Stromal Derived Factor-1 Promotes the Growth, Survival and Development of Human Bone Marrow Stromal Stem Cells

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ABSTRACT

The maintenance of bone marrow stromal stem cells (BMSSCs) is tightly controlled by the local microenvironment and by autocrine regulatory factors secreted by BMSSC. To identify such factors, a cDNA subtraction library was generated from purified BMSSC, based on their high expression of the STRO-1 antigen. Stromal derived factor-1 (SDF-1) was one differentially expressed gene highly expressed by purified BMSSC prior to culture. In vitro, immature pre-osteogenic cells expressed greater levels of SDF-1 when compared with mature cell types representative of osteoblasts and osteocytes/bone lining cells. Furthermore, SDF-1 expression was rapidly down-regulated when BMSSC were cultured under osteoinductive conditions. BMSSC were also shown to express functional cell surface SDF-1 receptors (CXCR4). Transduced BMSSC lines, secreting high SDF-1 levels, displayed an enhanced ability to form ectopic bone in vivo, in comparison with control BMSSC lines. Moreover, high SDF-1 expressing BMSSC displayed an increased capacity for cellular growth and protection against interleukin-4 induced apoptosis. Similarly, colony-forming unit-fibroblasts (CFU-F) also displayed increased growth and resistance to alpha-interferon-2a induced apoptosis, in synergy with PDGF-BB and SDF-1 in vitro. These studies indicate that the chemokine, SDF-1 may play a role in the maintenance, survival and osteogenic capacity of immature BMSSC populations.
INTRODUCTION

Postnatal human bone marrow stromal stem cells (BMSSC) or mesenchymal stem cells (MSC) have the capacity to regenerate a hematopoietic-supportive bone marrow organ and associated bone trabecular, when transplanted into immunocompromised mice \(^1\text{-}^5\). Recent studies have also reported that BMSSC are more plastic than first realized, by virtual of their ability to develop into diverse cell lineages such as myelo-supportive stroma, osteoblasts, chondrocytes, adipocytes, myoblasts, hepatocytes, cardiomyocytes and neural cells \(^6\text{-}^8\). These developments have prompted investigations into the possible use of ex vivo expanded BMSSC populations for a wide range of tissue engineering and gene therapy applications \(^9,\text{ }10\). Thus far, encouraging preliminary results have been reported for different human clinical trials \(^11\text{-}^15\). However, the progress of these studies has largely been restrained due to a lack of understanding of the critical factors that regulate the growth and differentiation of human multi-potential BMSSCs.

Postnatal stem cells have been identified in various tissues residing in specialised microenvironments or stem cell niches, endowed with the capacity to mediate stem cell proliferation, migration and differentiation \(^4,\text{ }16,\text{ }17\). These unique attributes involve a complex array of both paracrine and autocrine signaling molecules, specific cell-cell and cell-extracellular matrix interactions and physio-chemical and mechanical stimuli. We have recently reported that BMSSCs reside in a perivascular niche, based on a composite protein expression pattern of several markers associated with smooth muscle, pericytes and endothelial cells, using immunohistochemical staining and extensive FACS analysis \(^4,\text{ }5,\text{ }18,\text{ }19\). This notion is supported by studies that describe the phenotype of cultured BMSSC as smooth muscle cell-like in appearance \(^20\) while others propose that BMSSC may in fact be multipotential pericytes as identified in non-hematopoietic tissues.
Whilst, these studies have made substantial advances in determining the putative BMSSC niche, there still remains a distinct lack of knowledge concerning the factors and environmental cues that mediate the maintenance and development of multipotential BMSSC in situ.

To help address these issues, we have developed enrichment protocols to purify BMSSC from adult human bone marrow mononuclear preparations, based on their high expression of the MSC marker, STRO-1, and co-expression of the immunoglobulin superfamily members, CD106 and CD146. Genotypic analysis of purified preparations of STRO-1\textsuperscript{bright}/CD106\textsuperscript{+} or STRO-1\textsuperscript{bright}/CD146\textsuperscript{+} BMSSC has revealed a considerable difference in the gene expression profiles of these precursor cells when compared to culture expanded BMSSC. The resulting effect of normal culture was subsequently found to lead to a loss of telomerase activity and initiation of cell cycle progression by the normally quiescent BMSSC population following ex vivo expansion. This maturation phenomenon also correlated with a down regulation of the early stromal marker, STRO-1 over prolonged ex vivo expansion, with a concomitant up-regulation of genes associated with osteogenic commitment and differentiation including CBFA1, Osterix, osteopontin and osteocalcin. Therefore, the study of ex vivo expanded BMSSC poses a practical dilemma in relation to designing strategies to identify key regulatory molecules that may be secreted by early stromal stem cell populations with the capacity to modulate hematopoietic stem/precursor populations and perhaps BMSSC development.

The aim of this study was to identify critical factors, expressed by freshly sorted STRO-1\textsuperscript{bright} bone marrow cells, which play a role in regulating the growth and survival of BMSSC during ex vivo expansion. Given the rarity of BMSSC population, a PCR-based cDNA subtraction hybridization...
technology was employed to enrich and amplify transcripts uniquely expressed by the STRO-
1bright population. The present study identified a member of the CXC chemokine family, SDF-1 as
being highly expressed by purified preparations of STRO-1bright BMSSC. In humans, there are two
distinct isoforms of SDF-1 derived from a single gene (SDF-1α and β) with the alpha splice
variant being the most abundant 28-31. The SDF-1 receptor, CXCR4, is a G-coupled transmembrane
glycoprotein 32,33. Interactions between SDF-1 and CXCR4 have been shown to mediate blood cell
homeostasis, through the control of leukocyte development, migration and activation 34. Moreover,
SDF-1 has been shown to directly regulate the cell cycle and survival of HSC 35. Other studies
have also implicated SDF-1 as an important factor in promoting the survival and migration of
circulating tissue-specific progenitors identified for muscle, neural and liver, implicating this
chemokine as an important maintenance factor in postnatal tissue repair 36. SDF-1 has been
reported in the literature to be expressed by endothelial cells, perivascular cells, myelo- and B-
lympho-supportive stroma within the marrow spaces and near the endosteal surfaces in sections of
human bone marrow 26,37,38. In the present study we present functional data suggesting that SDF-1
is highly expressed by immature BMSSC and is a potential regulator of BMSSC growth and
survival.
Materials and Methods

Subjects and cell culture. Bone marrow (BM) aspirates were obtained from the posterior iliac crest of normal adult volunteers (19-35 years old) following informed consent, according to procedures approved by the ethics committee of the Royal Adelaide Hospital, South Australia. Bone marrow mononuclear cells (BMMNC) were prepared as previously described. Primary BMSSC cultures were established in α-MEM supplemented with 20% fetal calf serum, 2mM L-glutamine and 100 μM L-ascorbate-2-phosphate as previously described.

Primary antibodies. Primary antibodies used in this study: STRO-1 (mouse IgM), anti-human alkaline phosphatase antibody (B4-78, mouse IgG1; Hybridoma Studies Bank, University of Iowa, Iowa), anti-human CXCR4 antibody (mouse IgG2b; Chemicon, International, Temecula, CA, IgG2b), and anti-human Annexin V antibody (mouse IgG1; Chemicon,) were used as either tissue culture supernatant diluted 1:2 or as purified immunoglobulin 10 μg/ml, respectively. Isotype-matched control mouse monoclonal antibodies used in this study: 1A6.12, (IgM), 1B5 (IgG1) and 1A6.11 (IgG2b) (kindly provided by Prof. L.K. Ashman, University of Newcastle, NSW, Australia).

Purification of BMSSC. This was performed essentially as previously described. In brief, approximately 1-3 x 10^8 adult human BMMNC were incubated with blocking buffer (Hank’s balanced salt solution (HBSS) supplemented with 1% human serum, 1% bovine serum albumin and 5% fetal bovine serum), then sequentially incubated with STRO-1 supernatant, anti-IgM-biotin, streptavidin microbeads (Miltenyi Biotec Inc., Auburn, CA) and finally streptavidin FITC.
(Caltag Laboratories, Burlingame, CA) before being separated on a Mini MACS magnetic column (Miltenyi Biotec Inc.), according to the manufacturer’s recommendations. The MACS isolated STRO-1+ bone marrow mononuclear cells were subsequently sorted using a FACStarPLUS flow cytometer (Becton Dickinson, Sunnyvale, CA), based on their high (STRO-1bright) or low (STRO-1dull) STRO-1 expression (Figure 1A).

Isolation of STRO-1/alkaline phosphatase BMSSC subpopulations. Secondary cultures of human BMSSC were prepared as single cell suspensions by trypsin/EDTA digest and then incubated with antibodies identifying STRO-1 and the bone associated antigen alkaline phosphatase (AP), B4-78, as described by Gronthos et al. (1999) 40. Approximately, 2 x 10^7 cells were incubated with antibodies reactive to STRO-1 and alkaline phosphatase (B4-78) for one hour on ice. Replicate tubes were incubated with the corresponding single color and negative control antibodies. After washing the samples were incubated with goat anti-mouse IgG1-FITC and IgM-PE antibodies (Southern Biotechnology Associates Inc., Birmingham, AL) as secondary detection agents for 45 minutes on ice. Following washing, the cells were subsequently sorted to purity by double sorting, using a FACStarPLUS flow cytometer (Becton Dickinson), based on the four STRO-1/AP BMSSC subpopulations 40.

Calcium flux assays. Single cell suspensions of trypsin-detached secondary BMSSC cultures were resuspended to a concentration of 1 x 10^6 cells/ml in HBSS supplemented with 1% FCS and 1.25 mM CaCl₂. The cells were incubated with 2 μM fura-2-AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Excess fura-2-AM was removed by washing the cells twice, and the cells were resuspended in 2 ml of HBSS containing 1% FCS and 1.25 mM CaCl₂ to a final
concentration of 1 x10^6 cells/ml. [Ca^{2+}]_i was measured using spectrofluorometer (PerkinElmer, LS55 Luminescence spectrometer), with alternating excitation of 340 and 380 nm and fluorescence emission at 510 nm. After establishing a base line level of [Ca^{2+}]_i, the cells were treated with 30 ng/ml SDF-1α. When a stable peak of [Ca^{2+}]_i in response to SDF-1α was achieved, the BMSSC were permeabilised with 0.1 mM digitonin and EGTA added to a final concentration of 5mM. The digitonin and EGTA measurements were used to calibrate [Ca^{2+}]_i with regard to fura 2-AM fluorescence in each sample using a calibration equation as previously described^{41}.

**Colony efficiency assays.** Colony forming assays were performed using MACS/FACS isolated STRO-1^{bright} BMMNC then plated at a density of 5 x 10^4 per well in 24-well plates under serum-deprived conditions as previously described^5,39. Cells were plated in the presence of different cytokine combinations. Growth factors used in this study: alpha-interferon 2a (30,000 iu/ml) (F. Hoffmann-La Roche Ltd, Basel, Switzerland), platelet derived growth factor-BB (5 ng/ml), Interleukin-4 (30 ng/ml) and stromal derived factor-1 (30 ng/ml) (CytoLab/PeproTech, Rehovot, Israel). The cultures were terminated at day 14 and the number of CFU-F enumerated following staining with 0.1% (w/v) toluidine blue in 1% paraformaldehyde. Aggregates of >50 cells were scored as CFU-F derived colonies.

**Flow cytometric analysis.** BMSSC cultures were prepared by trypsin/EDTA digest then resuspended in blocking buffer for 30 minutes. Single cell suspensions were then incubated with either anti-CXCR4 antibody or 1A6.11 at a concentration of 10 µg/ml for one hour on ice. Similarly, high SDF-1 expressing BMSSC and vector control cells lines were prepared by
trypsin/EDTA treatment, blocked, then incubated with either anti-Annexin V antibody or the isotype matched control antibody, 1D4.5. After washing, the cells were incubated with the secondary detection reagents, goat anti-mouse IgG1- or IgG2b-FITC conjugated antibodies (1/50) (Southern Biotechnology Associates Inc.) for 45 minutes on ice. Following washing, the samples were analysed using an Epics®-XL-MCL flow cytometer (Beckman Coulter, Hialeah, FL).

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared from $2 \times 10^4$ STRO-1$^{bright}$, STRO-1$^{dull}$ and STRO-1$^{negative}$ sorted bone marrow mononuclear cells, cultured BMSSC STRO-1/alkaline phosphatase sorted subpopulations or the human osteosarcoma cell line, MG63, using the RNA STAT-60 system (TEL-TEST Inc. Friendswood TX). Total 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 previously described. Primers sets used in this study: SDF-1 (forward, 5'-gacccgcgctcgtccgcc-3'; reverse, 5'-gtctggactctactgttaagg-3'); CXCR4 (forward, 5'-tcctggagaaccagcggttac-3'; reverse, 5'-gacgccaacatagaccacct-3'); GAPDH (forward, 5'-catggagaaggctggggctc-3'; reverse, 5'-cactgacacgttggcagtgg-3'). Amplified products were analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining. Semi-quantitative analysis of transcript abundance was assessed relative to GAPDH expression using ImageQant software.

Generation of transduced BMSSC lines. Retroviral expression constructs were generated with the retroviral vector pLNCX2 (Clontech Laboratories Inc., Palo Alto, CA) encoding the full-length human SDF-1 cDNA amplified using the PCR forward (5'-aataactcggagacctgctgctgcgc-3') and
reverse (5’-aattagaagccgactactgaaggg-3’) primer set (underlined), constructed with Xho I and Not I (bold) restriction sites, respectively. The packaging cell line PT67 was transfected with either the SDF-1 containing constructs or pLNCX2 vector alone using Fugene-6-reagent (Boehringer Mannheim, Germany), then selected with 800 µg/ml of G418 (Sigma, Castle Hill, NSW). Harvested supernatant containing infectious particles from stable PT67 lines was used to transduce cultured BMSSC in the presence of 5 µg/ml polybrene (Sigma). Stable multi-colony derived BMSSC expressing high levels of SDF-1α and control cell lines were established following selection with 800 µg/ml of G418. Secreted SDF-1α concentrations were measured from supernatant filtered through a 0.2 µm filter using a standard SDF-1 ELISA kit according to the manufacturer’s specifications (R&D systems, USA).

Construction of a BMSSC cDNA subtraction hybridization library. In preliminary studies, STRO-1<sup>dull</sup> expressing marrow cells (Glycophorin-A<sup>+</sup> nucleated red cells) and STRO-1<sup>bright</sup> expressing cells (CFU-F population) were isolated by the MACS/FACS procedure as described above. Total RNA was prepared from STRO-1<sup>bright</sup> and STRO-1<sup>dull</sup> cells pooled from five different marrow samples (two males and three females aged 19-32) using the RNA STAT-60 system (TEL-TEST Inc.). First-strand synthesis was performed using the SMART cDNA synthesis kit (Clontech Laboratories Inc.). The resultant mRNA/single-stranded cDNA hybrid, was amplified by long-distance PCR (Clontech, Advantage 2 PCR kit) utilizing specific primer sites at the 3’ and 5’ prime ends formed during the initial RT process according to the manufacturer’s specifications. Following Rsa I digestion of the STRO-1<sup>bright</sup> cDNA, two aliquots were used to ligate different specific adaptor oligonucleotides using The Clontech PCR-Select cDNA subtraction kit. Two rounds of subtractive hybridization were performed using STRO-1<sup>bright</sup> (tester) and STRO-1<sup>dull</sup>
(driver) cDNA, and visa versa, according to the manufacturer's protocol. This procedure was also performed in reverse using STRO-1\textsuperscript{dull} tester cDNA hybridized against STRO-1\textsuperscript{bright} driver cDNA.

**Differential screening of BMSSC subtraction library.** To identify genes uniquely expressed by STRO-1\textsuperscript{bright} BMSSC population, STRO-1\textsuperscript{bright} subtracted cDNA was ligated into a T/A cloning vector (AdvaTAge PCR cloning kit, Clontech) then transformed into DH5\textalpha \textit{E. coli}. Two hundred randomly selected, ampicillin resistant bacterial clones, were amplified by PCR using the Clontech PCR-Select Differential Screening Kit according to the manufacturer's specifications. Briefly, the cDNA was used to construct replicate low-density microarray filters (zeta-probe GT membranes, BioRad) using a BRL Hybri-dot 96-well format manifold vacuum system as recommended by the manufacturer. Subtracted STRO-1\textsuperscript{bright} and subtracted STRO-1\textsuperscript{dull} cDNA were denatured at 95°C then labelled with 50 \mu Ci [\alpha-\textsuperscript{32}P] dCTP (3000 Ci/mmol ICN Radiochemicals) using Klenow enzyme (exo-; 5 units) for 40 minutes at 37°C. The DNA probes were hybridized to replicate filters overnight at 72°C, using Clontech Express Hyb. The filters were washed four times with 2X SSC/0.5% SDS and 2 times with 0.2X SSC/0.5% SDS at 68°C then screened using a PhosphoImager and analysed using ImageQant software (Molecular Dynamics Inc. Sunnyvale, CA).

**Differentiation of CFU-F in vitro.** We have previously reported the conditions for the induction of human BM stromal cells to develop a mineralized bone matrix in vitro cultured in \alpha\textsubscript{MEM} supplemented with 10% FCS, 100 \mu M L-ascorbate-2-phosphate, dexamethasone \textsuperscript{10}{-7} M and 3 mM inorganic phosphate \textsuperscript{42}. 

12
**Ectopic bone formation assay.** The adherent cells derived from STRO-1\(^{\text{bright}}\) sorted bone marrow mononuclear cells at passage 2-3 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 eight week old NOD/SCID mice as described previously \(^5\). These procedures were performed in accordance to specifications of an approved animal protocol (The University Adelaide AEC# M29/2002). Implants were recovered after 8 weeks, fixed in 4% paraformaldehyde for 2 days, then decalcified for a further 10 days in 10% EDTA prior to embedding in paraffin. Each transplant was cut into two pieces, then placed cut-surface down for paraffin embedding. For histological analysis, 5 µm sections of the implants were prepared and stained with H&E representative of the middle and either end of each transplant approximately 3-4 mm in length. The amount of new bone formation was calculated as a percentage of the total surface area present in twelve tissue sections. Measurement of new bone formation was assessed using Scion Imaging Software as previously described \(^24\).

**Statistics**

The Student's t-test was used for pairwise comparisons as indicated. Statistical significance was given at \(p<0.05\). One-way ANOVA analysis was employed for multiple comparisons as indicated. Statistical significance between the groups was determined using the Fisher's projected least significance difference test at \(p<0.05\).
Results

Purified Human BMSSC Express High Levels of SDF-1

Subtractive hybridization has previously been used to increase the frequency of differentially expressed genes in rare cell populations \(^{43,44}\). In the present study, STRO-1\(^{\text{dull}}\) (Glycophorin-A\(^{+}\) nucleated red cells) and the minor fraction of STRO-1\(^{\text{bright}}\) expressing marrow cells (which includes all colony-forming BMSSC) were isolated using a combined MACS/FACS procedure as previously described \(^5\) (Figure 1A). For each sorted STRO-1 population, total RNA was prepared from five individual bone marrow donors and pooled. Following first-strand synthesis, STRO-1\(^{\text{bright}}\) and STRO-1\(^{\text{dull}}\) cDNA was subjected to a series of subtractive hybridization steps as described in the Methods. To identify genes uniquely expressed by STRO-1\(^{\text{bright}}\) BMSSC population, STRO-1\(^{\text{bright}}\) subtracted cDNA was used to construct replicate low-density microarray filters comprised of two hundred randomly selected bacterial clones transformed with the STRO-1\(^{\text{bright}}\) subtracted cDNAs ligated into a T/A cloning vector. The microarrays were subsequently probed with either \([^{32}\text{P}]\) dCTP labelled STRO-1\(^{\text{bright}}\) or STRO-1\(^{\text{dull}}\) subtracted cDNA (Figure 1B, C). Differential screening identified a total of forty-four clones which were highly differentially expressed between the STRO-1\(^{\text{dull}}\) and STRO-1\(^{\text{bright}}\) subpopulations. DNA sequencing of all the differentially expressed clones revealed that only one clone was representative of a known stromal cell mitogen, namely platelet derived growth factor (PDGF) \(^{39}\). Interestingly, six out of the forty-four clones were found to contain DNA inserts corresponding to the chemokine, stromal-derived factor-1 (SDF-1). The high abundance of SDF-1 transcripts in human BMSSC was confirmed by semi-quantitative RT-PCR of total RNA prepared from freshly sorted STRO-1\(^{\text{bright}}\), STRO-1\(^{\text{dull}}\) and STRO-1\(^{\text{negative}}\) bone marrow sub-populations (Figure 1D).
SDF-1 is preferentially expressed by immature stromal populations in vitro

We next examined whether the expression of SDF-1 was correlated to the developmental stage of BMSSCs in vitro. SDF-1 expression levels were assessed in different stromal populations using an established in vitro model of osteogenic differentiation, based on the cell surface expression of STRO-1 and alkaline phosphatase (AP) \(^{40,45}\). Dual-colour fluorescence activated cell sorting (FACS) was used to partition the different BMSSC STRO-1/AP subfractions according to the sorting regions (R1-R4) depicted in Figure 2A. Each STRO-1/AP subfraction was double sorted to obtain >99.9% purity. Semi-quantative RT-PCR examining SDF-1 expression was subsequently performed on total RNA isolated from each STRO-1/AP sorted population. The analysis revealed that the most immature stromal population STRO-1\(^+\)/AP\(^-\) (osteoprogenitors) followed by STRO-1\(^+\)/AP\(^+\) (pre-osteoblasts) expressed higher levels of SDF-1 in comparison to the most mature cell populations, STRO-1\(^-\)/AP\(^+\) (osteoblasts) and STRO-1\(^-\)/AP\(^-\) (osteocytes, bone lining cells) when normalized to the house keeping gene GAPDH (Figure 2B).

In parallel experiments, secondary cultures of BMSSCs, supplemented with osteogenic inductive media (supplemented with L-ascorbate-2-phosphate, dexamethasone and inorganic phosphate), demonstrated a decrease in SDF-1 expression in a time dependent manner (Figure 3A). The data revealed that lower levels of SDF-1 expression were correlated with a higher proportion of pre-osteoblast-like cells (STRO-1\(^+\)/AP\(^+\)), following 48hrs stimulation with osteogenic induction medium (Figure 3B).
**BMSSC express the SDF-1 receptor, CXCR4**

In order to determine whether SDF-1 could act as an autocrine factor, preliminary experiments using RT-PCR analysis confirmed that BMSSC did in fact express the SDF-1 receptor, CXCR4, (Figure 4A). Examination of CXCR4 expression by normal cultured BMSSC and the human osteosarcoma cell line, MG63, revealed varying expression of the expected 568 base pair PCR product and a second, larger band. DNA sequence analysis confirmed the lower band as corresponding to the normal human CXCR4 isoform, while the larger band corresponded to a previously reported alternative splice variation 46. BMSSC were also shown to constitutively express low levels of CXCR4 protein at the cell surface as shown by flow cytometric analysis (Figure 4B). Calcium mobilization studies were carried out to determine whether CXCR4 expressed by BMSSC were functionally active. FURA-2 loaded BMSSC were challenged with 30 ng/ml of recombinant human SDF-1α (rhSDF-1α), resulting in a rapid and robust increase in intracellular calcium levels characteristic of SDF-1/CXCR4 signaling (Figure 4C).

**Overexpression of SDF-1 enhances the potential of BMSSC to form ectopic bone in vivo**

To determine if SDF-1 had any functional role in stromal cell development, retroviral expression constructs containing the full-length human SDF-1 cDNA, were used to transfect the packaging cell line PT67 as described in the Methods. Harvested supernatant containing infectious particles were then used to generate stable, multi-colony derived BMSSC cell lines expressing high levels of SDF-1α and corresponding control cell lines transduced with empty pLNCX2 vector (Figure 5A).
Cell lines derived from three individual bone marrow aspirates were implanted into immunocompromised mice in combination with hydroxyapatite/tricalcium phosphate particles, as described in the Methods. Scion Imaging analysis of histological sections from the harvested implants showed significantly greater levels (p< 0.05; t-test) of ectopic bone formation per area in those transplants containing high SDF-1 expressing BMSSC lines in comparison to the vector controls (Figure 5C).

Parallel studies were performed to identify potential mechanisms of the observed SDF-1 mediated enhanced bone formation capacity. Surprisingly, we failed to detect any statistical difference in the capacity of high SDF-1 expressing BMSC to form mineralized deposits of hydroxyapatite in vitro above the vector control cell lines (data not shown). Furthermore, we failed to detect any consistent differences in the expression of various bone-associated genes (BMP-2, BMP-4, CBFA-1, osterix, osteocalcin, AP) between the high SDF-1 expressing and matched vector control BMSSC lines (data not shown). Collectively, these data suggested that SDF-1 imposed an indirect effect on bone formation in vivo.

**SDF-1 Mediates BMSSC Growth and Survival**

We next examined the possibility that over-expression of SDF-1 may provide a growth or survival advantage to the transduced BMSSC. This notion was supported by proliferation studies demonstrating that high SDF-1 expressing BMSSC displayed a moderate but not significant increase in their growth capacity above that of the BMSSC vector control cell lines (Figure 6A). Furthermore, BMSSC lines over-expressing SDF-1 also exhibited a greater resistance to the apoptosis-inducing effects of the inflammatory cytokine, IL-4, previously shown to inhibit the
growth of BMSSC in vitro \(^{39}\), as assessed by the trypan blue uptake method (Figure 6B). In accord with these findings, living cultures of over-expressing SDF-1 BMSSC, also showed decreased cell surface staining of the early apoptosis marker, Annexin V, when challenged with IL-4 (Figure 6C, D).

Comparative experiments were subsequently performed to determine the effects of exogenous SDF-1 on the growth of normal BMSSC. Purified STRO-1 positive bone marrow cells were cultured under serum-deprived conditions, previously shown to enhance the formation of the earliest identifiable mesenchymal precursor cells, (CFU-F: colony forming unit-fibroblastic), in the presence of PDGF-BB to levels comparable to serum-replete cultures \(^{5,39}\). Whilst, exogenous rhSDF-1\(\alpha\) showed no inherit ability to stimulate colony production alone, an increase in CFU-F number was observed in combination with PDGF-BB (Figure 7). Moreover, addition of the known potent CFU-F inhibitor, alpha-interferon 2a \(^{39,47}\) demonstrated a typical decline in PDGF-BB induced colony formation, which was shown to be partially reversible in the presence of SDF-1. The observed response in the presence of SDF-1 was found to be optimal at 30 ng/ml over a concentration range 0.1 to 100 ng/ml (data not shown). Collectively, these data suggest that SDF-1 may play a role in promoting the self-renewal and survival capacity of BMSSC.
Discussion

The present study demonstrates for the first time that the earliest detectable BMSSC isolated directly from human bone marrow aspirates express high levels of SDF-1 prior to culture. We have previously reported that multipotential BMSSC are localised within the bone marrow microenvironment amongst the perivascular cells of large blood vessels 19. These observations correspond with the published distribution pattern of SDF-1 in human bone marrow, where the highest levels of SDF-1 are expressed by cells that surround blood vessels including, periarterial regions and blood capillaries of the bone, and by some bone marrow stromal cells near the endosteam at sites of early myelopoiesis and B-lympocyte development 26,37,38. Importantly, mature osteogenic cell populations located at the bone surfaces, or osteocytes within the bone matrix, appear to lack SDF-1 expression in situ 26.

Previous work by Stewart et al. (1999) 45 and our laboratory 40 have shown that early pre-osteogenic cells exist in normal stromal cultures that express the mesenchymal stem cell marker, STRO-1, but lack the expression of the osteoblast-associated marker, alkaline phosphatase. Progression of these precursor cell populations towards a mature and functional osteoblastic phenotype correlates to the loss of STRO-1 expression and an acquisition of AP cell surface expression 40,45. Utilizing this in vitro model of osteogenic cellular differentiation we have demonstrated that cultured BMSSC cells, representative of committed osteogenic populations, displayed decreased levels of SDF-1 when compared with more immature STRO-1 BMSSC fractions. Moreover, there was a significant diminution of SDF-1 expression following treatment of BMSSC with osteogenic induction media, providing further evidence that high SDF-1 expression is linked with a more primitive, less committed stage of pre-osteogenic differentiation.
Collectively, our data suggests that SDF-1 may act to localize primitive uncommitted BMSSC populations within their perivascular niche until required to proliferate and differentiate in response to environmental cues that may act to disrupt SDF-1/CXCR4 interactions, as has been suggested for other precursor populations

There is currently a large body of evidence describing the functional consequences of the SDF-1/CXCR4 interactions for various cellular processes including migration, chemotaxis, bone resorption, inflammation and metastasis. Both SDF-1 and CXCR4 have an unusually wide tissue distribution and a high degree of homology (>90%) between different species in comparison to other chemokine/receptor family members, underscoring the importance of these two molecules during embryonic development and tissue homeostasis in postnatal organisms. This is best seen during development in studies of SDF-1 and CXCR4 deficient mice, where both phenotypes, result in fetal mortality due to multiple defects affecting haematopoietic, cardiac, brain, vascular and gut tissues. Whilst, SDF-1 seems to be critical in normal haematopoiesis, inflammation and the metastasis of various tumours, little is known about the role of SDF-1 on the growth or differentiation of BMSSC.

SDF-1 mediates its effects through its receptor, CXCR4, a transmembrane glycoprotein, belonging to the family of G protein-coupled molecules, where CXCR4 also acts as the main co-receptor for human immunodeficiency virus type-1. Interestingly, we observed two CXCR4 splice variants both in normal cultured BMSSC and the human osteosarcoma cell line, MG63. DNA sequence analysis confirmed the smaller splice variant to correspond to the normal human CXCR4 cDNA spanning exons 1 and 2, the abundant form found in normal BMSSC. In contrast, the larger
splice variant, found to be highly abundant in MG63 cells, corresponded to a previously described alternative splice variation, generated through the inclusion of transcribed DNA sequence from the intersecting intron, resulting in the addition of a further nine amino acids \(^{46}\). Tissue distribution studies demonstrated that the smaller transcript was the predominant CXCR4 isoform found in normal tissues, while the larger transcript was highly expressed in various leukaemic and carcinoma cell lines \(^{46}\). While both splice variants are active, the functional significance of the larger CXCR4 transcript has not yet been determined, but may relate to the importance of SDF-1/CXCR4 in development as a mechanism to compensate for any errors that may occur in CXCR4 splicing during embryonic development.

In the present study, we demonstrated that BMSSC constitutively expressed low cell surface levels of functional CXCR4 protein as shown by flow cytometric analysis and calcium mobilisation studies. These data are consistent with the known signaling pathways triggered following SDF-1/CXCR4 interactions \(^{57,58}\). The expression of functional CXCR4 receptors on mesenchymal stem cells has also recently been demonstrated in studies showing SDF-1 dependent induction of MSC migration both in vitro \(^{59}\) and in vivo \(^{60}\). In addition, SDF-1/CXCR4 induced MSC migration appears to occur via a G-protein-dependent manner \(^{60}\). Furthermore, SDF-1 binding to CXCR4 leads to increased tyrosine phosphorylation of the receptor, leading to CXCR4 dimerization and internalization. Down-stream targets of SDF-1/CXCR4 interactions are known to mediate various cellular processes such as chemotactic migration involving cytoskeletal rearrangement and integrin-mediated adhesion, previously found to facilitate BMSSC attachment to basement membrane and other extracellular matrix components \(^{61}\). Therefore, SDF-1/CXCR4 signaling may play a critical role in regulating BMSSC growth and migration.
In the present study we showed that the majority of ex vivo expanded BMSSC begin to undergo partial osteogenic differentiation, which correlated with a decrease in SDF-1 expression. This maturation appeared to enhance the susceptibility of BMSSC to factors that induce apoptosis. Our studies showed that BMSSC lines over-expressing SDF-1 exhibited increased protection against the apoptotic effects of interleukin-4 (IL-4), previously shown to inhibit the growth of BMSSC in vitro. Similar experiments, demonstrated that high-SDF-1 expressing BMSSC lines were more resistant to the induction of early apoptosis in the presence of IL-4. The ability of SDF-1 to maintain stem cell survival has previously been reported, where over-expression of CXCR4 resulted in greater numbers and engraftment potential of purified haematopoietic stem cell preparations, following infusion into NOD/SCID mice. By analogy, our data showed that high SDF-1 expressing BMSSC lines exhibited an increased capacity to form ectopic bone in vivo when transplanted into NOD/SCID mice, due to BMSSC maintenance or viability, rather than as a consequence of induction of osteogenic commitment and differentiation. Moreover, other studies have shown that increased SDF-1 production in the bone marrow occurs following DNA induced damage, resulting in an improvement of hematopoietic reconstitution as a one potential mechanism to stimulate bone marrow recovery. Evidence is also emerging that SDF-1 may promote the survival and migration of different progenitor pools to facilitate tissue repair in the pancreas, liver, brain, skeletal muscle and heart following injury and may therefore have a significant role to play in bone cell remodelling.

The present study confirmed the survival and growth advantage conveyed by SDF-1 on the earliest identifiable mesenchymal precursor cells, freshly isolated STRO-1 bright bone marrow cells that contain the CFU-F population. Whilst exogenous rhSDF-1α showed no inherit ability to
stimulate colony production alone, an increase in CFU-F number was observed when added in combination with PDGF-BB. The varied effect of SDF-1 on the growth rates between different BMSSC populations may be due to differences in the developmental stage of the freshly isolated primitive BMSSC verses more mature ex vivo expanded stromal cells. Previous studies have reported that SDF-1 can function in synergy with various growth factors to increase cell survival of haematopoietic precursor cells.\textsuperscript{68,69} For example, SDF-1 was shown to help protect HSC directly from the apoptotic effects of gamma-irradiation in combination with other growth factors such as stem cell factor, GM-CSF, FLT-3 ligand, thrombopoietin and interleukin-3.\textsuperscript{68} Similarly, we found that SDF-1 in combination with PDGF-BB appeared to counteract the detrimental effects of alpha-interferon-2a, a known potent inhibitor of CFU-F formation.\textsuperscript{39,47} Therefore, PDGF and SDF-1 may act in synergy in promoting the self-renewal and survival capacity of BMSSC. While beyond the scope of the present study, it is anticipated that gene expression profiling studies comparing SDF-1 high expressing BMSSC lines with their corresponding control cell lines, will help identify critical SDF-1 stimulated signaling pathways that mediate the growth and survival of BMSSC. These studies lay the foundations for understanding the role of SDF-1 in BMSSC maintenance and development, and may help development of future strategies for managing diseases that effect bone remodelling, such as osteoporosis. In addition, these studies may facilitate greater understanding of haematopoietic recovery following high dose chemo- and radio-therapy, to potentially improve the treatment of those cancer patients receiving bone marrow/peripheral blood stem cell transplants.

Acknowledgments
We wish to acknowledge the generous support of Dr. Pamela Gerhon Robey, (National Institute of Dental & Craniofacial Research, NIH, Bethesda MD) for the construction of the cDNA subtraction library.
Figure Legends

**Figure 1. BMSSC express high levels of SDF-1.** (A) MACS isolated preparations of STRO-1\(^{+}\) BMMNC were partitioned into different STRO-1 subsets according to the regions, STRO-1\(^{\text{bright}}\) and STRO-1\(^{\text{dull}}\) using FACS. Total RNA was prepared from each STRO-1 subpopulation and used to construct a STRO-1\(^{\text{bright}}\) subtraction hybridization library as described in the Methods. (B) Replicate nitrocellulose filters, which have been blotted with representative PCR products amplified from bacterial clones transformed with STRO-1\(^{\text{bright}}\) subtracted cDNA. The filters were then probed with either \(^{32}\text{P}\) dCTP labeled STRO-1\(^{\text{bright}}\) or STRO-1\(^{\text{low}}\) subtracted cDNA. The arrows indicate differential expression of one clone containing a cDNA fragment corresponding to human SDF-1. (C) RT-PCR analysis demonstrating the relative expression of SDF-1 and GAPDH transcripts in total RNA prepared from freshly MACS/FACS isolated BMMNC STRO-1 populations prior to culture.

**Figure 2. Immature BMSSC express higher levels of SDF-1 than more mature populations.** (A) The dot plot represents dual flow cytometric analysis of single cell suspensions of ex vivo expanded BMSSC, grown under standard culture condition media, based on the cell surface expression of STRO-1 and alkaline phosphatase (AP) antigens. Total RNA was prepared from unsorted and the different sorted STRO-1/AP subpopulations isolated by FACS based on the sorting regions R1, R2, R3 and R4. (B) The graph depicts semi-quantitative RT-PCR analysis of the relative expression of SDF-1 in respect to GAPDH expression in the different FACS isolated cultured BMSSC populations (R1 to R4) based on their cell surface expression of STRO-1 and
AP. The most immature osteogenic precursor population (R1: STRO-1^+/AP^-) expressed higher levels than pre-osteoblasts (R2: STRO-1^+/AP^+), followed by more mature osteoblast (R4: STRO-1^+/AP^+) and osteocyte (R3: STRO-1^-/AP^-) populations. The data represent the mean values ± standard errors of two independent experiments using secondary BMSSC cultures established from two normal different bone marrow donors.

Figure 3. SDF-1 is down-regulated by BMSSC following osteoinduction. (A) Semi-quantitative RT-PCR of SDF-1 expression based on the relative GAPDH expression by cultured BMSSC over time, in the presence of osteoinductive media. The mean values ± standard errors represent four independent experiments. Osteoinductive conditions verses the corresponding controls were analysed using paired t-test at each time point with a significance value (*) of p<0.05. (B) The dot plot represents dual flow cytometric analysis of single cell suspensions of ex vivo expanded BMSSC, cultured for 48 hours in the presence of osteogenic induction media, based on the cell surface expression of STRO-1 and alkaline phosphatase antigens.

Figure 4. BMSSC express functional SDF-1 receptors. (A) RT-PCR analysis demonstrating the relative expression of CXCR4 and GAPDH transcripts in total RNA isolated from either primary BMSSC cultures or the human osteosarcoma cell line, MG63. (B) Single cell suspensions of cultured BMSSC were incubated with either a mouse anti-human CXCR4 antibody or the isotype-matched control antibody, 1A6.11 followed by a goat anti-mouse IgG1 FITC conjugated antibody. A representative histogram depicts the level of cell surface expression of CXCR4 (solid line) by BMSSC relative to the control samples (dotted line) as assessed by flow cytometric analysis. (C)
The graph demonstrates the levels of intracellular calcium measured in primary BMSSC cultures over-time following the addition of human recombinant SDF-1α (30 ng/ml).

**Figure 5. Enforced SDF-1 expression by BMSSC enhances their osteogenic potential.** (A) The retroviral packaging line PT67 was used to transduce secondary BMSSC cultures, derived from three different bone marrow aspirates, with a pLNCX2 construct containing either human SDF-1 cDNA or vector alone. Stable multi-colony derived high SDF-1 expressing BMSSC and corresponding control lines were generated after G418 selection. Triplicate samples of tissue culture supernatant were assessed for SDF-1α levels using a commercially available ELISA kit. The data represents the mean values ± standard errors generated from three different high SDF-1 expressing BMSSC cell lines versus the corresponding controls. (B) Single cell suspensions of each of the transduced BMSSC lines were mixed with hydroxyapatite (HA/TCP) particles and then implanted subcutaneously into NOD/SCID mice. The images represent cross sections of eight week old harvested transplants of new bone (b) formed by high SDF-1 expressing BMSSC (SDF-1) and control cell lines (LNCX2) stained with Hematoxylin & Eosin (200X). (C) Each graph represents a different high SDF-1 expressing BMSSC line and corresponding control cell line derived from three different bone marrow donors. The level of new bone formation is expressed as a percentage of the total tissue surface area analysed from twelve representative tissue sections, using Scion Imaging software. The data represent the mean values ± standard errors from duplicate transplants. Statistical differences (*) of p<0.05 between the SDF-1 high expressing BMSSC lines and corresponding controls was determined using the unpaired t-test.
**Figure 6. Enforced expression of SDF-1 by BMSSC mediates cell survival.** (A) Proliferation studies were performed by plating high SDF-1 expressing BMSSC and vector control cell lines in triplicate wells at a density of $5 \times 10^3$ cells per well in 96-well plates in regular growth medium for five days. Single cell suspensions were then prepared by trypsin/EDTA digest and counted to assess the total number of cells. (B) Parallel cultures were established in the presence of IL-4 (30 ng/ml) and the percentage of apoptotic cells was measured using trypan blue exclusion. (C) The histogram represents the level of cell surface Annexin V staining (solid line) by control cells lines compared with the isotype-matched control antibody (dotted line) cultured in the presence of IL-4. A representative image is shown of the intensity of fluorescence staining on living cells in situ (100X). (D) The histogram represents the level of cell surface Annexin V staining (solid line) by high SDF-1 expressing BMSSC lines compared with the isotype-matched control (dotted line) cultured in the presence of IL-4. A representative image is shown of the intensity of fluorescence staining on living cells in situ (100X). The data represent the mean values ± standard errors of triplicate experiments. Statistical differences (*) of p<0.05 between the SDF-1 high expressing BMSSC lines and corresponding controls was determined using the unpaired t-test.

**Figure 7. SDF-1 promotes the growth and survival of CFU-F.** The total number of CFU-F colonies derived from MACS/FACS isolated STRO-1bright BMMNC plated in serum-free media in the presence of different cytokine combinations were enumerated. Recombinant human PDGF-BB, SDF-1α, and alpha-interferon 2a were used at the optimal concentrations 5 ng/ml, 30,000 i.u./ml and 30 ng/ml respectively. The data represent the mean values ± standard errors of triplicate wells. Similar results were obtained using three different bone marrow aspirates. Statistical significance (p<0.01) was determined using one-way ANOVA for all treatments. The
Fisher's test was then used to determine the differences between all groups. Significant differences (p<0.05) were found between all treatments compared to PDGF-BB alone (*), and PDGF+IFN versus PDGF+SDF-1+IFN (**) at a significance of p<0.05.
References


60. Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells. 2004;22:415-427


1alpha/CXCR4 ligand-receptor axis is critical for progenitor survival and migration in the pancreas. J Cell Biol. 2003;163:859-869


Figure 1

A

STRO-1 Expression (FITC)

STRO-1Bright

STRO-1Dull

Relative Cell Number

20

40

60

80

10^0

10^1

10^2

10^3

10^4

B

STRO-1Bright

STRO-1Dull

C

D

bp

STRO-1Negative

STRO-1Dull

STRO-1Bright

SDF-1 (364)

GAPDH (417)
Figure 2

![Graph showing SDF-1/GAPDH ratio for different cell subsets.](image)

- **Presort**
- **STRO-1+/AP-**
- **STRO-1+/AP+**
- **STRO-1-/AP+**
- **STRO-1-/AP-**

The graph displays the % Unsorted Cells for each subset with bars representing SDF-1/GAPDH ratio.
Figure 3

A

Mean SDF-1 Expression (% of Control)

Time Point

4hr 24hr 48hr

B

STRO-1 (PE)

Alkaline Phosphatase (FITC)
Figure 4

A

bp

MG63

BMSSC

CXCR4 (568) ➔

GAPDH (417) ➔

B

Relative Cell Number

80

60

40

20

0

100 10^{1} 10^{2} 10^{3} 10^{4}

CXCR4 Expression (FITC)

C

\([\text{Ca}^{++}]_{i}\) (nM)

230

220

210

200

190

180

170

160

150

140

130

0 10 20 30 40 50 60 70 80 90 100

Time (seconds)
Figure 5

A. SDF-1α Levels (pg/mL)

- Media
- LNCX2
- SDF-1

B. SDF-1

- HA/TCP
- LNCX2

C. New bone formation (% total surface area)

- SDF-1
- LNCX2
Figure 6

A

Total Cell Number $\times 10^3$

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B

Trypan Blue Uptake (% Total Cells)

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* indicates a significant difference.

C

Relative Cell Number

Annexin V-FITC

30.8%

D

Relative Cell Number

Annexin V-FITC

12.5%
Figure 7

![Bar graph showing the number of CFU-F/10^5 Cells Plated for different treatments: PDGF, PDGF + IFN, PDGF + SDF-1, PDGF + IFN + SDF-1, SDF-1, and IFN. The graph illustrates statistical significance with stars (*) and double stars (**) denoting p-values.]
Stromal derived factor-1 promotes the growth, survival and development of human bone marrow stromal stem cells

Angela Kortesidis, Andrew Zannettino, Sandra Isenmann, Songtao Shi, Tsvee Lapidot and Stan Gronthos