

Stem cell procurement

Side population/ABCG2-positive cells represent a heterogeneous group of haemopoietic cells: implications for the use of adult stem cells in transplantation and plasticity protocols

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Summary:

Murine side population (SP) cells may have an increased ability to engraft lethally irradiated mice and lack CD34 expression. Strategies using CD34 as a primary marker of haemopoietic stem cells may therefore result in the exclusion of a primitive stem cell population. The molecular basis for the murine SP phenotype has been attributed to the multidrug-resistance transporter ABCG2. This study aimed to investigate ABCG2 expression from a variety of human sources and investigate the relationship between ABCG2 expression, the SP phenotype, and expression of markers such as CD34 and CD133. SP cells were observed in different haemopoietic sources, but a significant increase in the number of SP cells was observed in PB following granulocyte colony-stimulating factor mobilisation. No direct correlation between the frequency of SP cells and the expression of ABCG2 was observed. SP cells were identified in both lineage-positive and lineage-negative population and ABCG2 expression was enriched in lineage-negative SP cells. Lineage-negative SP cells were devoid of CD34 expression but enriched for CD133. Subsequent analysis revealed that ABCG2 and CD133 are coexpressed. Together, these data suggest that the ABCG2 transporter is neither required nor responsible for the SP phenotype in many human blood cells.

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The *in vivo* definition of human haemopoietic stem cells (HSCs) has hitherto been characterised by their ability to support haemopoiesis in humans receiving myeloablative

chemotherapy, or the ability to reconstitute human haemopoiesis in immunodeficient mice. Although cell surface expression of the CD34 antigen has been used traditionally to characterise HSCs,¹ it has recently been shown that the expression of CD34 in lineage-negative cells may not precisely define the 'stem cell compartment',² and CD34-negative cells may contribute to reconstitution of human haemopoiesis in NOD/SCID mice.³ The observation that cells of haemopoietic origin may contribute to other tissues (plasticity) further challenges the traditional perception of HSCs.^{4,5} Goodell *et al*⁶ have defined a population of cells termed side population (SP) cells characterised by their ability to efflux the fluorescent dye Hoechst 33342. Bone marrow-derived SP cells not only demonstrate potent haemopoietic engraftment in mice but also have the potential for plasticity.^{4,7,8} SP cells have since been identified in umbilical cord,⁹ adult bone marrow^{10,11} and adult human peripheral blood.¹² In contrast to observations in murine bone marrow, SP cells from human peripheral blood do not demonstrate a capacity for *in vitro* culture or engraftment in NOD/SCID mice. Although it could be considered that this may be, at least in part, attributable to the fact that the vast majority of SP cells in adult human peripheral blood are lineage-committed,¹³ it is interesting to note that lineage-negative SP cells from adult human peripheral blood also fail to support human haemopoiesis in NOD/SCID mice.

The molecular mechanism defining the SP phenotype has recently been attributed to the ATP-binding cassette (ABC) transporter ABCG2.^{13,14} High levels of ABCG2 mRNA were observed in SP cells derived from murine bone marrow, and enforced expression of ABCG2 in a bone-derived epithelial cell line conferred an SP phenotype. ABCG2 expression was also found to be sharply down-regulation upon lineage commitment. It has therefore been postulated that ABCG2 could be used as an alternative marker to CD34 for identification of HSCs. However, the relationship between ABCG2 expression and expression of other stem cell markers, including CD34 and CD133, remains to be defined. We therefore aimed to quantify ABCG2 expression in SP and mononuclear cell (MNC) populations from a variety of haematological sources and elucidate the relationship between ABCG2, CD34 and CD133 expression in an

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attempt to evaluate the role and potential clinical utility of ABCG2 as an HSC marker.

Methods and materials

Isolation of human cells

Human cord blood (CB) was obtained from normal, full-term deliveries ($n=6$). Peripheral blood stem cells (PBSC) samples were obtained from peripheral blood of patients following treatment with granulocyte colony-stimulating factor (G-CSF) and chemotherapy. PBSC were obtained from 22 patients with haematological malignancies in remission or plateau phase, including myeloma ($n=8$), lymphoma ($n=8$) and leukaemia ($n=4$). Mobilised peripheral blood samples were obtained from normal, healthy donor volunteers following administration of G-CSF ($n=8$). Additionally, nonmobilised samples were obtained from normal healthy donors in the absence of prior G-CSF treatment ($n=10$). Samples were processed within 24 h of collection. The study was conducted with approval from the Local Research Ethics Committee and all patients and healthy donor volunteers provided informed written consent.

Samples were centrifuged over Ficoll-Hypaque gradients (1.077 g/ml) (LymphoCyten Separation Medium, PAA Laboratories GmbH, Austria) for 25 min at 400 *g*. MNCs were recovered from the interface, washed twice in phosphate-buffered saline (PBS) and resuspended in ice-cold PBS containing 5 mM EDTA and 0.5% (w/v) bovine serum albumin (BSA).

Cell staining and flow cytometry

To analyse coexpression of ABCG2 with other cell surface markers, 10^6 MNCs were used for each staining procedure. Cells were resuspended in 100 μ l of PBS (supplemented with 5 mM EDTA and 0.5% BSA), blocked with 1 μ g of human IgG (Sandoglobulin, Sandoz, Austria), and then incubated for 15 min at room temperature. Conjugated antibody (10 μ l) was added to the cell suspension and incubated at 4°C for 30 min. The cells were then washed twice in 4 ml of PBS/EDTA/BSA and the pellets were resuspended in 300 μ l of PBS for final flow cytometric analysis. The following monoclonal antibodies were used to phenotype the cell populations: anti-human Bcrp1/ABCG2-PE (R&D System Europe, UK), CD3-FITC, CD34-FITC, CD56-FITC, CD45RA-FITC, CD45RO-FITC and CD71-FITC (BD Biosciences Pharmingen, UK), glycophorin A FITC and CD19-FITC (DAKO Ltd, Denmark) and CD133 APC (Miltenyi Biotec, UK). Additional aliquots of the cells were stained with the appropriate control antibodies: mouse IgG2b-PE (R&D System Europe), IgG1-FITC, IgG2a-FITC, IgG1-APC and IgG2b-FITC (BD Biosciences Pharmingen).

Analysis of the labelled cells was performed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). A range of $1-2 \times 10^5$ events were analysed for each test sample to ensure a sufficient number of positive staining cells. Data acquisition was performed using Winmdi 2.8 software (Scripps Research Institute, CA, USA). A gate was set around the whole population and

residual erythrocytes and dead cells were gated out using forward and side scatter profile.

Hoechst staining and SP cell analysis

The frequency of SP cells in peripheral blood was assessed in normal healthy donors ($n=10$), umbilical cord blood (UCB; $n=6$) and stem cell harvests from normal donors ($n=5$) and patients with haematological malignancies ($n=9$) following pretreatment with G-CSF. For SP analysis, MNCs were resuspended at a density of 10^6 cells/ml in RPMI supplemented with 10% fetal calf serum (FCS; Labtech International, UK). The cells were then incubated with Hoechst-33342 (Sigma, UK) at a final concentration of 5 μ g/ml for 90 min at 37°C. Cell suspensions were gently agitated every 30 min. Where necessary, the inhibitor Resperpine (Sigma) was added at 50 μ M, in order to demonstrate inhibition of the Hoechst-33342 efflux. After the incubation period, cells were washed twice in ice-cold Hoechst Buffer (Hanks Balanced Saline Solution (Gibco) supplemented with 2% FCS and 10 mM HEPES buffer (Gibco). After Hoechst staining, the cell preparations were centrifuged and the cells were resuspended in Hoechst buffer (10^6 cells/100 μ l) for antibody staining. Antibody staining was performed as described above, using anti-human Bcrp1/ABCG2- PE (R&D System Europe), CD3-PE, CD34-PE CD56-PE, CD19-PE, CD45RA-PE, CD45RO-PE and CD14-PE (BD Biosciences Pharmingen), glycophorin A-PE (DAKO, Denmark) and CD133-PE (Miltenyi Biotec), or appropriate control antibodies (mouse IgG2b-PE [R&D System Europe] IgG1-PE, IgG2a-PE (BD Biosciences Pharmingen, UK). Cells were maintained in Hoechst buffer at 4°C until analysed. A Vantage II flow cytometer was used for Hoechst-stained cell sorting. Red- and blue-Hoechst-stained cells were collected using 675/20 and 424/44 nm band pass filters, respectively. The fluorescence was detected using a 640 nm long pass dichroic beam.

Lineage depletion

Lineage-positive (Lin+) and lineage-negative (Lin-) cells were isolated using a commercially available kit (StemCell Technologies Inc., Vancouver, BC, Canada), which includes a cocktail of the following lineage-specific antibodies: anti-human CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin A. 100 μ l/ml of antibody cocktail were added to MNCs (5×10^7 cells/ml) and incubated at room temperature for 15 min. Magnetic colloid was added (60 μ l/ml of cells), mixed well and incubated for a further 15 min at room temperature. Cells were then separated by gravity flow chromatography using a G-5 column attached to a StemSep magnet. The purity of isolated Lin+ and Lin- cells was assessed by incubation with CD34-PE monoclonal antibody and flow cytometry. CD34+ cells routinely represented >85% of the Lin- fraction, whereas the Lin+ fraction was devoid of CD34 expression.

Isolation and enrichment of ABCG2+ and CD133+ cells

To obtain ABCG2+/CD133- and ABCG2+/CD133+ cells, MNC from CB and normal donor PBSC were

blocked with human IgG and labelled with conjugated ABCG2-PE-conjugated antibody. After incubation, cells were washed with cold PBS/BSA buffer and stained with anti-PE magnetic Micro beads (Miltenyi Biotec) at 4°C for 10–15 min in the dark. Following incubation, cells were washed and passed through MiniMACS columns (Miltenyi Biotec). The enriched ABCG2+ cells were collected by flushing the column with cold buffer, then labelled with CD133/1 APC-conjugated antibody (Miltenyi Biotec) prior to FACS sorting the ABCG2+/CD133 and ABCG2+/CD133+ cell populations. The ABCG2-negative cells remaining after ABCG2 enrichment were washed and labelled with CD133 Micro beads (Miltenyi Biotec) and sorted, by flow cytometry, for CD133+. Briefly, after magnetic separation, cells were labelled with CD133/2 APC-conjugated antibody (Miltenyi Biotec) and ABCG2 PE-conjugated antibody (R&D System Europe) and sorted by FACS for the CD133+/ABCG2 population. A sorting gate was set around the lymphocyte population using forward and side scatter profile and any remaining erythrocytes and dead cells were excluded from the sort. Isotype-matched controls conjugated to APC and PE were used to set the sorting gates.

Clonogenic assays

Sorted ABCG2+/CD133, ABCG2+/CD133+ and ABCG2/CD133+ cells were plated in a mixture containing 1.35% (v/v) methylcellulose (Sigma), 30% (v/v) FCS, 1% (v/v) deionised BSA, 10% (v/v) 5637-conditioned medium from the EJ bladder carcinoma cell line and 2 U/ml erythropoietin (EPO; Cilag AG, Switzerland). Cultures were plated in triplicate and incubated in a humidified atmosphere of 5% CO₂ and 5% O₂ at 37°C for 14 days. GM-CFC and BFU-E were counted according to standard criteria.

Results

SP cells in normal peripheral blood are predominantly lineage-committed

SP cells represented $0.42\% \pm 0.15$ (mean \pm s.e.m.) of the MNC population of normal healthy individuals (Figure 1ai). Confirmation of the SP phenotype was obtained by incubating the cells with the antagonist Reserpine (Figure 1aii). SP cells in circulating blood were demonstrated to be lineage specific, demonstrating $59\% \pm 5.6$ CD3+, $14.7\% \pm 6.6$ CD56+, $12.7\% \pm 2.19$ CD19+, $13\% \pm 4.17$ CD14+ and $17.1\% \pm 7.19$ glycoprotein A+ (Figure 1b). We were unable to identify any Lin⁻ SP cells circulating in normal individuals. The proportion of cells committed to a specific lineage within the SP tail consistently paralleled the proportions in the total MNC population. To ascertain whether the lineage-committed cells found within the SP tail were of a more naïve, less mature subgroup compared to the total MNC population, we investigated lymphocyte subgroups. The expression of the two isoforms of CD45, CD45RA and CD45 RO was examined. No significant difference was found between the proportion

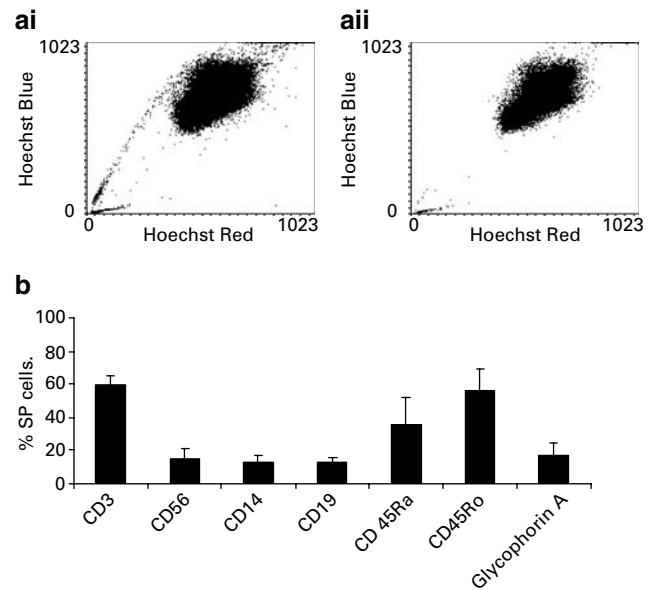


Figure 1 SP population observed in the peripheral blood of normal donors. Peripheral blood was obtained from normal healthy volunteers ($n = 10$). Mononuclear cells were separated by density gradient centrifugation and incubated with Hoechst 33342 to identify a circulating SP population (ai). Incubation with $50 \mu\text{M}$ Reserpine abrogated the SP tail (aii). Circulating SP cells were immunophenotyped by analysing for known cell surface markers (CD3, CD56, CD19, CD45RA and RO and Glycophorin A) using flow cytometry (b).

of CD45RA- and CD45RO-expressing cells in the SP population of normal peripheral blood ($47.2\% \pm 13.86$ and $35.71\% \pm 16.6$, respectively).

Frequency of SP cells in peripheral blood varies in response to G-CSF mobilisation

The frequency of SP cells found in peripheral blood from a variety of haematological sources was examined utilising the ability of cells to efflux Hoechst 33342. Sources examined included normal donors ($n = 10$), umbilical cord blood (UCB; $n = 6$) and PBSC harvests from G-CSF-treated normal donors ($n = 8$) and patients with haematological malignancies ($n = 12$). SP cells were observed at a lower frequency in normal peripheral blood ($6.7 \times 10^3/\text{ml} \pm 252$) and UCB ($1.96 \times 10^4/\text{ml} \pm 2421$) compared with post-G-CSF mobilisation samples from normal donors ($5.81 \times 10^5/\text{ml} \pm 3.5 \times 10^4$) and patients with malignancies ($1.37 \times 10^6/\text{ml} \pm 9.6 \times 10^4$) (Figure 2a). Lin⁺ cells were observed in the SP tail from UCB and G-CSF-mobilised normal donors and patients, confirming observations in normal peripheral blood (Figure 2b). The proportion of lineage cells was reduced in SP cells from UCB and G-CSF-mobilised peripheral blood compared with SP cells derived from 'nonmobilised' peripheral blood, suggesting that a subpopulation of Lin⁻ cells is present in the SP population derived from these sources. In accordance with these findings, a population of Lin⁻ SP cells was identified in both CB and mobilised peripheral blood (Figure 3).

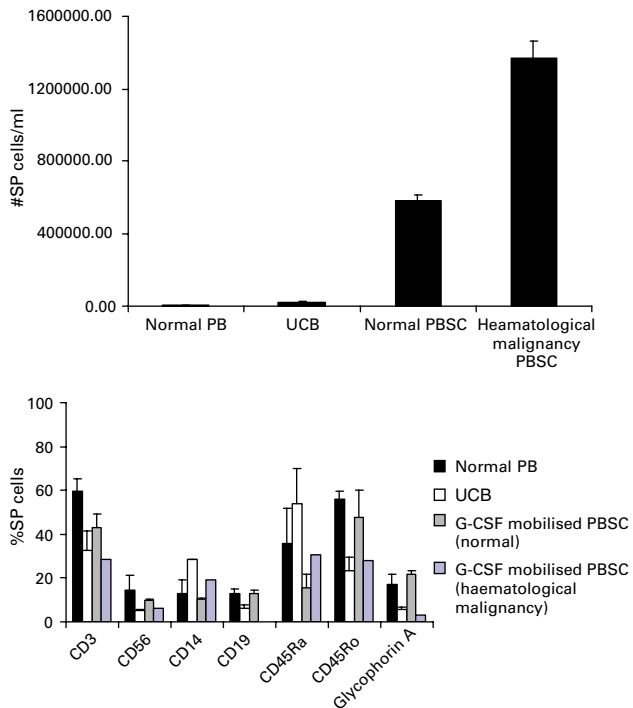


Figure 2 SP cells are found at a higher frequency in peripheral blood following G-CSF mobilisation in normal donors and in patients with haematological malignancies. SP cells were identified using Hoechst 33342 staining and flow cytometry in the peripheral blood of normal donors ($n=10$), umbilical cord blood ($n=6$) and from stem cell harvests from G-CSF-mobilised normal donors ($n=5$) and patients with haematological malignancies ($n=9$). The number of SP cells per ml was calculated as a proportion of the MNC population per ml of blood volume harvested (a). SP cells from UCB and G-CSF-mobilised donors and patients were further characterised by staining with a panel of PE-conjugated monoclonal antibodies including CD3, CD56, CD19, CD14, CD45RA, CD45RO and Glycophorin A (b).

ABCG2 is not an exclusive marker of peripheral blood-derived SP cells

Bcrp-1/ABCG2 has been defined as the molecular determinant of the SP phenotype in bone marrow and we therefore evaluated the expression of ABCG2 in peripheral blood-derived SP cells using flow cytometry. ABCG2 expression was observed in SP cells from all sources of peripheral blood examined. No significant enrichment for ABCG2 was observed in SP cells derived from normal donors ($4.62\% \pm 0.3$), UCB ($2.78\% \pm 1.7$) and mobilised PBSCs from patients with haematological malignancies ($1.78\% \pm 0.99$). However, a significant increase in the proportion of SP cells expressing ABCG2 was observed in stem cell harvests obtained from normal donors following G-CSF mobilisation; $18.8\% \pm 1.6$ of these SP cells demonstrated ABCG2 expression, representing a 10-fold increase compared with the frequency of ABCG2+ cells in the total MNC population.

Further characterisation of ABCG2+ cells was performed by analysing the coexpression of ABCG2 with a number of lineage-specific cell surface markers in the MNC population (Figure 4b). With the exception of the observed coexpression of ABCG2 and glycophorin A in $2.5\% \pm 1.5$

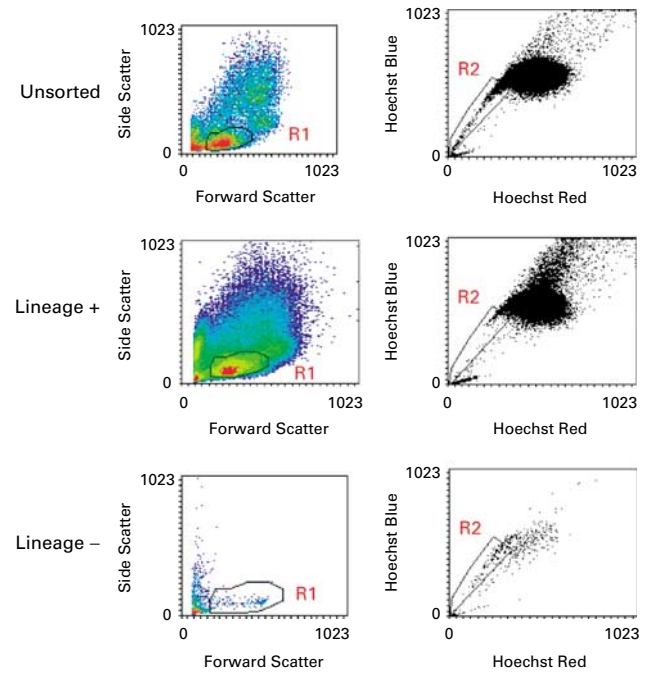


Figure 3 Identification of Lin+ and Lin- SP cells. MNCs from UCB or G-CSF-mobilised peripheral blood were subjected to lineage depletion. CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD45RO, CD56, CD66b and Glycophorin-A-positive cells were removed from the population and retained as the Lin+ fraction. The remaining cells were harvested as the Lin- fraction. The frequency of SP cells in the Lin+ and Lin- and total MNC populations was analysed using Hoechst 33342 efflux.

UCB CD71+ cells, ABCG2+ cells did not express lineage commitment markers. The difference in lineage commitment profile between peripheral blood SP and ABCG2 cells suggests that ABCG2 expression may not be an exclusive marker for Lin+ SP cells derived from peripheral blood and that alternative efflux pumps may define the SP phenotype.

ABCG2 expression is found in Lin- cells

In order to investigate this further, we examined the level of ABCG2 expression in Lin- cells and correlated this to the SP phenotype. MNCs derived from normal mobilised peripheral blood ($n=4$) were separated into Lin+ and Lin- fractions and the proportion of SP cells in the total MNC and Lin+ and Lin- fractions was assessed using Hoechst dye efflux (Figure 5a). SP cells represented $0.37\% \pm 0.06$ of total MNCs. The proportions of SP cells in the Lin+ and Lin- fractions were $0.19\% \pm 0.03$ and $6.33\% \pm 2.66$, respectively, representing a 17-fold increase in the Lin- population compared with the total MNC population. The proportion of SP cells expressing ABCG2, CD133 and CD34 was also assessed before and after lineage separation (Figure 5b). There was no expression of either CD34 or CD133 in Lin+ SP cells, but $0.5\% \pm 1.2$ Lin+ SP cells demonstrated detectable ABCG2 expression. Although no CD34 expression was detectable in Lin- SP cells, there was a striking increase in the proportions of

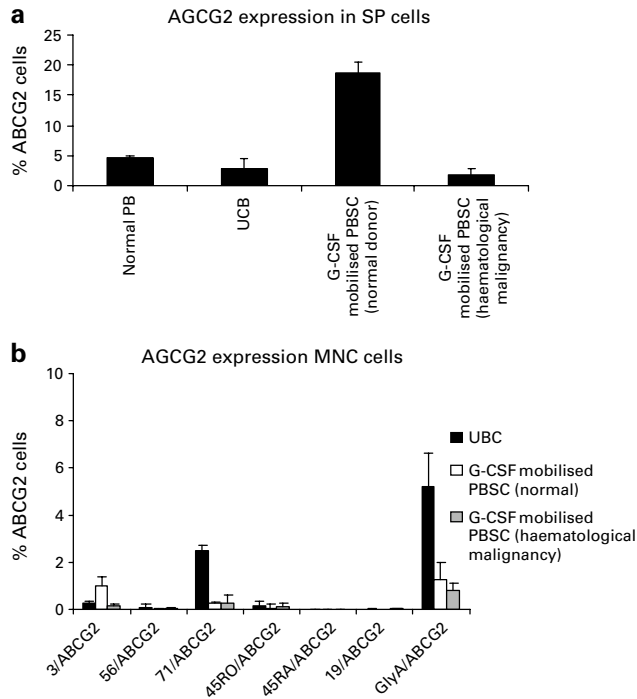


Figure 4 ABCG2 expression in SP and MNC populations. SP cells were identified using Hoechst 33342 staining and flow cytometry. The proportion of ABCG2-expressing cells was quantified in the SP population by costaining with a PE-conjugated mAb (a). The expression of ABCG2 in lineage-specific cells was assessed by analysing the coexpression of ABCG2 and lineage-specific markers (CD3, CD56, CD71, CD45RA, CD45RO, CD19 and glycophorin A) using dual-colour FACS scanning (b).

ABCG2+ and CD133+ (27.5% ± 3.6 and 38.46% ± 4.6) cells in the Lin- SP population compared with the total MNC population.

ABCG2 as a haemopoietic stem cell marker

The increase in the proportion of ABCG2- and CD133-positive cells in the Lin- SP cell population prompted us to examine whether ABCG2 and CD133 were expressed on the surface of the same cells. The coexpression of ABCG2 with CD34 and CD133 was analysed in the MNC populations of UCB and G-CSF-mobilised stem cell harvest (Figure 6). The proportion of ABCG2+/CD34+ and ABCG2+/CD133+ cells was compared to levels of CD34+/CD133+ cells, a known progenitor cell subpopulation. The frequencies of CD34+/CD133+ cells in the MNC populations from UCB, normal stem cell harvests and stem cell harvests from malignant donors were determined to be 0.46% ± 2.6, 0.74% ± 0.17 and 1.32% ± 0.53, respectively. Although ABCG2+/CD34+ cells were undetectable in all samples analysed, a small but distinct subgroup of ABCG2+/CD133+ cells was observed in the MNC populations of UCB (0.102% ± 0.06), PBSCs from normal mobilised donors (0.086% ± 0.03) and PBSCs from mobilised malignant donors (0.097% ± 0.07). Following enrichment for ABCG2+ cells, the coexpression of CD133 was clearly observed in a subgroup of ABCG2+ cells (Figure 6b, Region R2).

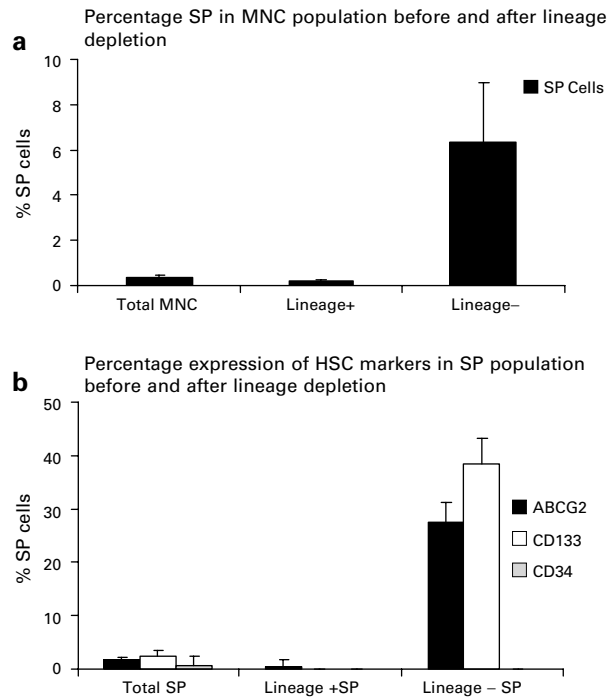


Figure 5 Frequency of SP cells increases following lineage depletion in UCB. MNCs from UCB were subjected to lineage depletion. The frequency of SP cells in the total MNC population and Lin+ and Lin- fractions was analysed using Hoechst 33342 efflux (a). The expression of ABCG2, CD133 and CD34 was analysed in the SP cells isolated from the total MNC population and Lin+ and Lin- fractions using Hoechst 33342 staining and PE-conjugated antibodies (b).

The clonogenic potential of isolated cell populations from G-CSF-mobilised normal donors ($n=5$) was determined using a CFU-mix assay to assess their ability to produce several distinct progenitor types. ABCG2-/CD133+ cells consistently produced both GM-CFC and BFU-E colonies (Table 1), although a degree of intersample variation was observed with respect to the number of colonies/200 cells plated. ABCG2+/CD133- cells were found to have little colony-forming potential, indeed only one out of the five samples plated produced detectable GM-CFC and BFU-E colonies. ABCG2+/CD133+ cells produced inconsistent results; three of the five samples produced both GM-CFC and BFU-E colonies within 1 week of plating, but the remaining two samples failed to produce any detectable colonies. Furthermore, we have thus far been unable to consistently support long-term culture of these cells on stromal layers. Overall, these findings suggest that isolated expression of ABCG2+ expression does not identify progenitor cells, but coexpression of ABCG2 and CD133 represents a novel subgroup of cells. Whether these cells represent a more primitive cell type than ABCG2-/CD133+ cells is yet to be definitively confirmed.

Discussion

The ability of a unique cell population to efflux the DNA-binding dye Hoechst 33324, so called SP cells, has recently

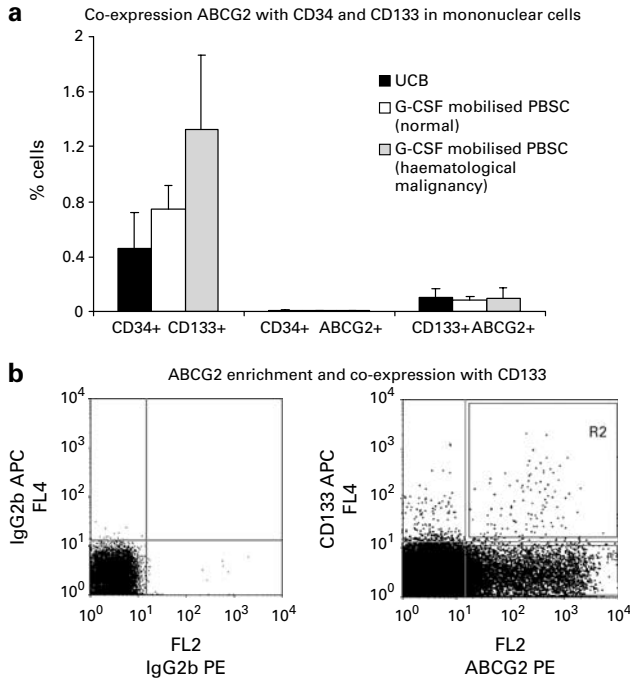


Figure 6 Coexpression of ABCG2 with CD34 and CD133. MNCs from UCB and G-CSF-mobilised PBSC (normal donors and donors with haematological malignancies) were analysed for the coexpression of ABCG2 with the HSC markers CD34 and CD133 using mAb conjugation and dual-colour FACS analysis (a). Cells were incubated with a PE-conjugated anti-ABCG2 or anti-CD34 mAb and then costained with an APC-conjugated anti-CD133 mAb. Where ABCG2 and CD34 were analysed together, a FITC-conjugated anti-CD34 antibody was utilised. ABCG2+/CD133+ cells were isolated following ABCG2 enrichment using magnetic microbead separation. Subsequent ABCG2-positive cells were costained with an anti-CD133 mAb and ABCG2+/CD133+ cells were harvested by cell sorting on a FACScan machine (Becton Dickinson). Sort gates were established using corresponding isotype control antibodies (b).

been the focus of attention in the quest to identify novel techniques to isolate primitive stem cells. Originally identified in murine bone marrow,⁶ SP analysis identified a population of cells capable of competitive repopulation in lethally irradiated mice. Cells isolated after dye efflux studies demonstrated over 1000-fold enrichment for cells capable of reconstitution, when compared to normal bone marrow cells. These SP cells were found to be CD34 negative, thus representing a novel stem cell population, which had previously been excluded from HSC studies and clinical protocols. Since then, SP cells have been identified in multiple species,¹⁵ and in multiple tissues including fetal

liver,⁸ breast, muscle, brain, spleen, kidney, heart, lung and small intestine.¹⁶ In these studies, SP cells isolated from fetal liver were demonstrated to be capable of full haemopoietic engraftment in NOD/SCID mice, and SP cells derived from all adult tissues studied were haemopoietic in origin and capable of differentiation into multiple haemopoietic lineages. SP cells identified in UCB were found to be CD34+ and CD34-. Many of the CD34- SP cells were mature lymphocytes and both CD34+ and CD34-, Lin- SP cells were incapable of complete *in vivo* engraftment and performed poorly in standard *in vitro* progenitor assays. Similarly, SP cells have been identified in human peripheral blood.¹² Again, these SP cells failed to demonstrate 'classic' *in vitro* and *in vivo* multipotent progenitor cell characteristics. In these studies, the authors alluded to a common lymphoid progenitor derived from SP analysis rather than a stem cell capable of multilineage differentiation.

In the current study, we have used FACS analysis to determine surface expression of a range of markers reporting haemopoietic progenitor cells. In the light of the low frequency of progenitor cells, a range of $1-2 \times 10^5$ events were analysed for each test sample. Our results are in accordance with previously published data demonstrating that SP cells derived from human peripheral blood are predominantly lineage-committed mature lymphocytes.¹² We too were unable to demonstrate multilineage differentiation of the SP cells, and contrary to early reports, we were unable to detect Lin-, SP cells from this source. These findings therefore suggest that an SP phenotype isolated by this technique does not automatically confer a progenitor cell status to the population. It implies that the ability of the SP cell to act as a multipotent stem cell would depend on the source from which the SP cell was derived.

We also examined the effect of G-CSF mobilisation on the frequency of SP cells found in the circulating blood. G-CSF treatment is used clinically to mobilise stem cells from their microenvironmental niche within the bone marrow to the circulating peripheral blood. If SP cells represent a unique progenitor population within the bone marrow, it would follow that these stem cells move into the periphery following mobilisation. We demonstrate that there is indeed a significant increase in the frequency of SP cells found in peripheral blood following mobilisation treatment. Unlike normal peripheral blood, we were able to identify Lin- SP cells in mobilised peripheral blood, suggesting that these cells with an SP phenotype may represent a novel circulating progenitor cell.

The molecular mechanism defining the SP phenotype has been attributed to the multidrug resistance transporter

Table 1 Clonogenic potential of cell populations defined by ABCG2 and CD133 expression

	Mean cell number	GM-CFC (%) (\pm s.e.m.)	BFU-E (%) (\pm s.e.m.)
ABCG2-/CD133+	200	56.4 \pm 23.8	13.6 \pm 5.2
ABCG2+/CD133+	200	28.8 \pm 15.2	25.2 \pm 15.3
ABCG2-/CD133-	200	0.58 \pm 0.5	0.16 \pm 0.2

In vitro progenitor cell assays were performed with cells harvested from UCB or normal G-CSF-mobilised peripheral blood stem cell harvests ($n=5$). ABCG2+ cells were enriched using magnetic bead selection. ABCG2+/CD133- and ABCG2+/CD133+ cells were subsequently FACS sorted and the clonogenic potential of these cell populations was determined using a CFU-mix assay.

ABCG2/bcrp-1.¹³ In a bcrp-1 knockout model, although the frequency of Lin⁻ SP cells decreased, residual SP cells were still identified and found to be nonrepopulating.¹⁷ The haemopoietic compartment in these mice was found to be more sensitive to chemotherapeutic drugs suggesting a role for ABCG2⁺ progenitor cells in chemoprotection. Interestingly, we observed a higher frequency of SP cells in patients who had received prior chemotherapy treatment for a variety of haematological malignancies. Our data show that patients who have received prior treatment with chemotherapeutic drugs have an amplified SP population that are potentially protected from the cytotoxic actions of the drugs used. These cells are mobilised into the periphery after G-CSF treatment and could represent a select population of cells that might have significant potential for clinical application.

The continued presence of SP cells in an ABCG2 knock out mouse implies that more than one drug efflux pump is responsible for the SP phenotype. We have utilised a commercially available anti-human ABCG2 antibody to assess the relationship between the SP phenotype and ABCG2 expression in human peripheral and UCB. We found no direction correlation between the frequency of SP cells and the number of ABCG2⁺ cells. This confirms the possibility that ABCG2 is not a unique marker for SP cells and that alternative efflux systems play a role in the SP phenotype. We have also demonstrated that ABCG2 expression is not exclusive to Lin⁻ cells, and as such ABCG2 expression alone should not be utilised as a marker for HSCs.

We have, however, demonstrated enrichment for ABCG2 expression in Lin⁻ SP cells derived from both UCB and mobilised peripheral blood. This enrichment for ABCG2 expression was found to be associated with a similar enrichment for CD133⁺ cells. CD34⁺ SP cells were not observed in this lineage-depleted population. These findings indicate that ABCG2⁺/CD133⁺ cells may represent a novel progenitor population. Indeed, we have been able to demonstrate that ABCG2 and CD133 are coexpressed on the surface of the same cell and that ABCG2⁺/CD133⁺ cells are capable of forming multilineage colonies when plated in *in vitro* progenitor cell assays. It is tempting to speculate that this population of cells is that described by Gallacher *et al.*¹⁸ These investigators report a rare subset of AC133⁺ CD34⁻ CD38⁻ Lin⁻ cells with substantial progenitor activity. Of note, some investigators have previously demonstrated high level gene expression ABCG2 in CD34⁺ cells.¹⁹ In accordance with these findings, our own preliminary data using mRNA expression suggest that the SP⁺ cells were positive for ABCG2, but FACs analysis demonstrated only very low or absent expression. This suggests that expression of CD34 in SP cells may be regulated, at least in part, by post-translational mechanisms, such as those described previously.²⁰

ABCG2⁺/CD133⁻ cells showed little potential for multilineage differentiation. Whether these ABCG2⁺/CD133⁺ cells represent a more primitive cell population than ABCG2⁻/CD133⁺, or indeed CD34⁺ cells, is yet to be determined. The better we become at identifying more primitive populations of progenitor cells, the more difficult

it becomes to characterise them using *in vitro* based assay systems. The true nature of a cell's ability to represent a stem cell can, ultimately, only be defined by its capacity to repopulate the entire haemopoietic system in conditioned recipients following transplantation. Further characterisation of cell hierarchy of cells is required in order to fully exploit the use of stem cells in the treatment of haematological malignancies and disorders.

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