 of the StatView package (Abacus Concepts, Berkeley, Calif., USA).

## Results

### Enrichment for CFU-F in antigen-positive BMMNC fractions

A “one-pass” immunoselection strategy was used to isolate fractions of BMMNC either enriched or depleted in cells bearing the antigen of interest. This proved sufficient to obtain a substantial increase in the proportion of antigen-positive cells (mean fold enrichment of 2, 3, 7 and 10 for HOP-26, CD166, STRO-1 and CD49a respectively; Table 1) whilst ensuring their recovery in numbers sufficient for functional studies of colony-forming efficiency. The purity of the recovered fractions for STRO-1, HOP-26 and CD166 was typically ≥70%, whereas that for CD49a was appreciably less at 55% (Table 1). When the recoveries were examined, however, it was clear that, for each antibody, only a minor proportion of antigen-positive BMMNC was being retained on the column (≤5%; Table 1). The data for the mean fluorescence intensity (MFI) suggested that this “bound fraction” represented the antigen “bright” cell population, and that, by inference, the antigen “dim” cells were being lost in the washes (Table 1).

Functional analysis of the MACS-separated populations of BMMNC revealed that, for each of the antibodies investigated, CFU-F were recovered almost exclusively in the antigen-positive fractions. When compared with each other and with freshly isolated BMMNC, CFE was consistently greatest in the CD49a<sup>+</sup> fraction and least in the CD166<sup>+</sup> fraction (Table 1).

**Table 1** Immunoselection of CFU-F from adult human bone marrow: an immunochemical and functional comparison of four antibodies with cells from three different donors. Freshly isolated, labelled BMMNC were separated into antibody-positive and antibody-negative fractions by MACS. Recovered cell fractions were analysed by flow cytometry (immunochemical data) or cultured for 14–17 days to assess colony forming efficiency (functional data). Immunochemical data (*Immunochemical*) are shown as the percentage of positively labelled cells with  $\geq 95\%$  control fluorescence or the mean fluorescence intensity (*MFI*);

*Recovery (%)* no. cells (positive fraction)/no. cells (starting fraction) $\times 100$ , *Enrichment factor* percentage of positive cells (positive fraction)/percentage positive cells (starting fraction). Functional data (*Functional*) is shown as total or AP<sup>+</sup> colonies/ $10^5$  cells; *Recovery (%)* total colonies (positive fraction)/(100/immunochemical recovery (%)) $\times$ total colonies (unfractionated BMMNC) $\times 100$ , *Enrichment factor* total colonies (positive fraction)/total colonies (unfractionated BMMNC). Values are the mean $\pm$ SEM

## Selection criteria

Immunochemical					Functional			
Cell fraction	% Positive cells	MFI	Recovery (%)	Enrichment factor	Colonies/ $10^5$ cells		Recovery (%)	Enrichment factor
					Total	AP <sup>+</sup>		
BMMNC					1.9 $\pm$ 0.6	0 $\pm$ 0	100	
STRO-1								
Starting	15 $\pm$ 9	6 $\pm$ 2						
Negative	10 $\pm$ 5	5 $\pm$ 1			0 $\pm$ 0	0 $\pm$ 0		
Positive	78 $\pm$ 0	45 $\pm$ 3	5 $\pm$ 2	7 $\pm$ 2.6	16 $\pm$ 2*	2 $\pm$ 1	49 $\pm$ 7**	9 $\pm$ 1.3
HOP-26								
Starting	24 $\pm$ 11	6 $\pm$ 1						
Negative	16 $\pm$ 9	6 $\pm$ 1			0 $\pm$ 0	0 $\pm$ 0		
Positive	70 $\pm$ 9	33 $\pm$ 5	1 $\pm$ 0	2 $\pm$ 0.1	12 $\pm$ 2*	1 $\pm$ 1	4 $\pm$ 0.6****	6 $\pm$ 0.9
CD166								
Starting	30 $\pm$ 6	6 $\pm$ 0						
Negative	27 $\pm$ 10	6 $\pm$ 1			1 $\pm$ 0.1	0 $\pm$ 0		
Positive	81 $\pm$ 3	25 $\pm$ 7	1 $\pm$ 0	3 $\pm$ 0.5	7 $\pm$ 1***	2 $\pm$ 1	4 $\pm$ 0.3****	4 $\pm$ 0.3***
CD49a								
Starting	7 $\pm$ 3	3 $\pm$ 1						
Negative	7 $\pm$ 3	3 $\pm$ 1			0 $\pm$ 0	0 $\pm$ 0		
Positive	55 $\pm$ 14	16 $\pm$ 5	2 $\pm$ 0	10 $\pm$ 3.6	33 $\pm$ 2****	3 $\pm$ 1	24 $\pm$ 1.8	18 $\pm$ 1.3**

\*  $P < 0.01$  when compared with the unfractionated cell population

\*\*  $P < 0.001$  when compared with all other groups

\*\*\*  $P < 0.01$  when compared with the STRO-1<sup>+</sup> fraction

\*\*\*\*  $P < 0.05$  when compared with the CD49a<sup>+</sup> fraction

## Antigen expression by culture-expanded BMSC

With the exception of CD166, which in all cases was expressed by the majority of cells, there was substantial inter-donor variation in the expression (% positive cells), by culture-expanded BMSC, of all the antigens investigated (Table 2). Analysis of the pooled data from multiple donors revealed that the proportion of cells expressing the HOP-26 antigen was similar in freshly isolated BMMNC and BMSC (40%–50%; Table 2). For the remaining antigens investigated, there was a tendency for the proportion of positive cells to increase with time in culture, although only in the cases of STRO-1 and CD166 were the increases of statistical significance ( $P < 0.05$  and 0.01 respectively; Table 2). The CD166-positive fraction of BMSC was also consistently brighter (greater MFI) than that of freshly isolated BMMNC ( $P < 0.01$ ; Table 2).

## Co-expression of STRO-1 and CD49a

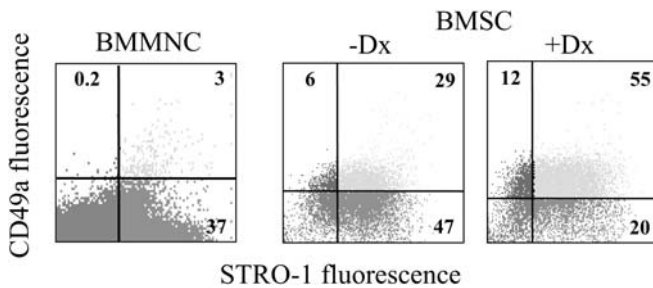
In contrast to the findings of Deschaseux and Charbord 2000, we found that essentially all CD49a<sup>+</sup> BMMNC co-expressed the STRO-1 antigen (Fig. 1). In culture, however, CD49a<sup>+</sup> BMSC were detected in the STRO-1<sup>+</sup> and STRO-1<sup>-</sup> fractions (Fig. 1). Treatment with dexamethasone (Dx), which promotes the differentiation and further maturation of cells of the osteogenic lineage in this cell culture system (Stewart et al. 1998; Walsh et al. 2001), increased the proportion of BMSC expressing CD49a<sup>+</sup> and the level (MFI) at which this antigen was expressed ( $P < 0.05$ ; Figs. 1, 2). The magnitude of this increase (approximately 1.8-fold) was similar in the STRO-1<sup>+</sup> and STRO-1<sup>-</sup> fractions (Fig. 1). The Dx-induced increase in the proportion of BMSC with the surface phenotypes CD49a<sup>+</sup>/STRO-1<sup>+</sup> and CD49a<sup>+</sup>/STRO-1<sup>-</sup> was associated with a decrease in the proportion of cells recovered in the STRO-1<sup>+</sup>/CD49a<sup>-</sup> fraction (Fig. 1).

**Table 2** Antigen expression by BMMNC and secondary cultures of BMSC. Freshly isolated BMMNC and confluent secondary cultures of BMSC were labelled with primary antibodies or isotype-matched controls and binding was detected by using a FITC-conjugated second antibody by flow cytometry. Data are shown as the percentage of positively labelled cells (% +ve) with  $\geq 95\%$  control fluorescence or the mean fluorescence intensity (MFI). Results are expressed as the mean  $\pm$  SEM (range), with the number of experiments (*n*)

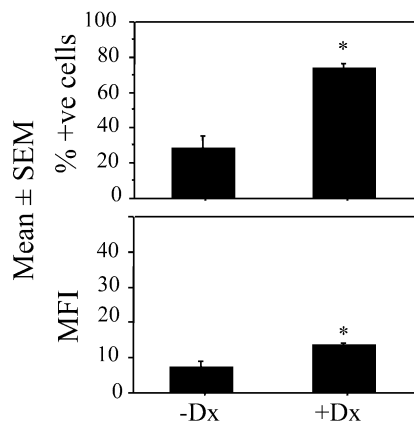
Marker	BMMNC			BMSC		
	% +ve	MFI	n	% +ve	MFI	n
IgM control	3 $\pm$ 0.3	3 $\pm$ 0.2	18	3 $\pm$ 0.2	3.4 $\pm$ 0.2	13
STRO-1	27 $\pm$ 3.0 (6–47)	12 $\pm$ 1.6 (4–24)	18	51 $\pm$ 8.1* (11–93)	28 $\pm$ 6.6 (5–89)	13
HOP-26	42 $\pm$ 5.1 (3–78)	16 $\pm$ 2.9 (4–48)	15	51 $\pm$ 8.4 (16–88)	16 $\pm$ 5.1 (7–23)	10
IgG1 control	3 $\pm$ 0.3	3 $\pm$ 0.2	14	2 $\pm$ 0.2	2 $\pm$ 0.2	13
CD49a	5 $\pm$ 1.3 (1–13)	3 $\pm$ 0.4 (2–5)	8	29 $\pm$ 6.6 (18–41)	7 $\pm$ 1.7 (5–11)	3
CD166	19 $\pm$ 4.0 (1–36)	7 $\pm$ 0.7 (3–10)	9	95 $\pm$ 2.5** (86–100)	81 $\pm$ 24.6** (18–192)	6

\* Significantly different from BMMNC,  $P < 0.01$  (ANOVA with Fisher's PLSD)

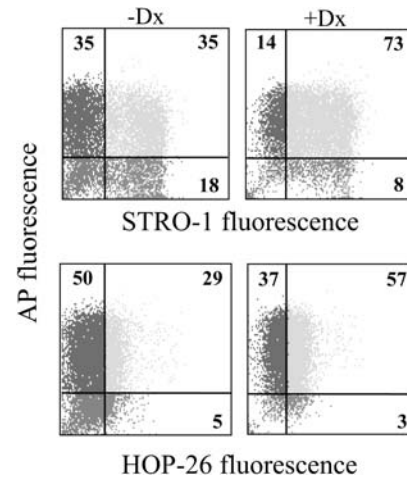
\*\* Significantly different from BMMNC,  $P < 0.0001$  (ANOVA with Fisher's PLSD)



**Fig. 1** Co-expression of STRO-1 and CD49a by BMMNC and confluent secondary cultures of BMSC with and without 10 nM dexamethasone (+Dx and -Dx, respectively). Flow cytometric data from representative experiments are shown as dot plots with CD49a fluorescence on the *y*-axis and STRO-1 fluorescence on the *x*-axis. Quadrants were set based on the fluorescence obtained with isotype-matched control antibodies. The percentage of positively labelled cells is given



**Fig. 2** Expression of CD49a in confluent secondary cultures of BMSC maintained in the absence (-Dx) or continuous presence (+Dx) of 10 nM Dx. Flow cytometric data is shown as mean  $\pm$  SEM percentage of positive cells with  $\geq 95\%$  control fluorescence or mean fluorescence intensity (MFI;  $n=3$ ; \* $P < 0.05$  Dx treated vs untreated, Mann-Whitney U-test)



**Fig. 3** Co-expression of STRO-1 or HOP-26 and AP in confluent secondary cultures of BMSC maintained in the absence (-Dx) or continuous presence (+Dx) of 10 nM Dx. Flow cytometric data from representative experiments are shown as dot plots with AP fluorescence on the *y*-axis and STRO-1 or HOP-26 fluorescence on the *x*-axis. Quadrants were set based on the fluorescence obtained with isotype-matched control antibodies. The percentage of positively labelled cells is given

#### Co-expression of HOP-26 and AP

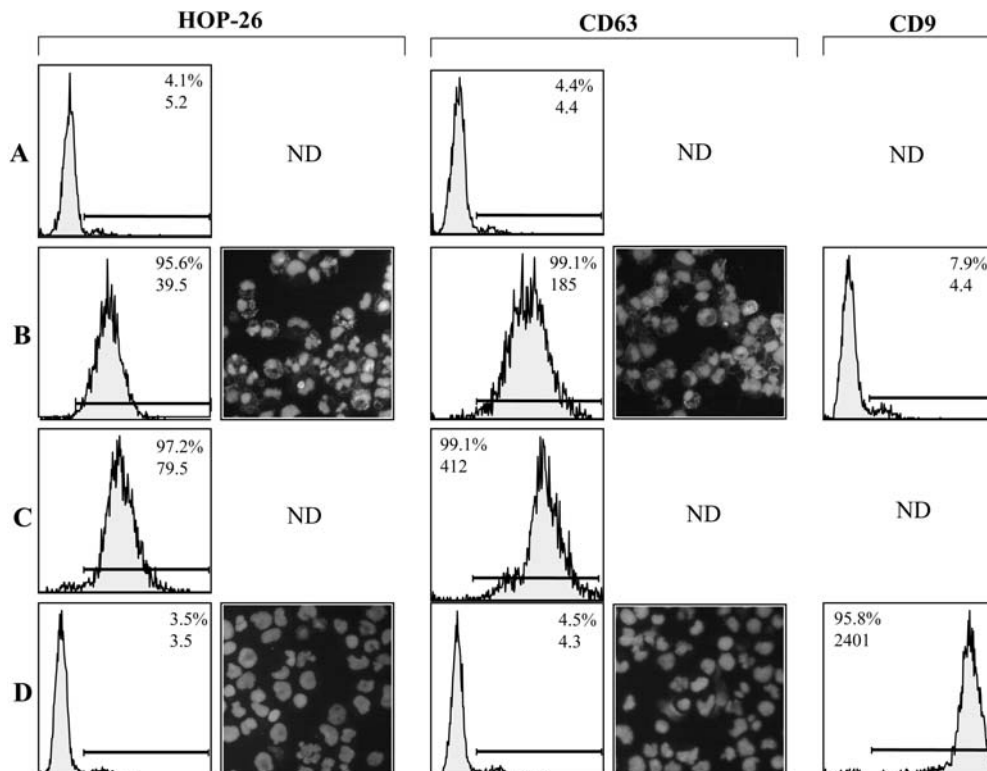
A significant proportion of HOP-26<sup>+</sup> BMSC were found to co-express AP, an early developmental marker for cells of the osteoblast lineage (Fig. 3). Treatment with Dx increased the size of the HOP-26<sup>+</sup>/AP<sup>+</sup> fraction without affecting that of the HOP-26<sup>+</sup>/AP<sup>-</sup> fraction (Fig. 3). Similarly, culture of BMSC in the continuous presence of 10 nM Dx resulted in an increase in the proportion of STRO-1<sup>+</sup>/AP<sup>+</sup> cells (Fig. 3).

#### Identification of HOP-26 as CD63

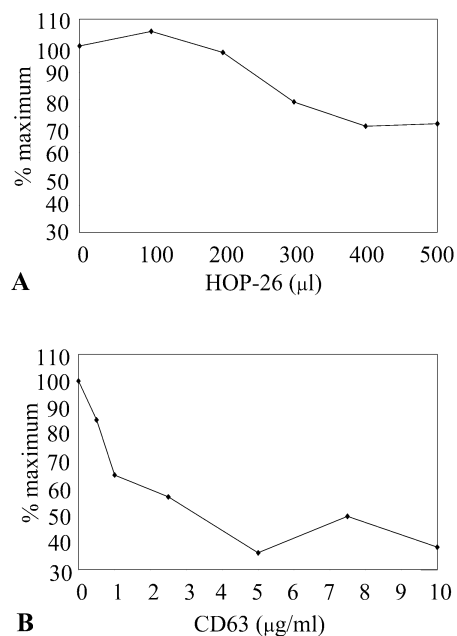
During the course of these studies, the antigen recognised by the antibody HOP-26 was identified as CD63. Preliminary screening with a panel of monoclonal anti-

**Fig. 4A–D** Expression of HOP-26, CD63 and CD9 by untransfected RBL-2H3 cells (A), and CD63-transfected (B), CD63-7-transfected (C) or CD9-transfected (D) RBL-2H3 cells.

Binding of the antibodies was detected by using an FITC-conjugated second antibody. Flow cytometric data are shown as frequency histograms of fluorescence intensity for representative experiments. The percentage of positively labelled cells with  $\geq 95\%$  control fluorescence and the mean fluorescence intensity are given. In immunohistochemical studies, cells were counterstained with ethidium bromide (ND not done).  $\times 400$



bodies recognising CD antigens revealed that the HOP-26 antigen and CD63 co-localised in freshly isolated BMMNC and in culture-expanded BMSC (data not shown). When saponin-permeabilised cells were analysed for HOP-26 binding by flow cytometry, high levels of intracellular binding sites were detected, an observation consistent with HOP-26 recognising CD63, a lysosomal membrane protein (data not shown). Subsequently, we found that CD63-transfected RBL-2H3 cells were both CD63<sup>+</sup> and HOP-26<sup>+</sup> (Fig. 4B). Neither HOP-26 nor H5C6 (anti-CD63) bound to untransfected cells (Fig. 4A), nor did cells transfected with the related tetraspanin CD9 (Fig. 4D). Cells transfected with a C-terminal deletion mutant of CD63 lacking the internalisation motif (CD63-7) showed an increased number of cell-surface binding sites (MFI) for both CD63 and HOP-26 (Fig. 4C). Competition studies revealed that H5C6 could inhibit the binding of HOP-26 to CD63-transfected RBL cells (Fig. 5A) and that the reverse was also true (Fig. 5B). Consistent with HOP-26 being of the IgM subclass, and therefore of lower affinity, the degree of inhibition obtained was less than that observed with H5C6, an antibody of the IgG<sub>1</sub> subclass. Finally, the results of preliminary experiments showed that the CFE of the CD63<sup>+</sup> and HOP-26<sup>+</sup> fractions of BMMNC obtained from the same donor were essentially the same (data not shown).



**Fig. 5A, B** Competition of HOP-26 antibody (A) or CD63 antibody (B) binding to CD63-transfected RBL-2H3 cells with either CD63 or HOP-26 antibodies, respectively. Binding was detected by using an FITC-conjugated second antibody by flow cytometry. Data are shown as the percentage of inhibition based on the percentage of positively labelled cells recorded

## Discussion

The bone marrow stroma comprises a developmentally heterogeneous population of cells that show a wide variation in their potential for proliferation and differentiation (Mankani and Robey 1998; Owen 1998; Phinney et al. 1999; Bianco et al. 2001). An antibody-based stromal classification system, akin to the CD nomenclature that exists for the haematopoietic system, would have a number of important benefits. It would lead to an improved understanding of the relationships that exist between the different lineages that form a part of this complex hierarchy. It would enable the effects of therapeutic agents (e.g. bone anabolics) or treatment regimens (chemotherapy, radiotherapy) on the cellular dynamics of the marrow stromal system to be assessed. Most importantly, it would provide a rational basis for realising the full clinical potential of *ex vivo* expanded subsets of BMSC of defined developmental potential in tissue reconstruction and repair, transplantation medicine and gene therapy.

The isolation of CFU-F from adult human bone marrow by using the monoclonal antibody STRO-1 was the first indication that this rare population of clonogenic precursors might be distinguishable from other BMMNC on the basis of their cell surface phenotype (Simmons and Torok-Storb 1991). It is important to appreciate, however, that, although relatively enriched in CFU-F, the STRO-1<sup>+</sup> fraction also contains other cell types of both stromal and haematopoietic origin (Simmons and Torok-Storb 1991; Gronthos et al. 1994). Consequently, there is an urgent need for additional antibody reagents that recognise this clinically important cell population. Based on the literature, HOP-26 (Joyner et al. 1997) and those antibodies recognising CD166 (Bruder et al. 1997, 1998c) and CD49a (Deschaseaux and Charbord 2000) might satisfy this need. Prior to this investigation, however, no attempt had been made to compare directly their utility as reagents for the immunoselection of CFU-F or as developmental markers of culture-expanded BMSC.

In this investigation, in which a one-pass immunoselection strategy was employed, the incidence of CFU-F was consistently greatest in the CD49a<sup>+</sup> fraction, although the degree of enrichment obtained was markedly donor-dependant. Absolute recovery of CFU-F, however, was consistently greatest in the STRO-1<sup>+</sup> fraction (49% compared with 24% for the CD49a<sup>+</sup> fraction). No correlation was observed between the degree of enrichment obtained and the purity of the antigen-positive fraction. Thus, the purity of the CD49a<sup>+</sup> fraction was the least of all the antibodies investigated and yet it contained the highest proportion of CFU-F. Whereas the purity of this fraction could have been increased by passage over a second column, this, in our experience, would have been achieved only at the expense of a significant reduction in the absolute number of antigen-positive cells recovered (data not shown).

The relative enrichment of CFU-F in the CD49a<sup>+</sup> fraction was approximately 18-fold, which was in good

agreement with the findings of Deschaseaux and Charbord (2000). In contrast to them, however, we found that essentially all CD49a<sup>+</sup> BMMNC co-expressed STRO-1. This discrepancy may be related to the use of marrow sampled from different anatomical sites and from donors of different ages.

The recovery of CFU-F in the STRO-1, CD49a and HOP-26 antigen-positive fractions was considerably less than that reported previously by other investigators (Simmons and Torok-Storb 1991; Joyner et al. 1997; Deschaseaux and Charbord 2000). We suspect that this discrepancy relates to our use of the MACS protocol, which, based on a comparison of the pre- and post-selection values of MFI, appears to be selective for cells in the "antigen-bright" subpopulation. The absence of "antigen-dim" cells in the unbound fraction suggests that they are lost in the column washes. It will be important to establish whether this population contains additional CFU-F and, if so, whether their developmental potential differs from those recovered in the "antigen-bright" population.

The incidence of HOP-26<sup>+</sup> BMMNC in this investigation (approximately 42%) is considerably greater than that reported by Joyner et al. (1997;  $\leq 1\%$ ). This discrepancy most likely reflects the greater sensitivity of flow cytometry when compared with the immunostaining protocol used by those authors. A further factor is their use of fixed cell populations; we have found that the choice of fixative markedly alters the sensitivity of the subsequent staining procedure for a number of stromal antigens, including HOP-26 (data not shown). It seems reasonable to assume, therefore, that Joyner et al. (1997) detected only the "antigen-bright" subpopulation of HOP-26<sup>+</sup> cells: a possibility supported by our observation that this fraction does indeed comprise only 1% of BMMNC.

There is widespread interest in the possibility of exploiting the developmental potential of CFU-F for tissue reconstruction and repair. The low incidence of this cell type, even in the bones of young healthy adults, has led many to propose the use of culture-expanded populations of BMSC in their stead. A major, and as yet unresolved, limitation of this approach is that, whilst capable of generating large numbers of cells, the cultures obtained remain poorly defined in terms of the extent and nature of their cellular heterogeneity. In consequence, the prospective evaluation of the likely clinical impact of the cultured cell population, although highly desirable, is currently not feasible. Establishing whether any of the antigens investigated has the potential to sub-type culture-expanded BMSC is considered, therefore, to have a high priority.

When cultured *ex vivo*, BMMNC gave rise consistently to populations of BMSC expressing the antigens STRO-1, CD49a, HOP-26 and CD166. Irrespective of the donor of origin, CD166 was expressed by  $\geq 95\%$  of BMMNC. A similar result was obtained by Bruder et al. (1997) and, on this basis, it was concluded that culture-expanded BMSC comprise an homogeneous population. The results presented here suggest that this conclusion was premature

and illustrate the potential pitfalls of assigning identity on the basis of the expression of a single antigenic marker.

In contrast to that of CD166, the expression of STRO-1, CD49a and HOP-26 *in vitro* was markedly donor-dependent. We and others have shown that the potential of BMSC for proliferation and differentiation is similarly donor-dependent and that this variation is unrelated to differences in age or gender (Gundle and Beresford 1995; Phinney et al. 1999; Walsh et al. 2000). In future studies, it will be important to establish whether such differences in the developmental potential of BMSC can be linked to differences in the expression of these or other cell surface antigens.

In culture, the expression of STRO-1 and CD49a remains restricted to a subset of cells, albeit a higher proportion than are present in freshly isolated preparations of BMMNC. To assess whether the expression of these antigens by BMSC is developmentally regulated, cells were cultured in the absence or presence of the synthetic glucocorticoid Dx (10 nM), which, as we have shown previously, promotes the differentiation and further maturation of cells of the osteogenic lineage in this human cell culture system (Walsh et al. 2001). As expected, under these conditions, there was a marked increase in the proportion of BMSC present in the putative osteoprogenitor fraction (STRO-1<sup>+</sup>/AP<sup>+</sup>) and a decline in the proportion of cells present in the primitive precursor fraction (STRO-1<sup>+</sup>/AP<sup>-</sup>; Stewart et al. 1998; Walsh et al. 2001). This was associated with a significant increase in expression of CD49a<sup>+</sup> (%-positive cells and MFI, an indirect measure of the number of binding sites/cell) in both the STRO-1<sup>+</sup> and STRO-1<sup>-</sup> subpopulations. One interpretation of these findings is that the expression of CD49a *in vitro*, like that of STRO-1, is developmentally regulated and linked to the differentiation and/or further maturation of cells of the osteoblast lineage. Further experiments, including the co-localisation of CD49a and other osteoblast markers *in vitro* and *in vivo*, will be required to confirm or refute this interesting possibility.

Joyner and co-workers (1997) have reported that the expression of HOP-26 in culture is initially low ( $\leq 10\%$ ), reaches a maximum at day 3 (95% positive) but declines thereafter, such that, by day 28, less than 1% of BMSC are antigen-positive. Evidence for such temporal regulation of HOP-26 expression is lacking in our investigation. Indeed, its level of expression (%-positive cells and MFI) in BMMNC and BMSC is remarkably similar at 40%–50% of total. We suspect that this discrepancy relates to the use of different techniques (flow cytometry versus immunocytochemistry) to enumerate the fraction of antigen-positive cells.

Treatment with Dx had no consistent effect on the size of the HOP-26<sup>+</sup> cell population but did alter the proportion of cells that co-expressed the developmental marker AP, the expression of which is widely accepted as an early indicator that a cell has entered the osteogenic pathway of differentiation. *In vivo*, the majority of cells, including osteoblasts, are HOP26<sup>-</sup>, but small numbers of

HOP-26<sup>+</sup> cells are located close to bone surfaces. On this basis, Joyner et al. (1997) have hypothesised that osteogenic precursors might be included within the HOP-26<sup>+</sup> fraction but they did not explore this possibility further by attempting to co-localise the expression of HOP26 and AP. Our demonstration that HOP26<sup>+</sup>/AP<sup>+</sup> BMSC are found *in vitro* and that their number is increased by treatment with Dx is consistent with the hypothesis of Joyner et al. (1997), but determining the exact developmental status of these cells will require their isolation and further characterisation.

In the course of this investigation, evidence was obtained to support the conclusion that HOP-26 recognises CD63, a lysosomal membrane glycoprotein that belongs to the tetraspanin or transmembrane 4 superfamily (TMS4; Metzelaar et al. 1991; Berditchevski 2001). Our observation of a high level of HOP-26 reactivity in freshly isolated BMMNC is consistent with this possibility, as CD63 is known to be expressed by cells of both haematopoietic and non-haematopoietic origin (Barclay et al. 1993). Molecular cloning of the cell surface antigen recognised by HOP-26 has since provided confirmation of its identity as CD63 (Zannettino et al. 2003).

The functional significance of CD63 expression by primitive cells of the osteoblast (bone-forming) lineage is currently unknown but merits further investigation. TMS4 proteins, including CD63, have been implicated in a wide range of cellular functions including migration, adhesion (both cell-cell and cell-matrix), proliferation and differentiation, possibly reflecting their ability to form complexes with integrins (Smith et al. 1995; McCullough et al. 1996; Berditchevski 2001). In adherent cells, integrin-TMS4 complexes are clustered into a distinct type of adhesion structure at the cell periphery. The presence of the TMS4 moiety is thought to modulate integrin signalling, compartmentalisation on the cell surface and intracellular trafficking and recycling (Berditchevski 2001). Nevertheless, the precise biochemical function of the TMS4 proteins remains undefined and no membrane or soluble protein that might serve as a physiological receptor/ligand has been identified.

Targeting of cell surface markers expressed selectively by primitive cells is potentially a fast and clinically efficient means of purifying a heterogeneous marrow sample. The results that we have presented here suggest that this is a realistic possibility but that further screening of antibodies characterised to date and the generation of antibodies recognising novel antigens is still required, as is an analysis of the antibody-defined cell sub-populations recovered both *in vitro* and *in vivo*. Identification of novel differentially expressed markers would further extend the phenotypic classification of the stromal cell lineage and hence enable greater standardisation of research carried out with these cells (Stewart et al. 1996, 2001; Letchford et al. 2001). The more that we learn of the complexities of the stromal system, the more likely it seems that the use of “cocktails” rather than single antibodies will provide the most effective means of isolating primitive marrow



stromal cells and defining the developmental potential of the progeny that they give rise to when cultured *ex vivo*.

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