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Role of the ABC transporter ABCG2 in human haematopoiesis

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**Aus der Medizinischen Klinik und Poliklinik III am Klinikum Großhadern
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Die funktionelle Bedeutung des ATP – bindenden Transportproteins ABCG2 für die Hämatopoese

**Dissertation
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This work is dedicated to my grandfathers,
late Dr. Fakhrul Hasan and late Hafiz Abdur-Rahman

1 Introduction

1.1 Haematopoiesis

Blood contains several different cell types that can be classified into two main classes, lymphoid cells (T, B, and natural killer cells) and myeloid cells (granulocytes, monocytes, erythrocytes, and megakaryocytes). Each of these has a unique set of specialized properties and, in most cases, important life-supporting functions. All blood cells have a limited lifespan: several hours for granulocytes, several weeks for erythrocytes, and up to several years for memory T-cells. Each day the body produces billions of new cells to replace blood cells lost to normal turnover processes as well as to illness or trauma. A variety of homeostatic mechanisms allow blood cell production to respond quickly to stresses such as bleeding or infection and then return to normal levels when the stress is resolved. The highly orchestrated process of blood cell production and homeostasis is termed *haematopoiesis* (fig 1.1).

1.1.1 Haematopoietic Stem Cells

All blood cells are produced from a small common pool of pluripotent cells called haematopoietic stem cells (HSC) and oligo-potent progenitors by differentiation (Morrison et al., 1995). The production of mature blood cells from such a pluripotent HSC involves a highly regulated progression through successive stages as commitment to a specific lineage, terminal differentiation of lineage restricted progenitor, growth arrest and apoptosis. The expression of different molecules on the surface of haematopoietic progenitors permits the interaction with various regulatory elements present in their environment, which includes stromal cells, extracellular matrix molecules and soluble regulatory cytokines as growth and differentiation factors. HSC are endowed with two characteristics: they give rise to additional HSC through self-renewal and also undergo differentiation to progenitor cells that become variously committed to different haematopoietic lineages (Weissman, 2000). These processes are under the tight control of distinct genetic programs with specific nuclear factors playing a key role. Operationally, HSC are best described as those cells capable of reconstituting the haematopoietic system of a recipient individual.

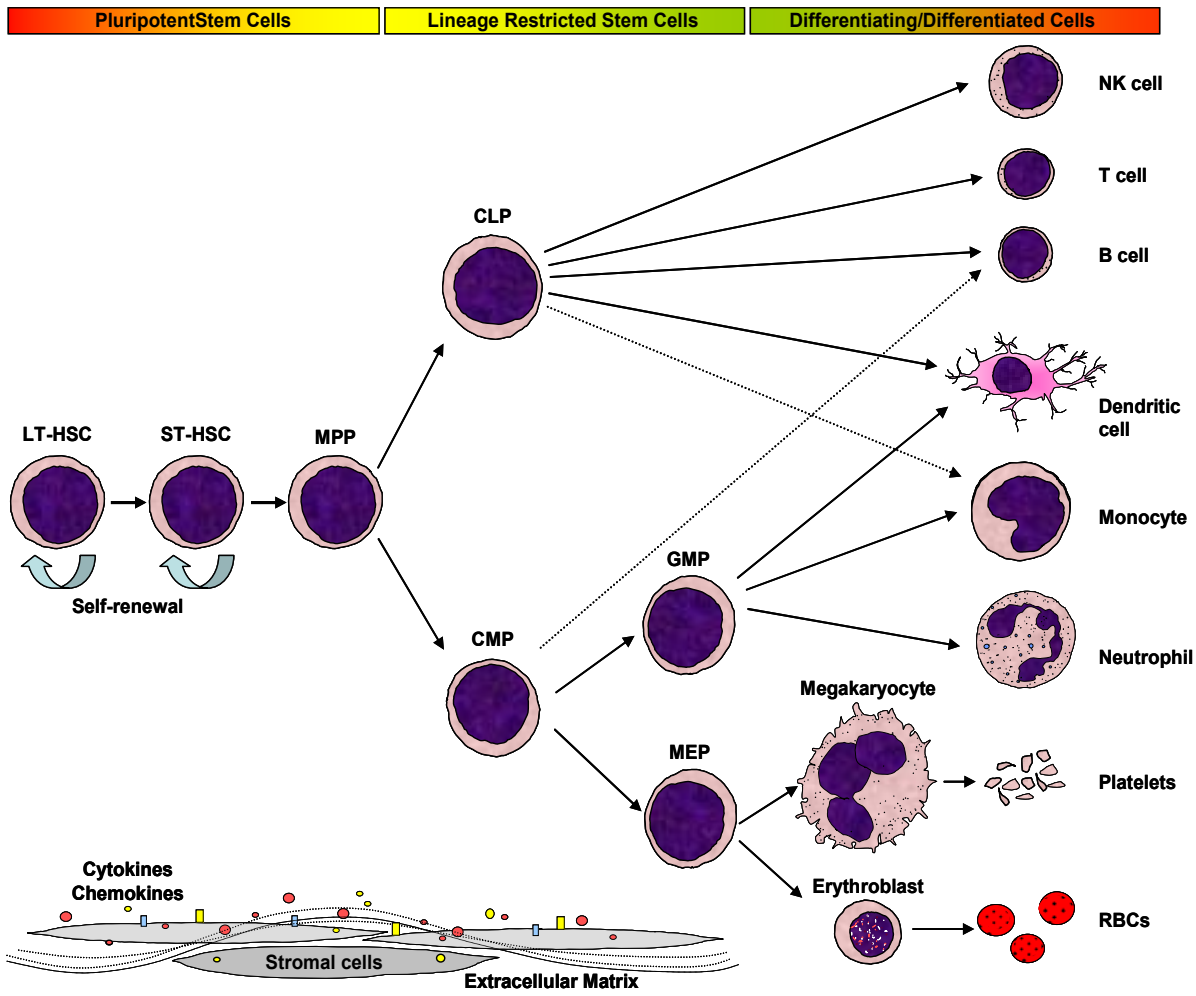

Figure 1.1 Ontogeny of haematopoietic cells from HSC. HSC can be subdivided into long-term repopulating HSC (LT-HSC), short-term repopulating HSC (ST-HSC) and multipotent progenitors (MPP). They give rise to common lymphoid progenitors (CLPs; the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells). These progenitors differentiate into progressively more restricted, committed progenitors and become the mature haematopoietic cells of the various lineages (indicated on the right hand side).

1.1.2 Origin of HSC

In vertebrates, haematopoiesis occurs in successive waves during development. Broadly, it occurs in two phases: a transient embryonic ('primitive') phase of haematopoiesis and a subsequent definitive ('adult') phase. The embryonic phase of haematopoiesis is probably utilized to provide the embryo with its initial blood cells and capillary network to the yolk. The definitive phase of haematopoiesis is used to generate more cell types and to provide the stem cells that will last for the lifetime of the individual. The yolk sac is the primary site for haematopoiesis in mouse and human embryos and the first blood cells arise in the yolk sac blood islands (D

Metcalf and MAS Moore, 1971). Primitive haematopoiesis is also observed in the para-aortic splanchnopleura (P-SP) and the aorta-gonad-mesonephrous region (AGM). In contrast to the splanchnopleura, which generates multipotent haematopoietic cells including lymphoid progeny, the yolk sac generates only erythroid colonies and the CFU-mix, but no lymphoid progeny (Cumano et al., 2001). It has been demonstrated that the P-SP and the AGM gives rise to definitive multilineage haematopoiesis independently from the yolk sac. These haematopoietic stem cells later colonise the fetal liver, and around the time of birth, stem cells from the liver populate the bone marrow (BM), which then becomes a major site of blood formation throughout adult life.

1.1.3 Genetic programs specifying HSC

The production of mature blood cells from HSC requires three distinct genetic programs. These include: a) the specification of HSC, b) their self-renewal and c) their commitment/proliferation/differentiation (fig 1.2). The possible fates of haematopoietic stem cells are shown in figure 1.3.

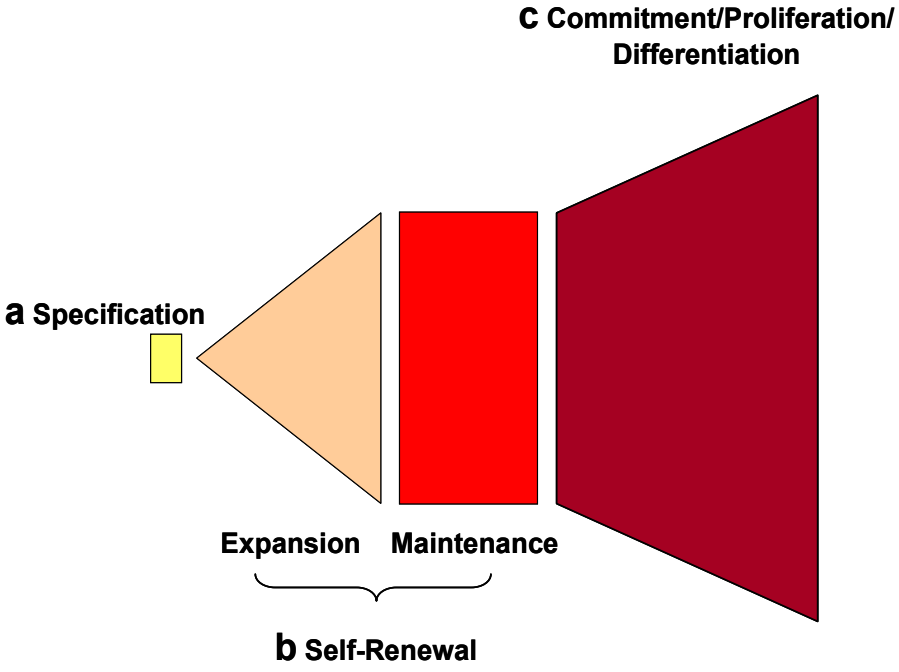

Figure 1.2 Genetic programs in haematopoiesis. At least three genetic programs are required for the development of the blood system (a) The specification of HSC (genesis), (b) the self-renewal, including expansion and maintenance of HSC, and (c) the commitment, proliferation and differentiation of HSC.

The transcriptional machinery governing early HSC function is very complex. Despite the progress that has been made in the recent years in identifying and obtaining enriched HSC populations, analysis of the population dynamics and cell cycle kinetics of HSCs remains difficult. Most of the studies leading to the knowledge of genes involved in HSC genetic programs have been carried by assaying haematopoietic cells from animals deficient for the gene of interest. More recently, expression profiling strategies have been used to determine genetic and molecular signatures of HSC (Ivanova et al., 2002).

Genes involved in specifying HSC during early embryogenesis include: SCL and Rbtn2/Lmo-2 which are necessary for primitive and definitive haematopoiesis (Shivdasani et al., 1995; Warren et al., 1994). GATA-2 and AML1 are specifically required for definitive haematopoiesis (Okuda et al., 1996). Some other factors appear to be more lineage-specific in action such as GATA-3, Ikaros, PU.1, GATA-1, CBP, Atf4, c-myb, and E2A, and their absence affects specific haematopoietic lineages (Bain et al 1994, Scott 1994, Wang et al 1996 Mauoka 2002, Wang 1997, Emambokus 2003). After their specification in early ontogeny, HSC undergo two rounds of mobilization: first, to the fetal liver where they expand and second, to the bone marrow where they are maintained throughout adult life. Expansion of the HSC in the fetal liver is necessary to increase the pool size of the mobilized stem cell population. The genetic factors involved in regulating fetal liver HSC are: *Meis1*, which is highly expressed in fetal liver Sca-1 $^{+}$ Lin- cells that are enriched for HSC activity (Pineault et al., 2002) and *Hoxb4*, the overexpression of which causes *in vivo* and *ex vivo* expansion of HSC (Antonchuk et al., 2001; Buske et al., 2002). Hox proteins interact with another transcription factor Pbx which itself interacts with Meis1 and forms a trimeric nuclear complex which is involved in target gene regulation (Liu et al., 2001; Swift et al., 1998). HSC self-renewal maintenance in adult BM is regulated by a different set of genes. A number of recent studies point out to nuclear factors such as the *Polycomb-Group (PcG*) genes *Bmi-1* and *Rae-28*, *GATA-2* and *TEL* for potentially regulating this process. It has been observed that *Bmi-1* levels decline during haematopoietic development, and that *Bmi-1* deficient mice develop hypocellular BM and die at less than 2 months of age. This led to the speculation that Bmi-1 is involved in maintenance of the HSC pool (Lessard et al., 2004). *Rae-28*, a known nuclear partner of Bmi-1 also plays a crucial role in maintaining the activity of HSC during fetal haematopoiesis (Ohta et al., 2002). The zinc-finger transcription factor GATA-2, a member of GATA family, plays a critical role in

maintaining the pool of multipotent progenitors and HSC, both during embryogenesis and in the adult (Tsai et al., 1994).

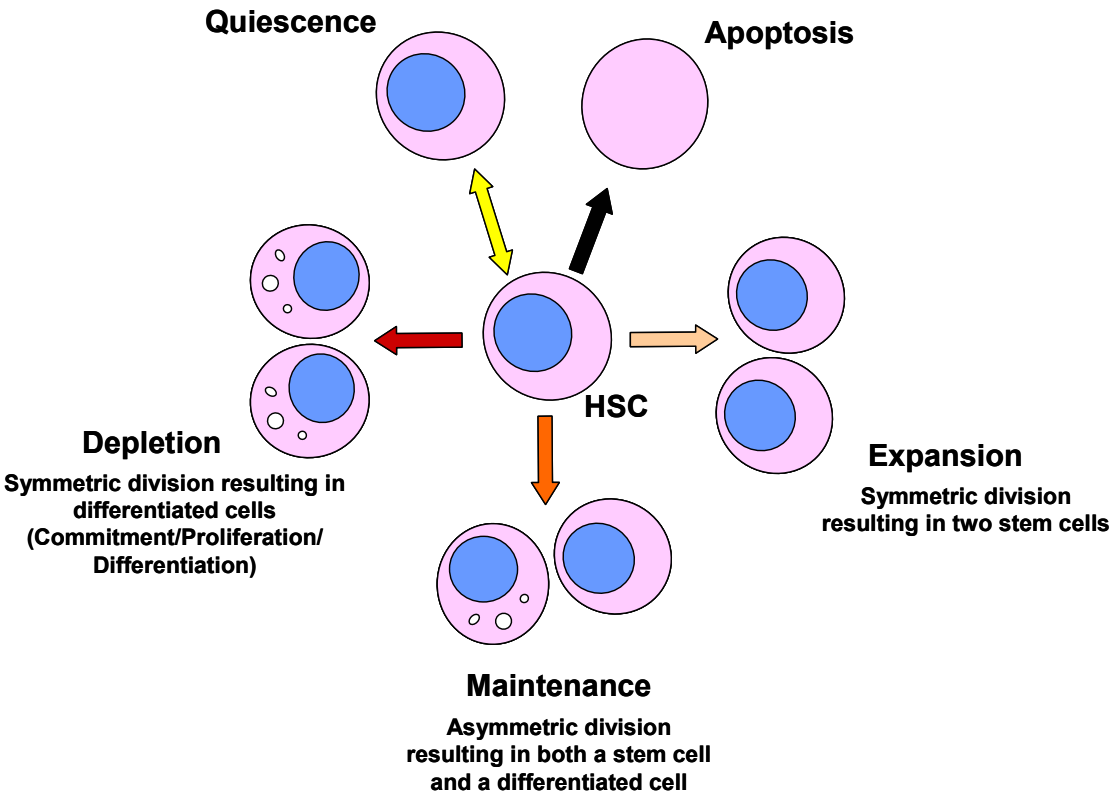

Figure 1.3 Possible HSC fates. HSCs are characterized by increased cell cycle quiescence compared to other cells. They can remain in quiescence, enter cell cycle to undergo symmetric or asymmetric division, or undergo apoptotic death. Division results in daughter stem cells or mature cells through symmetric division or both through asymmetric division.

The commitment and differentiation of haematopoietic lineages from common multi-potential progenitors cells require the role played by lineage-specific transcription factors. The zinc-finger transcription factor GATA-1 and its transcriptional cofactor called Friend of GATA-1 (FOG-1), have been found to be essential for erythroid and megakaryocytic differentiation (Pevny et al., 1991; Tsang et al., 1998; Vyas et al., 1999). PU.1 is a member of the Ets family of transcription factors and is essential in the development of cells of the monocytic, granulocytic and lymphoid lineages (Scott et al., 1994).

1.1.4 Characterization of HSC

HSC are rare during the postnatal life, with estimates varying from less than 0.05% to up to 0.5% of the total cells in the bone marrow. The majority of them normally

remains quiescent, as shown by their resistance to treatment with 5-fluorouracil, which eliminates dividing cells without adversely affecting the long-term repopulating capability of the bone marrow. Identification and isolation of HSC relies on the development of quantitative and specific assays for these cells. A proof of the existence of HSC requires the demonstration of its ability to produce a long-lasting multilineage clone *in vivo*. Various types of syngenic and xenogenic models have been developed to detect human and animal HSC based on this definition (Dick et al., 1997; Eaves et al., 1997; Zanjani, 1997). Although HSC can be defined using *in vivo* models, there is as yet no *in vitro* assay that specifically detects HSC. The existing *in vitro* assays are only able to detect the intermediate progenitors that develop from the HSC ((fig 1.4).

***In vivo* models:** Haematopoietic stem cells are defined in repopulation assays based on their functional ability to home to the bone marrow microenvironment and to repopulate transplanted recipients durably with both myeloid and lymphoid cell populations (Moore, 1997). In order to study human haematopoiesis using *in vivo* models, two essential prerequisites need to be met: The host should not eliminate the xenograft via an immune reaction and should provide a permissive microenvironment for engraftment and multilineage differentiation of donor cells. Various animal models such as fetal lambs, dogs, and immunodeficient mice have been tried for *in vivo* functional assays of human HSC (Berenson et al., 1987; Berenson et al., 1988; Eaves et al., 1997; Zanjani et al., 1997). Spontaneous mutant mouse models, having multiple defects in immunity, partially meet these criteria and have been modified to improve their model function. The NOD[1]/ LtSz-SCID mouse, generated by crossing the *SCID*[2] mutation from C.B-17- *scid* mice onto the NOD background, is widely being used to study reconstitution with human haematopoietic cells (Larochelle et al., 1996; Conneally et al., 1997). The cells capable of multilineage repopulation of transplanted NOD/SCID mice are termed as SCID

[1] Non obese diabetic (NOD) mice are animal models of spontaneous autoimmune T-cell mediated insulin dependent diabetes mellitus (IDDM).

[2] Severe combined immunodeficiency disease. (SCID). SCID mice fail to develop T and B cells. This defect is due to failure in VDJ recombination. Since the mice lack T and B cells of their own, they do not reject the transplanted human tissues and therefore could be used for such studies.

repopulating cells (SRC) (Peled et al., 1999) and occur at low frequencies in the order of 1 in 9.3 x 10^5 mononuclear cord blood cells and 1 in 3.0 x 10^6 normal bone marrow cells (Wang et al., 1997). There are some limitations with this assay as NOD/SCID mice develop thymic lymphomas at an early age and consequently mortality increases from the 3rd month of their life. For this reason long term studies are not possible unless serial transplantation is performed. In addition, the differentiation of human haematopoietic cells is skewed towards the B-lymphoid lineage raising some concerns about the normal development of human stem cells in the murine environment. The recently developed β2 microglobulin knockout NOD/LtSz-SCID B2m^{null} mice (NOD/SCID/B2m^{null}) and the NOD/SCID/γ_c^{null} both lacking natural killer (NK) cell activity show better engraftment of human cells than NOD/SCID mice, but have shorter survival (Christianson et al., 1997). NOD/SCID/B2m^{null} and NOD/LtSz-SCID transgenic for human growth factors have been studied for the engraftment of human AML cells (Feuring-Buske et al., 2003). The NOD/SCID/B2m^{null} mouse has been reported to require more than 4 to 30-fold less human cells to reach similar levels of human engraftment (Kollet et al., 2000). However, the SRC giving human reconstitution in these mice has been shown to include cells expressing CD38, while SRCs in the NOD/SCID model are exclusively CD38 negative. These observations suggest that less primitive progenitors represent the predominant human repopulating cells in NOD/SCID/B2m^{null} mice (Glimm et al., 2000).

The fetal sheep model provides an alternative to the murine xenotransplantation models. Zanjani et al, showed that human HSC can be transplanted intraperitoneally into unconditioned, early gestational sheep foetuses (Zanjani et al., 1995; Zanjani, 2000). In this model low numbers of selected progenitors can engraft, and myelo-erythroid as well as T and B lymphoid read-out can be monitored over several years.

***In vitro* assays:** The first in vitro assays for haematopoietic progenitors were developed way back in the 1960's. Most of these assays were originally developed for the murine stem cells but subsequently adapted for the detection of similar human cell populations. All of the assays measure some aspect of stem cell activity and identify cells with one or more stem cell attributes. Some of these assays may detect similar or at least overlapping populations.

Long term culture initiating cell (LTC-IC) assay: Perhaps the most frequently used method for assessing the frequency of primitive cells *in vitro* is the long-term culture initiating cell (LTC-IC) assay. This assay is based on the observation that the bone marrow stromal cells can support the survival of primitive haematopoietic cells for several weeks. Candidate cells are primarily cultured for 5-8 weeks on adherent, bone marrow-derived stromal cells that presumably resemble a bone marrow-like environment (Dexter et al., 1977). In a second step cells are transferred into semisolid medium containing cytokines. As the clonogenic cells initially present in a cell suspension are not able to survive a period of more than ~3 weeks, the clonogenic cell output after 5 to 8 weeks can be used to quantify the number of primitive LTC-IC present at the time of culture initiation. LTC-IC assays can detect some but not all HSC. Although the murine LTC-IC can regenerate haematopoiesis in an irradiated mouse (Ploemacher et al., 1991), this property has not been demonstrated for human LTC-IC. Part of the human LTC-IC compartment is considered to be quite distinct from cells with marrow repopulating ability as shown by cell fractionation and some gene marking techniques (Dexter et al., 1977; Larochelle et al., 1996). The human LTC-IC population therefore probably represents a less primitive cell within the stem cell compartment.

Colony Assay: Another extensively used assay, the colony forming cell (CFC) assay, allows the enumeration of more mature progenitor cells capable of forming colonies when cultured in semisolid media. These semisolid media reduce cell mobility and allow individual cells to develop into colonies of differentiated daughter cells. The morphology of colonies is distinct from one another and helps in identifying the type of colony being assessed. Since the HSC divide poorly in semisolid media and are themselves unable to form colonies, the CFC are considered to comprise a large, intermediate progenitor compartment that spans the entire stepwise process of lineage restriction.

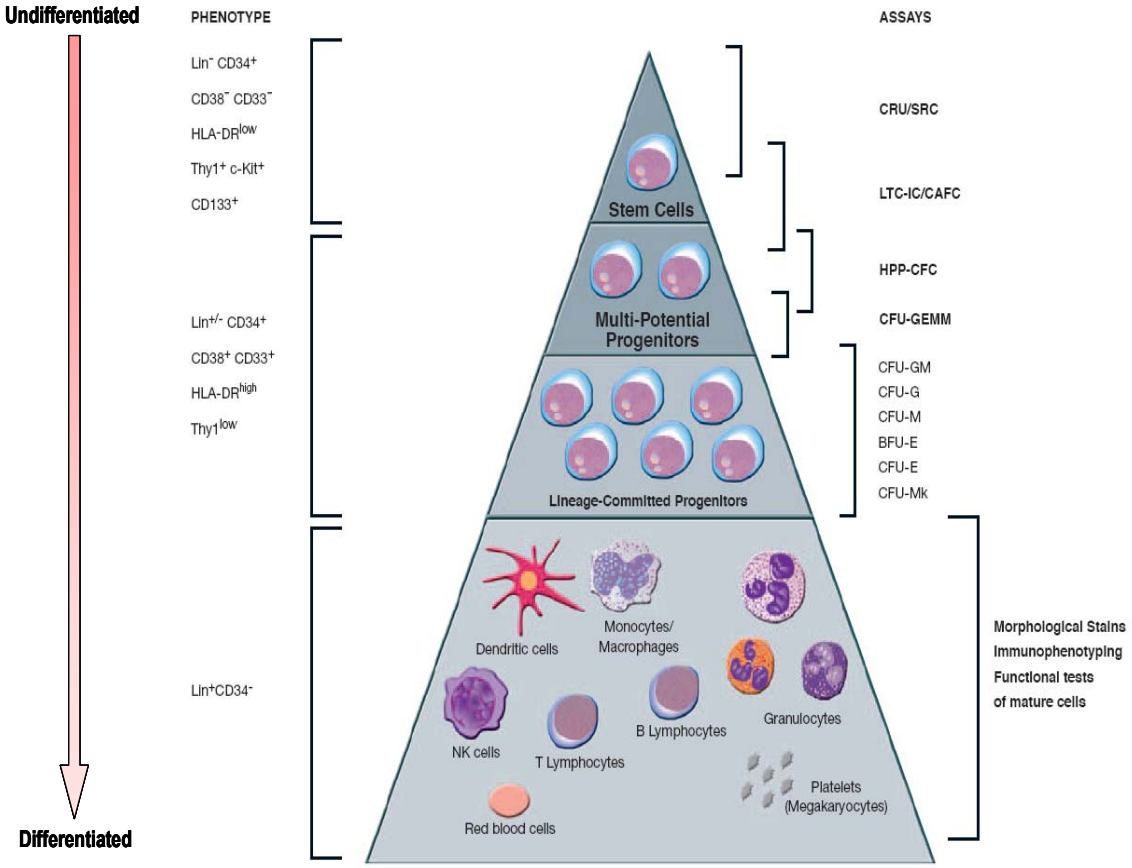

Figure 1.4 Various classes of haematopoietic stem/progenitor cells identified *in vitro* and *in vivo* assays. Cells have been placed on the vertical axis according to their maturation stage**.** Examples of commonly used combinations of cell surface antigens for identifying cells at distinct stages of differentiation are shown on the left. Assays for competitive repopulating unit (CRU) and NOD/SCID mouse repopulating cell (SRC) identify repopulating stem cells. Assays for Long-Term Culture-Initiating Cells (LTC-IC) and Cobblestone Area Forming-Cells (CAFC) identify similar populations of very primitive progenitor cells. High Proliferative Potential Colony-Forming Cells (HPP-CFC) and Colony-Forming Unit- Granulocyte, Erythrocyte, Macrophage, Megakaryocyte (CFU-GEMM) are multi-potential progenitors and are considered more primitive than the more lineage-restricted CFU-GM, CFU-G, CFU-M, CFU-Megakaryocyte (CFU-Mk), Burst-Forming Unit-Erythroid (BFU-E) and CFU-E. Mature cells are identified by their morphology, functional properties and expression of lineage-specific antigens (Adapted from (Coulombel, 2004) and Stem Cell Technologies Catalogue).

1.1.5 Surface marker analysis of HSC

Despite many exhaustive studies, researchers have not been able to identify a reliable direct marker for HSC. However, there are several markers whose expression is gained or lost at different rates as HSC differentiate (Wognum et al.,

2003). By targeting various combinations of markers, it has thus become possible to obtain fractions of cells with HSC characteristics. Some of the important markers currently used for HSC specification are:

CD34: The CD34 antigen is the major positive marker for human haematopoietic and progenitor cells and has been widely used for the isolation of these cells. Among non-haematopoietic tissues, CD34 is expressed on endothelial cells of microvessels and is the ligand for L-selectin (CD62L) (Fina et al., 1990). Of haematopoietic cells in human fetal liver, cord blood, and bone marrow, 0.5-5% express CD34 (Civin et al., 1984; Krause et al., 1996). However, the $CD34^+$ cells are heterogeneous and include committed progenitors (Hao et al., 1995). By assessing the coexpression of other cell surface markers, it is possible to obtain cell fractions that are more highly enriched for primitive cells. $CD34^+$ cells that do not express mature lineage markers (Lin-, as CD3, CD4, CD8, CD19, CD20, CD56, CD11b, CD14, and CD15) and CD38 contains single cells with *in vitro* bilineage, lymphoid (B/NK) and myeloid differential potential (Bhatia et al., 1997; Hao et al., 1995; Waller et al., 1995). Expression of CD38 is correlated with increased differentiation; only a small fraction (1-10%) of $CD34^+$ cells do not express CD38 (Hao et al., 1995). Both the $CD34^+$ CD38- and the $CD34^+$ $CD38^+$ fractions contain CFC and the LTC-ICs. The SRC are exclusively found in the cell fraction that expresses high levels of CD34 and no CD38 (Conneally et al., 1997).

CD133: CD133 (Prominin-1 or AC133) is a 120-kDa cholesterol-binding glycoprotein (Roper et al., 2000; Weigmann et al., 1997) that belongs to a growing family of pentaspan membrane proteins expressed throughout the animal kingdom (Fargeas et al., 2003). CD133 is expressed on several primitive cells such as haematopoietic stem and progenitor cells derived from bone marrow, fetal liver, and peripheral blood (Yin et al., 1997), as well as neural and endothelial stem cells (Peichev et al., 2000; Uchida et al., 2000). CD133 is expressed on a majority of, but not all, $CD34^+$ cells. These include repopulating cells, immature progenitors, and monocytes/granulocyte progenitors, but not most erythroid progenitors. A subpopulation of $CD34^-$ $CD133^+$ lineage- cells has been described in human cord blood which might be hierarchically more primitive than $CD34^+$ cells (Gallacher et al., 2000).

C-KIT: C-KIT or CD117 is the receptor for stem cell factor. C-KIT is expressed on ~2-3 % of $CD34^+$ cells including most lineage committed progenitor cells, and is selectively upregulated in terminally differentiating erythroid cells but is absent from virtually all circulating mature blood cells (Kawashima et al., 1996). However, the level of expression of C-KIT on $CD34^+$ cells is usually high enough to allow its use in haematopoietic cell isolation methods.

1.1.6 Other features of haematopoietic stem cells

Aldehyde dehydrogenase expression:

The detoxifying enzyme aldehyde dehydrogenase (ALDH) is expressed at high levels in the human haematopoietic progenitors, and increased cytosolic ALDH activity confers resistance of HSC to alkylating agents such as cyclophosphamide (Kastan et al., 1990; Gordon et al., 1985). Aldefluor, a fluorescent substrate of ALDH has been used to isolate cells with increased ALDH activity by FACS (Storms et al., 1999). Using a 2-step strategy consisting of lineage depletion followed by the selection of cells with high and low ALDH activity (ALDH ^{hi} Lin^- and ALDH ^{low} Lin^-), Hess et al isolated a functional population of haematopoietic stem and progenitor cells from human UCB (Hess et al., 2004) (fig 1.5). ALDH ^{hi} Lin^- cells highly expressed primitive cell surface markers ($CD34^+$ CD38- and $CD34^+$ $CD133^+$) and demonstrated enhanced *in vitro* clonogenic progenitor capacity similar to that of lineage depleted $CD34^+$ $CD38^-$ cells. Transplantation of purified ALDH ^{hi} Lin^- cells into NOD/SCID and NOD/LtSz-SCID $B2m^{null}$ mice resulted in multilineage haematopoietic engraftment (Hess et al., 2004).

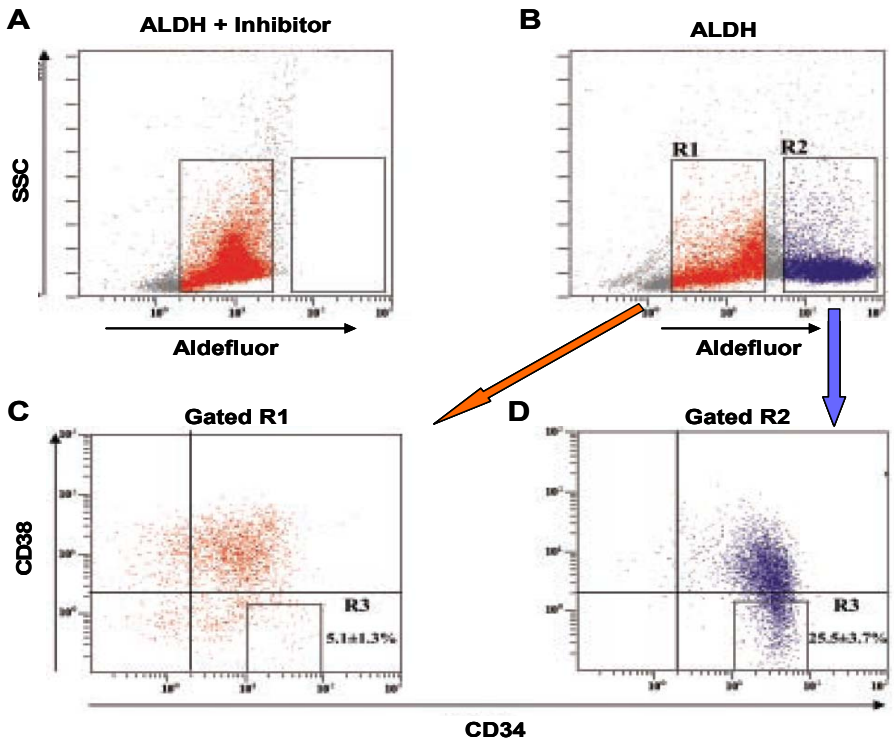

Figure 1.5 Phenotypic analysis of purified ALDH^{hi}Lin^{-} and ALDH^{low} Lin^{-} populations (Adapted from Hess et al., 2005).

Side Population phenotype and the expression of ATP Binding Cassette Proteins:

Primitive haematopoietic cells from mouse, humans and other species have also been identified and isolated based on their ability to efflux certain fluorescent dyes, such as Rhodamine-123 (Rho) and Hoechst 33342. Adult human HSC are mostly Rho$^{-/low}$ and this phenotype has been attributed to the expression of P-glycoprotein, an ABC transporter expressed on the cell surface. Mice lacking P-glycoprotein are functionally normal but are Rho^{-} (Uchida et al., 2002). Adult BM of many species have been shown to contain a rare population of Hoechst 33342$^{-/low}$ cells that have been designated as side population (SP) cells because they form a characteristic cluster of events off to the lower left side in dual wavelength FACS dot-plot profiles of Hoechst 33342 stained cells (fig 1.6). The ability of SP cells to efflux Hoechst 33342 is due to the activity of another ABC transporter, the ABCG2. SP cells are highly enriched for HSC activity and represent about 0.05% of adult nucleated bone marrow cells in mice (Goodell et al., 1996). SP stem cells have been identified in haematopoietic compartments of humans, rhesus monkeys and swine (Storms et al., 2000; Uchida et al., 2001). In human fetal liver, all of the cells that are capable of engrafting NOD/SCID mice were located in the 10% of CD34^{+} CD38- that display an

SP phenotype (Uchida et al., 2001). SP cells have also been demonstrated in non-haematopoietic tissues such as skeletal muscles (Gussoni et al., 1999; Jackson et al., 1999) and neuronal tissues (Islam et al., 2005). This suggested that SP cells represent a distinct class of tissue-specific stem cells, perhaps present several different tissues (Zhou et al., 2001) and that this phenotype could be used as stem cell purification marker.

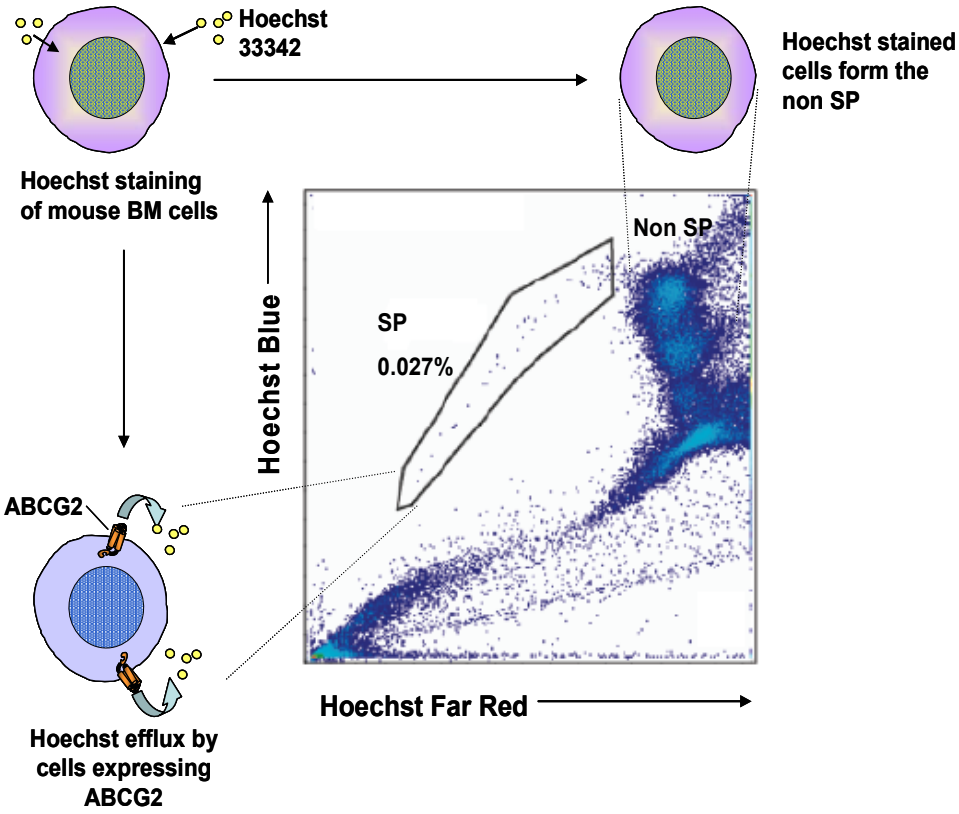

Figure 1.6 Hoechst SP analysis of mouse BM cells. Whole BM of mice is labelled with membrane permeating DNA-binding dye Hoechst 33342 and excited with 50 mW of UV (351–364 nm), and then the emission was detected through a 450/20-nm (Hoechst blue) band-pass filter and a 675-nm (Hoechst red) long-pass filter on an ultraviolet laser equipped flow cytometer. All of the parameters were collected using linear amplification in list mode and displayed in a Hoechst blue versus Hoechst red dotplot to visualize SP. A very small population of cells extrudes this dye via the ABC transporter ABCG2 and forms a dim tail extending from the normal G1 cell population. This population is termed as the side population (SP).

In section 1.2 ABC proteins will be described in detail with particular emphasis on ABCG2, the molecular determinant of SP phenotype.

1.1.7 Sources of HSC

Although BM is the primary source of HSC during the postnatal life, various alternative sources are available for clinical and experimental use of HSC. These

alternative sources of HSC are mobilized peripheral blood, umbilical cord blood (UCB) and fetal liver.

Bone Marrow:

In adults, haematopoiesis is exclusively accomplished by HSC residing in the bone marrow. Till and McCulloch demonstrated more than 40 years ago, that a single precursor cell exists in the bone marrow (BM) of adult animals that is capable of both extensive self-renewal and multi-lineage differentiation (TILL and McCULLOCH, 1961). With this began the understanding of both bone marrow transplantation and BM derived stem cells. For more than 30 years, bone marrow transplantation has been used to treat blood diseases such as leukemia and other disorders of the immune system. Although in an adult human the amount of active bone marrow is about 2,600 g, with approximately 1.3 x 10^{12} marrow cells, HSC constitute only up to 0.5% of the cells. The majority of the HSC in BM remains normally quiescent, as demonstrated by their resistance to treatment by 5-fluorouracil or 4-hydroxyperoxycyclophosphamide, which eliminates dividing cells without adversely affecting the long-term repopulating capability of the BM. HSC from bone marrow are easy to isolate and can be transferred from the donor to the recipient with virtually no manipulation because the haematopoietic system has a high rate of renewal normally. For any type of stem cell transplantation, however, certain considerations must be taken into account if they are to obtain a successful graft. These factors include matching the HLA type of the donor with that of the recipient, determining the critical numbers of stem cells required and the use of accessory cells or factors.

Peripheral Blood:

Normal peripheral blood contains about 0.06% circulating HSC. However, temporarily shifting HSC, from extravascular BM sites into the circulating blood by using cytokine treatment dramatically increases the circulating stem cell concentration and easily compensates for the low baseline circulating $CD34^+$ cell concentration. When healthy donors are treated with recombinant human granulocyte colony stimulating factor (rhG-CSF; 12 mg/kg per day) over 3 days, with another rhG-CSF dose given on the fourth day before the stem cell apheresis procedure, the mean peripheral blood (PB) $CD34^+$ cell concentration increases from 3.8 x 10^9/L to 61.9 x 10^9/L, a 16.3-fold increase over baseline. The increase in early $CD34^+38^-$ progenitor cells is, at 23.2-fold, even greater (Korbling et al., 1995).

Autologous and allogenic mobilized PB HSC is commonly used for transplantation therapy as it has the advantage of a more rapid haematopoietic recovery compared to bone marrow transplantation.

Umbilical Cord Blood:

Umbilical cord blood (UCB) is another important source of stem cells. Approximately 30% to 45% of all blood associated with a fetus is circulating in the umbilical cord and in the placenta. At the time of birth this blood is usually discarded. Being rich in HSC it is however, collected for experimental and clinical use. Multiple studies exploring the nature of UCB HSC have shown a higher proportion of primitive haematopoietic progenitors in UCB, with superior *in vitro* proliferative responses and *in vivo* engraftment capacity compared to adult BM (Leung et al., 1998; Lewis and Verfaillie, 2000; Mayani and Lansdorp, 1998). UCB is considered a valuable source for stem cell-based therapies, particularly because it is easily available, non-invasive, and less immunogenic compared to other sources for stem cell therapy such as bone marrow (Roncarolo et al., 1994). However, a single cord may not contain sufficient total number of HSC for fast and durable engraftment in adult patients (Rubinstein et al., 1998). Methods for *ex vivo* expansion of these HSC are being evaluated.

1.2 ABC proteins

The ATP-binding cassette (ABC) proteins represent the largest family of transmembrane proteins. These proteins bind ATP and use the energy to translocate various molecules across extra- and intracellular membranes (Higgins, 1992). These are classified as ABC transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide binding domains (NBD). The NBD are highly conserved and contain characteristic Walker A and B motifs, separated by about 90-120 amino acids. They also contain an additional element, the signature (C) motif, located close to the Walker B motif. The eukaryotic ABC proteins are organized either as full transporters or as half transporters (Hyde et al., 1990) (fig 1.7). The translocator component of a full ABC transporter is composed of two multi-transmembrane domains (TMD) and two NBD. Half transporters consist of one TMD and NBD, and require the formation of a homo- or heterodimer to form a functional transporter. The TMD consist of 6-11 membrane spanning α-helices and provide the specificity for the substrate. The conformational changes within the TMD are

believed to be responsible for the transport of molecules through these transporters. The NBDs are located on the cytoplasmic side and hydrolyse the cytoplasmic ATP to derive energy for the active transport of substrates across the membranes.

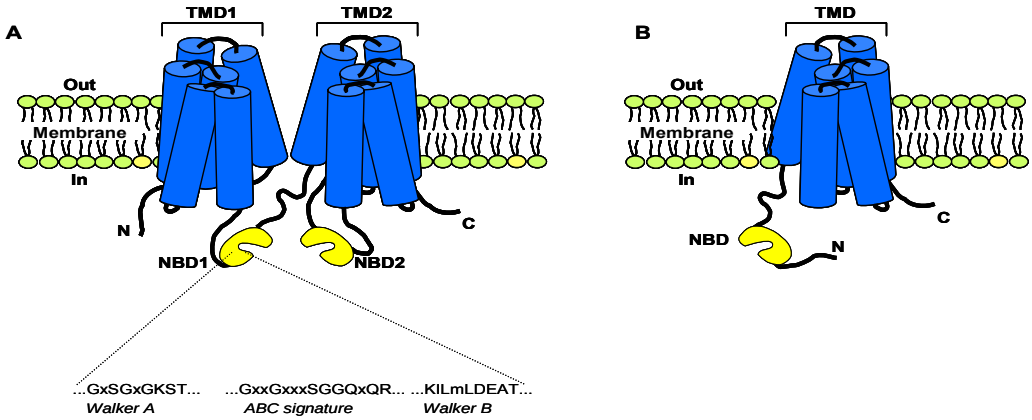

Figure 1.7 Structural organisation of ABC Transporters. (A) ABC transporters having two TMD and two NBD. The NBD are located at the cytoplasmic surface of the membrane and contain the highly conserved ABC signature and the Walker A and B motifs which are involved in ATP hydrolysis. The most conserved residues in these motifs are indicated. (B) ABC half transporter having one NBD and one TMD. (Modified from H Bolhuis et al., FEMS 1997)

These transporters are mostly unidirectional and the transport can take place against considerable concentration gradients. In bacteria, they are predominantly involved in the import of essential compounds that cannot be obtained by diffusion such as sugars, vitamins, metal ions, etc., into the cells. In eukaryotes, most ABC transporters move the compounds from the cytoplasm, to the outside of the cell or into an intracellular compartment. Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds either within the cell as part of a metabolic process, or outside the cell for transport to other organs or for secretion from the body.

The human ABC protein family currently comprises 48 members and can be subdivided into seven distinct subfamilies, based on similarity in gene structure, organization of domains as well as sequence homology in the NBD and the TM domains (Dean et al., 2001). As per the HUGO Gene Nomenclature, the 7 subfamilies of human ABC genes are termed ABCA (12 members), ABCB (11 members), ABCC (13 members), ABCD (4 members), ABCE (1 member), ABCF (3 members), and ABCG (5 members). A list of all known human ABC genes including a concise summary of each subfamily is displayed in table 1.1.

Table 1.1 List of Human ABC Genes, Chromosomal location, and Function (Dean et al., 2001).

Gene	Alias	Location	Subfamily	Expression	Function
ABCA1	*ABC1*	9q31.1	ABC1	Ubiquitous	Cholesterol efflux onto HDL
ABCA2	*ABC2*	9q34	ABC1	Brain	Drug resistance
ABCA3	*ABC3, ABCC*	16p13.3	ABC1	Lung	
ABCA4	*ABCR*	1p22.1-p21	ABC1	Photoreceptors	N-retinylidene-PE efflux
ABCA5		17q24	ABC1	Muscle, heart, testes	
ABCA6		17q24	ABC1	Liver	
ABCA7		19p13.3	ABC1	Spleen, thymus	
ABCA8		17q24	ABC1	Ovary	
ABCA9		17q24	ABC1	Heart	
ABCA10		17q24	ABC1	Muscle, heart	
ABCA12		2q34	ABC1	Stomach	
ABCA13		7p11-q11	ABC1	Low in all tissues	
ABCB1	*MDR1, PGY1, PGP*	7p21	MDR	HSC, Adrenal, kidney	Multidrug resistance
ABCB2	*TAP1*	6p21	MDR	All cells	Peptide transport
ABCB3	*TAP2*	6p21	MDR	All cells	Peptide transport
ABCB4	*PGY3*	7q21.1	MDR	Liver	PC transport
ABCB5		7p14	MDR	Ubiquitous	
ABCB6	*MTABC3*	2q36	MDR	Mitochondria	Iron transport
ABCB7	*ABC7*	Xq12-q13	MDR	Mitochondria	Fe/S cluster transport
ABCB8	*MABC1*	7q36	MDR	Mitochondria	
ABCB9		12q24	MDR	Heart, brain	
ABCB10	*MTABC2*	1q42	MDR	Mitochondria	
ABCB11	*SPGP*	2q24	MDR	Liver	Bile salt transport
ABCC1	*MRP1*	16p13.1	CF/MRP	Lung, testes, PBMC	Drug resistance
ABCC2	*MRP2*	10q24	CF/MRP	Liver	Organic anion efflux
ABCC3	*MRP3*	17q21.3	CF/MRP	Lung, intestine, liver	Drug resistance
ABCC4	*MRP4*	13q32	CF/MRP	Prostrate	Nucleoside transport
ABCC5	*MRP5*	3q27	CF/MRP	Ubiquitous	Nucleoside transport
ABCC6	*MRP6*	16p13.1	CF/MRP	Kidney, liver	
CFTR	*ABCC7*	7q31.2	CF/MRP	Exocrine tissue	Chloride ion channel
ABCC8	*SUR*	11p15.1	CF/MRP	Pancreas	Sulfonylurea receptor
ABCC9	*SUR2*	12p12.1	CF/MRP	Heart, muscle	
ABCC10	*MRP7*	6p21	CF/MRP	Low in all tissues	
ABCC11	*MRP8*	16q11-q12	CF/MRP	Low in all tissues	
ABCC12	*MRP9*	16q11-q12	CF/MRP	Low in all tissues	
ABCD1	*ALD*	Xq28	ALD	Peroxisomes	VLCFA transport regulation
ABCD2	*ALDL1, ALDR*	12q11-q12	ALD	Peroxisomes	
ABCD3	*PXMP1, PMP70*	1p22-p21	ALD	Peroxisomes	
ABCD4	*PMP69, P70R*	14q24.3	ALD	Peroxisomes	
ABCE1	*OABP, RNS41*	4q31	OABP	Ovary, testes, spleen	Oligoadenylate binding protein
ABCF1	*ABC50*	6p21.33	GCN20	Ubiquitous	
ABCF2		7q36	GCN20	Ubiquitous	
ABCF3		3q25	GCN20	Ubiquitous	
ABCG1	*ABC8, White*	21q22.3	White	Ubiquitous	Cholesterol transport
ABCG2	*ABCP, MXR, BCRP*	4q22	White	Placenta, intestine, HSC	Toxin efflux, sterol transport
ABCG4	*White2*	11q23	White	Liver	
ABCG5	*White3*	2p21	White	Liver, intestine	Sterol transport
ABCG8		2p21	White	Liver, intestine	Sterol transport

ABC genes that are over-expressed in HSC and determine the drug and dye efflux properties of stem cells are *MDR1* and *ABCG2*:

1.2.1 Multidrug resistance 1 (ABCB1):

Multidrug resistance1 (MDR1; *ABCB1*), formerly known as PGP was the first human ABC transporter cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells that had developed resistance to chemotherapy drugs (Juliano and Ling, 1976). MDR1 has been demonstrated to be a promiscuous transporter of hydrophobic substrates, hydrophobic drugs including colchicine, VP16, adriamycin and vinblastine as well as lipids, steroids, xenobiotics, and peptides(Ambudkar, 1998). The gene is thought to have an important role in removing toxic metabolites from cells, but is also expressed in cells at the blood–brain barrier and presumably has a role in transporting compounds into the brain that cannot be delivered by diffusion. MDR1 has been shown to affect the pharmacology of the drugs that are substrates (Hoffmeyer et al., 2000). Chaudhary and Roninson were the first to demonstrate that the MDR1 is highly expressed in $CD34^+$ haematopoietic stem cells (Chaudhary and Roninson, 1991), suggesting that efflux pump activity could be responsible for the low retention of Rho123 in primitive haematopoietic cells. Experiments involving overexpression of MDR1 in HSC demonstrate an expansion of transduced cells in 12-day cultures as well as increased repopulating activity *in vivo* (Bunting et al., 2000). Mice receiving MDR1-transduced cells developed a myeloproliferative disorder characterised by high peripheral white blood cell counts and splenomegaly due to myeloid progenitor accumulation at both sites (Bunting et al., 1998; Bunting et al., 2000), indicating that ABC proteins might play a role in haematopoietic development.

1.2.2 ABCG2:

ABCG2, commonly known as Breast Cancer Resistance Protein (BCRP), is a member of the G-subfamily of ABC proteins. A brief introduction to G-subfamily is essential for understanding the uniqueness of ABCG2: The human ABCG subfamily comprises five members (ABCG1, ABCG2, ABCG4, ABCG5 and ABCG8). Members of this family are unique in their domain organization; they are "reverse" half transporters that have a single NBD at the N terminus and a TMD at the C terminus. Most of the human ABCG proteins seem to be involved in lipid and/or sterol

transport. The ubiquitously expressed ABCG1 and the liver specific ABCG4 are thought to be involved in cellular cholesterol transport. ABCG5 and ABCG8 arranged head-to-head with each other on chromosome 2p15-p16, heterodimerise together and are thought to transport non-cholesterol sterols (sitosterols). ABCG2 mediates resistance to multiple chemotherapeutic agents by pumping them out of the cells and thus lowering their cytotoxic effects. More recently ABCG2 has been shown to transport protoporphyrins and sulphated estrogens (Jonker et al., 2002; Imai et al., 2003).

1.2.3 Organisation of *ABCG2* gene and regulation:

ABCG2 was first described by Allikmets et al. as an ABC transporter gene highly expressed in placenta and was named ABCP (Allikmets et al., 1998). The *ABCG2* gene contains 16 exons spanning 66 kb and was mapped to human chromosome 4q22 (fig 1.8). It produces two transcripts that differ at the 5'-end, but encode the same protein consisting of 655 amino acids. Characterization of the *ABCG2* promoter revealed that it is a TATA-less promoter with several Sp1, AP1 and AP2 sites and a CCAAT box downstream of a putative CpG island (Bailey-Dell et al., 2001). A putative estrogen response element (ERE) has been found recently. It has been demonstrated that *ABCG2* mRNA expression was induced by 17β-estradiol (E2) in estrogen receptor (ER)-positive cell lines and that this effect can be reversed by the antiestrogens (Ee et al., 2004).

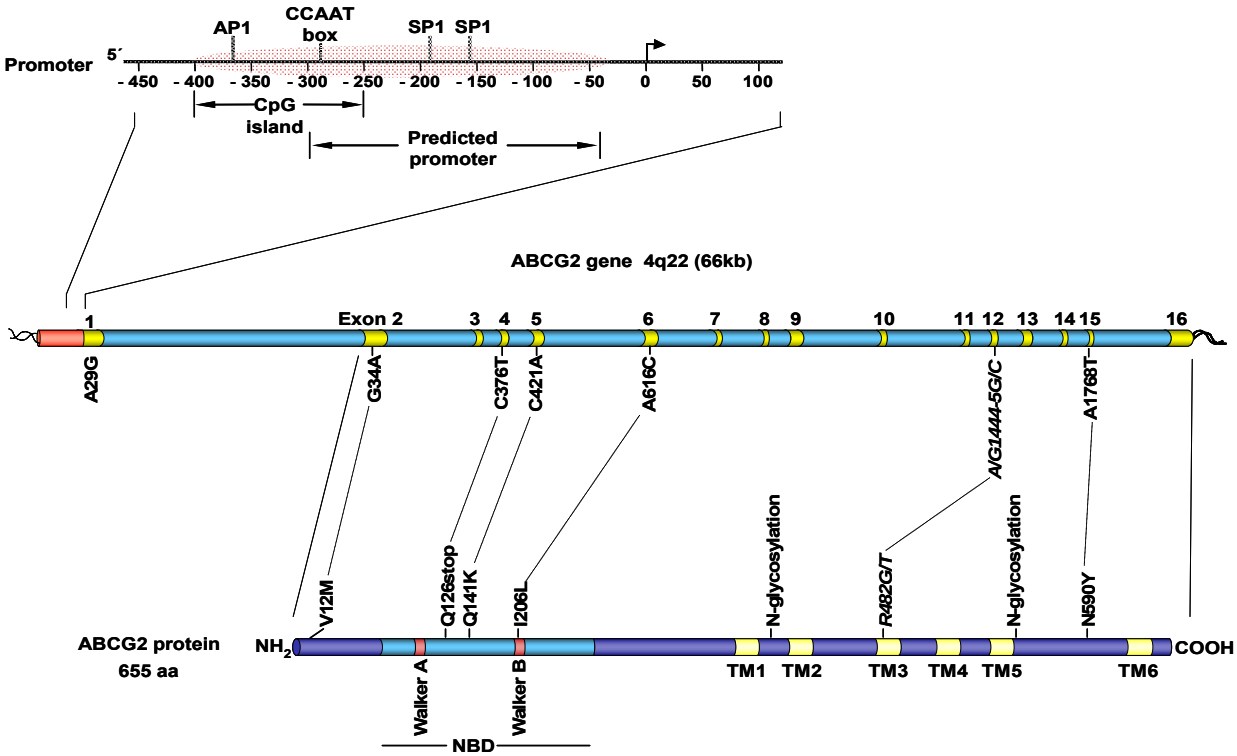

Figure 1.8 Diagram of genomic organisation of the *ABCG2* and primary structure of ABCG2 protein. The exons are numbered. The lower panel shows relations between coding regions and the protein domain organisation. Positions of single nucleotide polymorphisms (SNP) are indicated. NBD indicates the nucleotide binding domain; TM represents transmembrane region. Acquired mutations observed in some drug selected cell lines are indicated in italics. (Adapted from Doyle and Ross, 2003).

Recent evidence suggests that ABCG2 functions as a homodimer bridged by disulphide bonds. Under reducing conditions in the SDS-PAGE, ABCG2 protein migrates as a 70 kDa band but in non-reducing conditions, as a 140 kDa complex. The 140 kDa ABCG2 complex dissociates into 70 kDa polypeptides with the addition of 2-mercaptoethanol indicating the presence of ABCG2 as homodimer. The functional activity of ABCG2 in transfected insect cells, using a baculovirus-Sf9 cell system, indicates the activity of the protein as a homodimer as in this heterologous system, it is unlikely that any possible heterodimeric partners of ABCG2 are expressed (Ozvegy et al., 2001; Ozvegy et al., 2002).

1.2.4 Tissue distribution of ABCG2:

Cellular localization studies using monoclonal and polyclonal antibody probes confirm the predominant plasma membrane localization of ABCG2 transporter (Rocchi et al., 2000; Scheffer et al., 2000). ABCG2 expression analysis in normal

tissues using Northern blots reveal high expression in placental tissue, parts of brain and liver (Doyle et al., 1998). Using commercially available multiple tissue dot blots and *ABCG2* cDNA as probe, Doyle *et al.* showed highest level of expression in placenta, followed by liver and small intestine. Certain parts of the brain (putamen, substantia nigra, pituitary gland, thalamus, amygdala, caudate and subthalamic nuclei) also show relatively high expression of *ABCG2* mRNA. Immunohistochemical studies with BXP-21 and BXP-34 show moderate to strong expression in many normal tissues, for instance in the apical membrane of the placental syncytiotrophoblasts, on the bile canalicular membrane of the hepatocytes, on the apical side of the villous epithelial cells in the intestine and colon, in cardiac muscle, in pancreatic endocrine cells and in the endothelial cells of almost all tissues including the brain microvessels (Cooray et al., 2002; Diestra et al., 2002; Maliepaard et al., 2001).

1.2.5 Multidrug resistance:

Cells exposed to toxic compounds can develop resistance by a number of mechanisms including decreased uptake, increased detoxification, alteration of target proteins, or increased excretion. Several of these pathways can lead to multidrug resistance (MDR), in which the cell is resistant to several drugs in addition to the initial compound. This is a particular limitation to cancer chemotherapy. ABC genes have an important role in MDR and at least six genes are associated with drug transport (table 1.1). The overexpression of ABCG2 was observed in certain drug resistance cell lines and tumours, providing a multidrug resistance phenotype in these cancer cells. ABCG2 has the ability to confer high levels of resistance to mitoxantrone, anthracyclines, doxorubicin, daunorubicin, bisantrene, and the camptothecins topotecan and SN-38. ABCG2 expression also results in diminished intracellular accumulation of Hoechst 33342 (Kim et al., 2002). In certain drug-selected cell lines, overexpressing human or mouse ABCG2, a single amino acid change in amino acid position 482 occurred (Allen et al., 2002; Honjo et al., 2001). The mutants having R482G or R482T showed altered substrate specificity as compared to the wild-type protein, i.e. they conferred increased mitoxantrone or doxorubicin resistance and rhodamine 123 export capacities (fig 1.9). These R482 mutants show increased transport and ATP hydrolytic activity, therefore they are considered as “gain of function” mutants (Ozvegy et al., 2002).

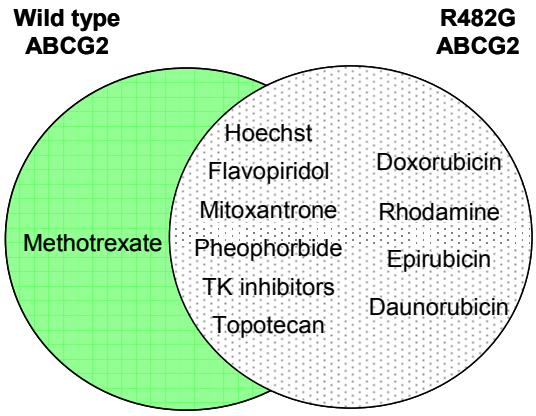

Figure 1.9 ABCG2 substrates. Transported substrates of the wild-type and R482G ABCG2 protein (Sarkadi et al., 2004).

ABCG2 is relevant in several pharmacological areas similar to the well defined ABCB1 (P-glycoprotein). By efflux of some cytotoxic agents from cancer cells it renders these cells resistant to chemotherapy. Through the same efflux mechanism, ABCG2 affects pharmacokinetic parameters of substrate drugs, such as intestinal absorption/bioavailability, hepatic/renal elimination and plasma clearance. It also helps protect sensitive body compartments from potentially harmful xenobiotics.

1.2.6 ABCG2 is the molecular determinant of side population (SP) phenotype:

Goodell et al identified murine bone marrow cells having stem cell characteristics by their rapid efflux of the Hoechst 33342 (Goodell et al., 1996). When unpurified murine bone marrow cells were labelled with the membrane-permeating, DNA binding vital dye Hoechst 33342, a very small fraction of cells extrudes this dye via a membrane pump. Analysis of these cells on a flow cytometer equipped with an ultraviolet (UV) laser source permits detection of these cells; when Hoechst-stained cells are analysed simultaneously through blue and red emission filters, the SP forms a dim tail extending from the normal G1 cell populations. These cells can reconstitute the bone marrow of lethally irradiated mice even when injected in low numbers, indicating that the SP cells are enriched for totipotent stem cells (Goodell et al., 1996). High levels of ABCG2 transcripts in SP cells from various sources and induction of SP phenotype by expression of ABCG2 indicate that ABCG2 is the molecular determinant of SP (Zhou et al., 2001) (fig 1.10). Specific inhibitors of ABCG2 abrogate the SP phenotype. *Abcg2* $^{-/-}$ *(Bcrp1* $^{-/-}$) mice had significant defect in the SP compartment in bone marrow and skeletal muscle cells (Zhou et al., 2002).

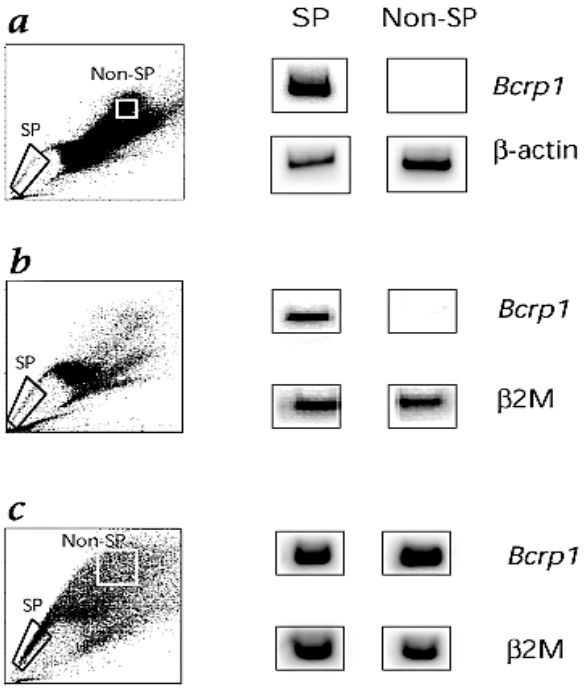

Figure 1.10 ABCG2 is the molecular determinant of SP. RT-PCR analyses for Bcrp1/ABCG2 expression in sorted SP cells from rhesus monkey bone marrow(a), mouse skeletal muscle (b) and murine ES cells (c) demonstrating that ABCG2 is the molecular determinant of SP (Zhou et al., 2001).

1.2.7 *ABCG2* in stem cells:

Expression of *ABCG2* is highly conserved in primitive stem cells from a variety of sources suggesting its role in the regulation of stem cell biology. In the haematopoietic compartment *ABCG2* expression is restricted to the most immature haematopoietic progenitors in bone marrow and is sharply down-regulated at the committed progenitor level, suggesting an important role of this ABC transporter in the earliest stages of haematopoietic development (Scharenberg et al., 2002). The enforced expression of MDR1 (ABCB1) has been shown to cause stem cell expansion and an associated myeloproliferative syndrome in recipient mice. This indicates endogenously expressed transporters might be conferring functional effects in stem cells. The expression of human *ABCG2* in mouse bone marrow cells expanded the SP population, and caused a reduction in the mature cell numbers, suggesting that *ABCG2* is both a stem cell marker and an important determinant of the SP phenotype (Zhou et al., 2002). Expression of murine *Abcg2* was demonstrated in $CD34^{+}/CD45^{-}$ murine myogenic-endothelial progenitors, which are resident in the interstitial spaces of mammalian skeletal muscle (Tamaki et al., 2002). ABCG2 expression has also been demonstrated in the multipotent progenitor

cells in the islets isolated form adult human pancreas (Lechner et al., 2002). Recently Du et al, reported the presence of an *ABCG2* expressing population of cells in human corneal stroma that can be isolated as a side population with Hoechst labelling. These SP cells are clonogenic, lack expression of differentiated corneal cells or fibroblasts, could be induced to express differentiation markers of corneal keratocytes and markers of chondrogenesis and neural cells (Du et al., 2005). ABCG2-expressing side-population cells have been reported by several other groups to display the ability to express differentiated markers of multiple tissues in response to different environmental stimuli (Cai et al., 2004; Hierlihy et al., 2002; Jiang et al., 2002; Lechner et al., 2002; Scharenberg et al., 2002; Suva et al., 2004; Zhou et al., 2001). Although *ABCG2* is clearly a stem cell specific gene, its physiological function in these cells is not yet clear. A possible function of ABCG2 in stem cell differentiation and protection against toxins is being investigated.

1.2.8 Expression of ABCG2 in human cancers and the concept of cancer stem cells

Doyle et al were the first to describe ABCG2 as a novel multidrug resistance transporter, overexpressed in drug selected breast cancer cell lines (Doyle et al., 1998). Using monoclonal antibody BXP-21, ABCG2 has been demonstrated in over 40% of 21 different types of solid tumors tested (Diestra et al., 2002). Since ABCG2 has been demonstrated to transport various anti-cancer agents, most of the studies of ABCG2 expression in human cancers have focussed on its prognostic impact and the use of ABCG2 inhibitors that can effectively reverse chemotherapy resistance. Ross et al examined blast cells from 21 acute leukaemia patients (ALL: 1 patient, AML: 20 patients) for ABCG2 expression with quantitative RT-PCR. Blast cells in seven patients with high clinical drug-resistance disease had relatively high expression of *ABCG2* mRNA. ABCG2 expression was low or barely detectable in blast cells from the remaining 14 patients (Ross et al., 2000). An additional study on *ABCG2* mRNA expression, using 20 paired clinical AML samples from diagnosis and relapse or refractory disease, demonstrated that ABCG2 was the only drug resistance protein that was expressed at a significantly higher RNA level (median 1.7-fold, P=0.04) in the relapsed/refractory state relative to that at diagnosis (van den Heuvel-Eibrink MM et al., 2002), suggesting that ABCG2 may be involved in resistance to one of the agents commonly used in front-line treatment of AML. An

upregulation of *ABCG2* mRNA at relapse was also observed by another group, who studied 59 samples of children with newly diagnosed AML, of which 9 were also analysed in relapse. The median *ABCG2* expression was more than 10 times higher in patients who did not achieve remission after the first chemotherapy course versus patients who did achieve remission (Steinbach et al., 2002). ABCG2 may also play a role in treatment failure in acute lymphoblastic leukemia (ALL), since ALL patients are treated with ABCG2 substrates such as methotrexate, daunorubicin, doxorubicin and mitoxantrone. ABCG2 is expressed and is functionally active in B-lineage ALL, and at lower levels in T-lineage ALL indicating that ABCG2 plays a small role in drug resistance in B-ALL (Stam et al., 2004).

ABCG2 expression in tumor cells may also be demonstrated with the help of SP cells as ABCG2 is the primary determinant of SP. Since tumors contain cells that are heterogeneous in phenotype and proliferative potential, it could be reasoned that they might harbour SP cells, whose intrinsic dye efflux properties would be expected to be associated with the ability to export many cytotoxic drugs, hence leading to treatment failure or the risk of early relapse. The SP cells from human primary tumours have characteristics that support their consideration as “primitive” cells. Both stem cells and cancer cells are thought to be capable of unlimited proliferation. It has been observed that even in long-term cultured cancer cells, in general, sufficient numbers of cells have to be injected to initiate an orthotopic tumor in recipient animals (Passegue et al., 2003), suggesting that in long-term tumor cell cultures not all cells are equal and only a small population of cells is tumorigenic. There is accumulating evidence that cancers, like normal organs, may be maintained by a hierarchical organisation that includes stem-like cells (SLC), precursor cells, and differentiated cells (Reya et al., 2001; Tu et al., 2002). The stem-like cells that feed the bulk cancer cells have been termed as cancer stem cells (CSCs). Most of these tumorigenic SLCs were identified using markers that identify putative normal stem cells. Interestingly, SLCs have also been identified in immortalized cell lines (Benchaouir et al., 2004), long-term cultured cancer cells (Hirschmann-Jax et al., 2004; Kondo et al., 2004) or patient samples (Hirschmann-Jax et al., 2004) using the side population technique.

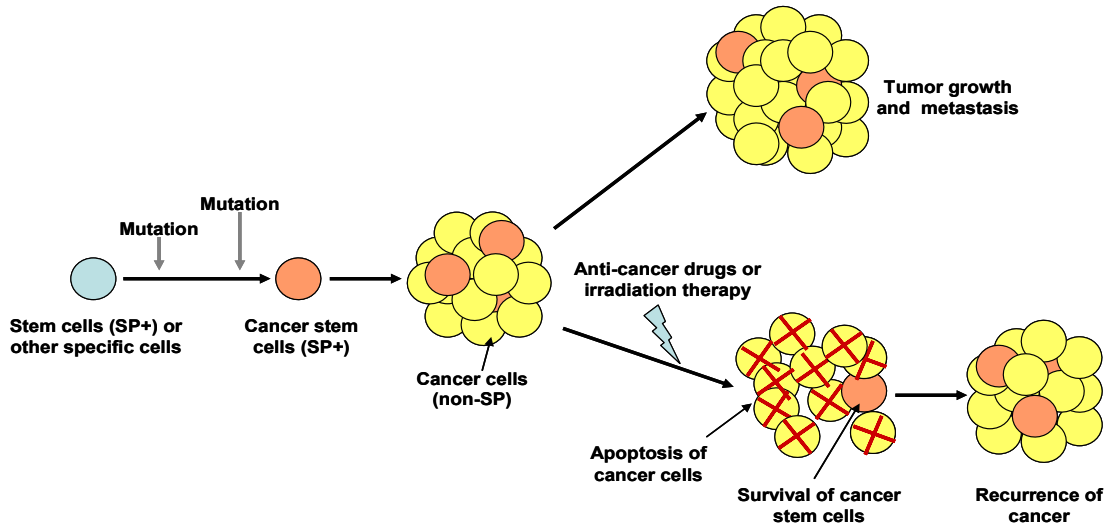

Figure 1.11 Role of cancer stem cells in malignancy. Cancer stem cells are thought to arise from either stem cells or some specific cells, which can revert to stem cells. The cancer stem cells self-renew, generate cancer cells and form huge malignant tumors. Small cell aggregates containing cancer stem cells transfer through blood vessels, invade into other tissues, and form metastatic tumors. Both anti-cancer drugs and irradiation cause cancer cells to die by apoptosis, however cancer stem cells might survive and regenerate cancer. (Adapted from Takao Setoguchi, Cell Cycle 2004).

Efforts to demonstrate the functional properties of SP in AML have revealed the heterogeneous nature of SP cells in terms of expression of known haematopoietic markers (Feuring-Buske and Hogge, 2001; Wulf et al., 2001). The concept of CSCs within the malignant SP cells has been strengthened by the following studies: Wulf et al detected a malignant $CD45^+$ $CD34^{low/neg}$ SP cell subset in more than 80% of the AML patients studied. These cells generated $CD45^+$ $CD34^+$ malignant haematopoietic stem cells as well as committed myeloid progenitors (Wulf et al., 2001). A much more detail study based on Hoechst 33342 SP phenotype, performed by Feuring-Buske et al, demonstrated the presence of normal progenitors within the SP^+ $CD34^+CD38^-$ compartment of samples from AML patients. When SP^+ cells were sorted according to their expression of CD34 and CD38 markers, $CD34^+CD38^-$ cells from 9 out of 15 AML samples were entirely cytogenetically normal, as were most CFC, LTC-IC and SRC from this population. In contrast, leukemic cells were detected among SP^+ $CD34^-CD38^-$ and among $CD34^+CD38^-$ cells outside the side population (Feuring-Buske and Hogge, 2001), demonstrating that CSCs can be located within a subset of SP, and that SP alone is perhaps not a sufficient marker for the tumorigenic stem-like cells in cancers.

In this study we have made an effort to elucidate the functions of *ABCG2* in HSC.

2 Aim of the study.

HSC are required to repopulate all blood cell lineages throughout the lifespan of an individual. This process of blood formation requires both stem cell divisions that produce lineage committed progenitors, and self renewal divisions that maintain stem cell pool *in vivo*. The cellular controls that regulate these decisions remain largely unknown, and are a major focus in the field of haematopoietic research. The expression of ABC proteins and their ability to transport Hoechst and Rhodamine dye as well as a number of chemotherapeutic drugs is another important feature observed in HSC. The expression pattern of the ABCG2 and the related ABC transporter MDR1 is stem cell associated. The overexpression of human *MDR1* in murine BM resulted in HSC expansion and a myeloproliferative disorder in mice. Enforced expression of human ABCG2 in murine BM cells significantly blocked haematopoietic development (Zhou et al., 2001). These studies collectively suggest a role for *ABCG2* in early haematopoiesis. However, till date there is no study demonstrating the role of *ABCG2* in normal human haematopoiesis.

The aim of the current study is to understand the biological role of *ABCG2* in primitive human haematopoiesis. In this regard we have tried to address the following specific questions:

- Does the constitutive expression of *ABCG2* induce an abnormal proliferation or differentiation block as the *MDR1*?
- What are the effects of constitutive expression of *ABCG2* in haematopoietic stem cells at the level of the stem cell and early progenitors?

To answer these questions, we have used a retroviral gene transfer approach to constitutively express *ABCG2* in candidate human HSC.

3 Materials & Methods

3.1 Materials

3.1.1 Plasmids

MSCV-IRES-YFP: A modified form of the MSCV vector that contains a bicistronic EYFP expression cassette with an internal ribosomal entry site (IRES) from the encephalomyocarditis virus (ECM). This vector (called MIY) was kindly provided by Keith Humphries (Terry Fox Laboratory, Vancouver, Canada).

***ABCG2*:** Human *ABCG2* gene was purchased from the RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany.

3.1.2 Antibodies

ABCG2: Monoclonal antibody against human ABCG2 (Clone BXP-21) was purchased from Kamiya Biomedical Company, Seattle, WA.

Goat anti-mouse: PE conjugated goat anti-mouse secondary antibody was purchased from R & D Systems GmBH, Germany

Table 3.1 Antibody combinations used to study the transplanted human cells in NOD/SCID mice.

No.	Antibodies	Target
PE conjugated		
1	CD45	Lymphocytes
2	CD34	HSC
3	CD33	Myeloid
4	CD15	Neutrophils & Eosinophils
5	Glyco A	Mature erythroid cells
6	CD41a	Megkaryocytes
7	CD 117	HSC
8	CD16	NK Cells
9	Isotype	Control
APC conjugated		
10	CD71	Lympocytes/erythroid precursors

11	CD38	Haematopoietic progenitors
12	CD19	B-cells
13	CD36	Early erythroid cells
14	CD56	NK Cells
15	CD133	HSC
16	Isotype	Control

3.1.3 Primers

Oligonucleotide	Sequence 5' to 3'
ABCG2 forward	ATTGAAGGCAAAGGCAGATG
ABCG2 reverse	TGAGTCCTGGGCAGAAGTTT
β-ACTIN forward	CTTCAACACCCCAGCCAT
β-ACTIN reverse	TAATGTCACGCACGATTTCC

3.1.4 Mammalian cell lines

Phoenix Ampho: Packaging cell line is used for transient, episomal stable, and library generation for retroviral gene transfer experiments (Stanford University, Medical Centre, USA).

PG13: PG13 is a mouse embryonic fibroblast used as a retroviral packaging cell line. It was purchased from ATCC. Introduction of retroviral vectors into PG13 cells results in the production of retrovirus virions capable of infecting cells from many species excluding mice.

K562: An erythroleukemia cell line derived from a chronic myeloid leukemia patient in blast crisis. K562 cells were purchased from ATCC.

M2-10B4 j-GCSF-tkneo j-IL-3-hytk: Murine M2-10B4 fibroblasts engineered to produce high levels of both human granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3; 190 and 4 ng/mll, respectively), referred henceforth as M2-10B4 G-CSF / IL-3, were provided courteously provided by Connie Eaves (Terry Fox Laboratory, Vancouver, Canada).

Sl/Sl j-SF-tkneo j-IL-3-hytk: Sl/Sl fibroblasts engineered to produce high levels of soluble Steel factor (SF), with or without production of the transmembrane form of SF (60 and 4 ng/ ml, respectively), referred henceforth as Sl/Sl SF / IL-3, were provided kindly by Connie Eaves (Terry Fox Laboratory, Vancouver, Canada).

3.1.5 Bacterial strain

- E. coli DH5α

3.1.6 The NOD-SCID Mice:

The NOD/LtSz-*scid* strain was generated by crossing the *SCID* mutation from C.B-17- *scid* mice onto the NOD background. C.B-17-*scid* mice lack functional T & B lymphocytes. The NOD strain mouse is an animal model of spontaneous autoimmune T-cell mediated insulin dependent diabetes mellitus (IDDM); however they have multiple defects in innate immunity. They are deficient in NK cell activity; display defects in myeloid development and function, and cannot generate either the classical or alternative pathways of haemolytic complement activation. The NOD/LtSz-*scid* lacks an adaptive immune system; due to the absence of T cells, they do not develop autoimmune IDDM and remain insulitis- and diabetes free throughout life. However they carry the innate immune defects present in the parental NOD/Lt stock of mice (Lowry et al., 1996). NOD/SCID mice were bred from the breeding pairs originally obtained from Taconic Europe and maintained in the animal facility located at the GSF-Haematology, Munich. All animals were handled under sterile conditions and maintained under micro isolators.

3.1.7 Mice related reagents and equipment:

Avertin solution: Stock solution was prepared by adding 15.5 ml tert-amyl alcohol to 25 grams Avertin (2-2-2 Tribromoethanol), both procured from Sigma-Aldrich, St. Louis, MO and dissolved overnight. For working solution, 0.5 ml stock solution was added to 39.5 ml of cell culture grade phosphate buffered saline (PBS) and dissolved with a magnetic stirrer.

Formalin: 10% solution of formaldehyde from Sigma-Aldrich, St. Louis, MO in water was used for fixing parts of animal tissues.

Sterile Syringes: BD Plastipak 1 ml syringe (BD Biosciences, Palo Alto, CA) for injection of cells in mice and Kendall Monoject 3 ml syringes (Tyco Healthcare, UK) for bone marrow flushing and plating of CFC.

Sterile needles: 0.5 x 25 mm for *intra venous* injection of cells in mice and 0.55 x 25 mm (BD Microlance, Drogheda, Ireland) for bone marrow aspiration from living

mice and flushing of bone marrow from extracted bones. 16 X 1.5 inch needles for dispensing and plating Methocult (CFC) media (Stem Cell Technologies, Vancouver, Canada)

Ammonium Chloride solution: For erythrocyte lysis 0.8% NH_4Cl with 0.1 mM EDTA (Stem Cell Technologies, Vancouver, Canada)

Heparinized capillaries: (Microvette CB 300) plastic capillaries for collection of blood, containing 15 units (I.E) Lithium heparin per ml of blood (Sarstedt, Numbrecht, Germany)

3.1.8 Commercial kits

MACS CD34 and CD133 cell Isolation kits: CD34 and CD133 HSC were isolated from UCB using MACS kits from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany.

DNeasy mini kit: Genomic DNA extraction kit from small cell numbers purchased from Qiagen GmbH, Hilden, Germany.

Small-scale plasmid preparation: GFX miniprep kit for isolation of plasmid DNA from bacteria purchased from Amersham Biosciences GmbH, Freiburg, Germany.

Gel elution of DNA and PCR product or DNA cleanup: GFX gel elution and PCR purification kit for DNA elution from gels and clean up of PCRs purchased from Amersham Biosciences GmbH, Freiburg, Germany.

Total RNA and genomic DNA isolation: Total RNA isolation reagent (TRIZOL) and Genomic DNA isolation reagent DNAZOL were purchased from Invitrogen, Carlsbad, CA.

Molecular weight markers: Nucleic acid size standards, 1 kb and 100 bp ladder were purchased from New England Biolabs (NEB, Beverly, MA).

Enzymes: T4 DNA ligase, *Xho I, Eco RV, Eco RI, Hpa I,* and *Pme I* were purchased from New England Biolabs (NEB, Beverly, MA)

RT and PCR: Platinum Taq DNA polymerase kit, ThermoScript RT-PCR kit and DNaseI DNA inactivating enzyme kit were purchased from Invitrogen, Carlsbad, CA

Real time PCR kit: LightCycler FastStart DNA Master SYBR green I kit were purchased from Roche Diagnostics, Mannheim, Germany. LightCycler Carousel, carousel centrifuge and the LightCycler data analysis software from Roche Diagnostics were used.

3.1.9 Buffers and special medium

All buffers were prepared in deionized water.

TAE buffer: (for agarose gel electrophoresis)

- Tris-HCl (pH 8.2) 40 mM
- Acetic Acid 20 mM
- EDTA (pH 7.6) 2 mM

FACS buffer:

- Phosphate buffered saline (PBS)
- FBS 3%
- Propidium Iodide 1 µg/ml

MACS buffer:

- PBS (pH 7.4)
- FBS 3%
- EDTA 2 mM

Serum free medium (SFM):

IMDM (with L-glutamine) supplemented with the following:

- BIT 9500 (BSA, insulin and transferrin) 20%
- 2-Mercaptoethanol 10^{-4} M
- Low-density lipoproteins (LDL) 40 µg/ml

Haematopoietic cell washing buffer:

IMDM supplemented with 2% FBS was used for washing of haematopoietic cells. The same buffer was used as a medium for injection of cells in mice.

3.1.10 Microscopes

- Axiovert 25 Inverted light microscope from Carl Zeiss, Germany, was used for normal visualization of cells in culture and for scoring colonies in CFC assays.
- Axiostar upright light microscope from Carl Zeiss, Germany, was used for cell counting and visualisation of cytospin preparations.

3.1.11 Fluorescence activated cell sorting and analysis

BD FACS Vantage SE System from BD Biosciences, Palo Alto, CA was used for fluorescence activated cell sorting. BD FACS Calibur System was used for acquisition and analysis of fluorochrome labelled cells.

3.1.12 Reagents

No.	Reagents	Company
1	Acetic acid	Sigma-Aldrich, Germany
2	Agar	Sigma-Aldrich, Germany
3	Agarose	Sigma-Aldrich, Germany
4	Avertin	Sigma-Aldrich, Germany
5	BIT	Stem Cell Technologies, Canada
6	Bromphenolblue	Sigma-Aldrich, Germany
7	BSA	Sigma-Aldrich, Germany
8	Calcium Chloride	Sigma-Aldrich, Germany
9	Cytofix (Cell fixation reagent)	BD Pharmingen, Germany
10	DMEM	PAN Biotech, Germany
11	DMSO	Sigma-Aldrich, Germany
12	Ethanol	Sigma-Aldrich, Germany
13	Ethidium Bromide	Sigma-Aldrich, Germany
14	Fetal Bovine Serum	PAN Biotech, Germany
15	Formaldehyde	Sigma-Aldrich, Germany
16	G-418 (Geneticin)	GIBCO, Invitrogen Corporation,Germany
17	HEPES	GIBCO, Invitrogen Corporation,Germany
18	HLTM (Myelocult H5100)	Stem Cell Technologies, Canada
19	Hydrocortisone (solucortef)	Stem Cell Technologies, Canada
20	IMDM	GIBCO, Invitrogen Corporation, Germany
21	Isopropanol	Sigma-Aldrich, Germany

22	Methanol	Sigma-Aldrich, Germany
23	Methocult H4434 (Methylcellulose)	Stem Cell Technologies, Canada
24	Pancoll	PAN Biotech, Germany
25	PBS	PAN Biotech, Germany
26	Penicillin/Streptomycin	GIBCO, Invitrogen Corporation, Germany
27	Protamine sulfate	Sigma-Aldrich, Germany
28	RPMI	PAN Biotech, Germany
29	SDS	Sigma-Aldrich, Germany
30	SOC medium	Peqlab, Germany
31	Sodium Chloride	Sigma-Aldrich, Germany
32	Trypsin/EDTA	GIBCO, Invitrogen Corporation, Germany
33	β- Mercaptoethanol	Sigma-Aldrich, Germany

3.1.13 Cytokines

No.	**Cytokine**	**Company**
1	Flt-3-ligand	PAN Biotech GmbH, Germany
2	G-CSF	Immuno Tools, Germany
3	IL3	ImmunoTools, Germany
4	IL6	Tebu-bio, Germany
5	SF Steel factor	ImmunoTools, Germany

3.1.14 Apparatus

No.	**Apparatus**	**Company**
1	Biophotometer	Eppendorf, Germany
2	Cell culture dishes (3, 6, 10, 15 cm)	Sarstedt, Germany
3	Cell culture pipettes (2, 5, 10, 25 ml)	Corning Inc., NY
4	Cell scrapers	Sarstedt, Germany
5	Cell strainers	BD Biosciences, CA
6	Centrifuge (5810R)	Eppendorf, Germany
7	Centrifuge (Megafuge1.0)	Herraus, Germany
8	CO2 Incubator	UniEquip, Germany
9	Cryotubes	Nunc

10	Cytospin 2 Shandon apparatus	Thermo Electron Corp., USA
11	Cytospin filter cards	Histocom AG, Switzerland
12	Cytospin slides	Marienfield, Germany
13	PCR cycler	Peqlab, Germany
14	PCR soft tubes	Biozyme, Germany
15	Pipetteman	Eppendorf, Germany
16	Thermomixer	Eppendorf, Germany
17	UV transilluminator	Vilber Lourmat, France
18	Electrophoresis system-Horizontal	Peqlab, Germany

3.2 Methods

3.2.1 Cell culture

- PG13 cell lines were cultured in DMEM supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% Glutamax at 37°C in 5% CO_2 atmosphere.
- K562 cell were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin at 37°C in 5% CO_2 atmosphere.
- Phoenix Ampho packaging cells were cultured in DMEM supplemented 15% FBS, 1% penicillin/streptomycin and 1% Glutamax at 37°C in 5% CO_2 atmosphere.
- M2-10B4 j-GCSF-tkneo j-IL-3-hytk cells were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, the selection antibiotics G418 (0.4 mg/ml) and Hygromycin B (0.06 mg/ml) at 37°C in 5% CO_2 atmosphere.
- Sl/Sl j-SF-tkneo j-IL-3-hytk cells were cultured in DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, the selection antibiotics G418 (0.8 mg/ml) and Hygromycin B (0.125 mg/ml) at 37°C in 5% CO_2 atmosphere

3.2.2 Freezing of mammalian cells

Cells grown to a confluency of 90-95% in 10 cm cell culture dishes were washed with warm PBS and trypsinized. Trypsinized cells were washed using their respective cell culture medium and pelleted down at 400 x g for 5 min. The pellets were resuspended in 0.9 ml FBS. DMSO was added drop wise to the cells in FBS to a

final concentration of 10%. Aliquots were transferred to cryotubes and stored in freezing containers at -80°C for 24 hours and transferred to liquid nitrogen for long term storage.

3.2.3 Thawing of cells

Frozen cryotubes were thawed in a water bath at 37°C and immediately transferred to a 15 ml tube. Warm 2 ml culture medium (DMEM or IMDM with 10%FBS) was added and mixed gently. This was followed by addition of 8 ml culture medium and centrifugation at 400 x g for 5 minutes. The cell pellet obtained is resuspended in culture medium and placed into culture dishes or flasks and placed in CO_2 incubator. A small aliquot of cells were taken apart for cell counting. For thawing UCB mononuclear cells, DNase I was used (0.1 mg/ml) to prevent cell loss through clumping.

3.2.4 ABCG2 cloning

The full-length *ABCG2* (2.2 Kb) was sub cloned as *Hpa I* fragment upstream of the IRES sequence linked to the gene encoding the enhanced yellow fluorescent protein (EYFP) in the MIY vector. The resultant vector (*ABCG2*-YFP) was used for the preparation of stable packaging cell lines. As a control, the MSCV vector carrying only the IRES yellow fluorescent protein cassette (YFP virus) was used (fig 3.1). High-titre, helper-free recombinant retrovirus was generated by first transfecting the Phoenix Ampho packaging cell line and subsequently transducing PG13 packaging cells. High-titre producer clones were isolated for each virus.

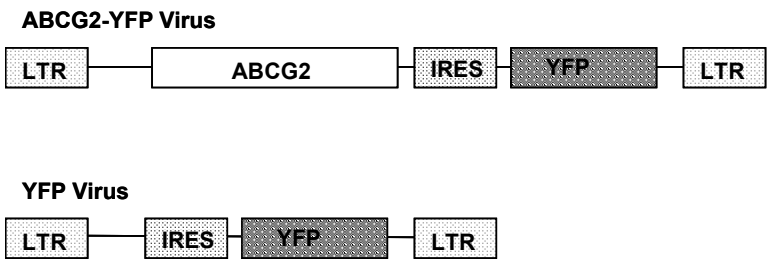

Figure 3.1 Retroviral constructs. Schematic representation of the retroviral constructs used for transduction of UCB Lin^- cells.

3.2.5 Developing high titre producer cell lines

Phoenix Ampho cells cultured in DMEM with 10% fetal bovine serum were plated at 2.5 x 10^6 cells per 10 cm plate one day before transfection. Medium was changed 4 hours prior to transfection. In a 5 ml tube 20 µg of plasmid DNA, 62.5 µl of 2M $CaCl_2$ and dH_2O to make up to 500 µl were mixed together. 500 µl of 2x HBS was added drop wise to form a precipitate within the next few minutes. This mixture was then added to the cells drop wise. After about 12 hours, the medium was replaced with fresh medium to collect virus particles. The virus containing medium (VCM) was collected after 24 hours, filtered trough 0.45 µm filter, supplemented with protamine sulphate to give a final concentration of 5 µg/ml, and layered on PG13 packaging cells for viral infection. After repeated infections (3-5 times), PG13 cells were allowed to express the YFP contained in the plasmid vector for a period of about 48 hours & then YFP expressing cells were sorted out using FACS and cultured for up to 2 weeks. From these YFP^+ PG13 cells, single cells were sorted into 96 well plates, expanded & viral production was titred using K562 cells. Individual YFP^+ PG13 clones were tested & the clone that was producing the highest viral titre was identified & used for infecting umbilical cord blood derived haematopoietic cells.

3.2.6 Purification of human UCB $CD133^+$ and $CD34^+$ cells

Density Gradient Centrifugation:

Umbilical cord blood was collected in heparinised syringes according to institutional guidelines following normal full-term deliveries. Informed consent was obtained in all cases. Mononuclear cells (MNC) were separated using density gradient centrifugation. Fresh umbilical cord blood, not older than 12 hours, was diluted with 2 volumes of PBS and layered over Pancoll. Usually 35 ml of diluted blood was layered over 15 ml Pancoll in a 50 ml conical tube. This was centrifuged at 400x g for 30 minutes at 20°C in a swinging-bucket rotor without brakes. The upper layer was aspirated and discarded, leaving the interphase undisturbed. The interphase containing MNC such as lymphocytes, monocytes and thrombocytes was then transferred to a new 50 ml tube, washed twice with large volumes of PBS, and then counted before labelling with magnetic bead or fluorochrome conjugated antibodies.

Magnetic Separation:

$CD133^+$ cell purification was conducted using MACS CD133 Cell Isolation Kit that uses positive selection method. Cells were resuspended in a volume of 300 µl per 10^8 cells, blocked with 100 µl of FcR Blocking Reagent and labelled with 100 µl of

CD133 Microbeads. When working with higher cell number, all the reagent volumes & the total volume was scaled up accordingly. This was followed by incubation for 30 minutes at 4-8°C. Cells were then washed twice by adding 10x the labelling volume of buffer and centrifuged at 300 x g for 15 minutes. The resultant cell pellet was then resuspended in 500 µl of MACS buffer and loaded into MS Column mounted on magnetic separator. The negative cells were allowed to pass through and the column was washed at least three times with 2 ml buffer. The column was then removed from the separator, placed on a collection tube, loaded with fresh buffer, and the magnetically labelled cells flushed out using the plunger. The magnetic separation was usually repeated to get a purity of more than 95%. Purified cells were then frozen in FBS with 10% DMSO and thawed when needed for pre-stimulation and transduction.

FACS sorting:

$CD34^+$ cell enrichment was done either by MACS as done for $CD133^+$ cells or by FACS. For separation by FACS, MNCs were thawed from frozen stocks or prepared freshly from UCB and labelled using anti CD34-PE antibody (100 µL per 10^8 cells), for 30 minutes on ice. Labelled cells were then washed twice with PBS, resuspended in FACS buffer and sorted. The sorted cells with purity above 95% were used for 48 hour pre-stimulation followed by transduction.

3.2.7 Pre-stimulation and transduction of human cells

Frozen $CD133^+$ or $CD34^+$ cells were thawed and briefly prestimulated before transduction: Cells at 2×10^5/ ml were cultured for 48 hours in serum-free medium (SFM), comprising of Iscoves modified Dulbecco medium (IMDM) supplemented with the serum substitute (BIT),10^{-4} M mercaptoethanol and 40 µg/ml low-density lipoproteins (LDL). Following recombinant human cytokines were added to the SFM: 100 ng/ml Flt-3 ligand, 100 ng/ml steel factor (SCF), 20 ng/ml interleukin-3 (IL-3), 20 ng/ml IL-6, and 20 ng/ml granulocyte colony-stimulating factor (G-CSF). After 48 hours, cells were resuspended in filtered VCM supplemented with the same cytokine combination and plated on tissue culture dishes that were preloaded twice with VCM, each time for 30 minutes. Protamine sulphate (5 µg/ml) was added to enhance viral infection. This procedure was repeated on the next 2 consecutive days for a total of 3 infections. For *in vitro* studies, aliquots of these cells were transferred to fresh SFM plus the same additives and cytokines and then incubated for an additional 48 hours prior to being stained with PE-labelled anti-CD34 antibody and

isolation of the $YFP^+/CD34^+$ cells on a FACSVantage (Becton Dickinson) sorter. For *in vivo* studies, transduced cells were injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice after final transduction (< 6 hours after the last exposure to fresh VCM) without pre-selection (fig 3.2).

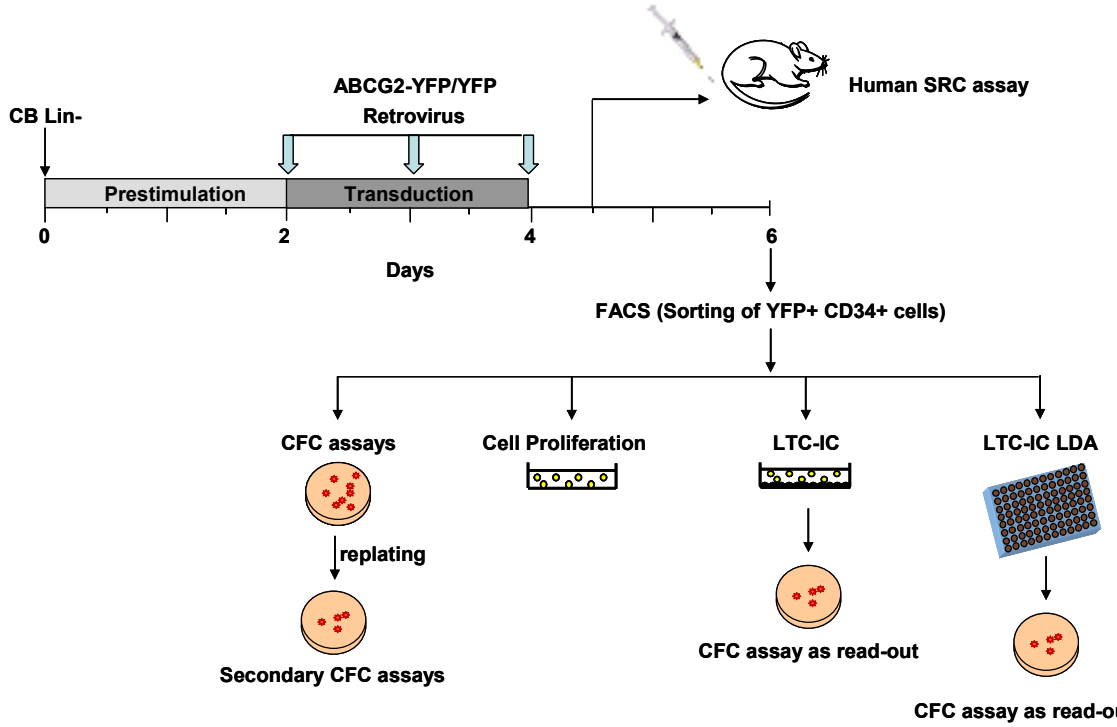

Figure 3.2 Experimental Plan. Human UCB derived $CD133^+$ cells ($CB\ Lin^-$) are prestimulated and retrovirally transduced with *ABCG2*-YFP and YFP alone (control) thrice. Transduced CB cells that express CD34 are sorted out using FACS and placed into various *in vitro* assays 2 days after the final transduction. For the SRC assay, cells are injected in the NOD/SCID mice without any preselection 6-10 hours after the final transduction.

3.2.8 Human CFC Assay

Haematopoietic colony-forming cells (CFC) were assayed using methylcellulose-based medium (MethoCult; H 4434) (fig 3.3). Required number of pre-aliquoted tubes of MethoCult medium was thawed overnight under refrigeration (2-8°C) or at room temperature. The cells were diluted with IMDM + 2% FBS to 10X the final concentration(s) required for plating. For a duplicate assay 0.3 ml of diluted cells was added to 3 ml MethoCult tube and the contents vortexed vigorously. After about 5 minutes, 1.1 ml of cell: methylcellulose mixture was dispensed into 35 mm culture dishes using sterile a 3 ml syringe and16-gauge bunt-end needle. The 35 mm culture dishes were placed into 10 cm petri dish along with an extra 35 mm dish containing sterile water to maintain humidity and placed in a CO_2 incubator at 37°C and >95%

humidity. CFC numbers were evaluated after an incubation period of 12-14 days and distinguished into following classes:

Colony-forming unit-erythroid (CFU-E): Produces 1-2 cell clusters containing a total of 8-200 erythroblasts. A CFU-E consists of mature erythroid progenitors that require erythropoietin (EPO) for differentiation.

Burst-forming unit-erythroid (BFU-E): Produces a colony containing >200 erythroblasts in a single or multiple clusters. A BFU-E consists of more immature progenitors than CFU-E and require EPO and cytokines with burst-promoting activity such as Interleukin-3 (IL-3) and Stem Cell Factor (SCF) for optimal colony growth.

Colony-forming unit-granulocyte, macrophage (CFU-GM): Produces a colony containing at least 20 granulocyte cells (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM). CFU-GM colonies arising from primitive progenitors may contain thousands of cells in single or multiple clusters.

Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM): A multi-potential progenitor that produces a colony containing erythroblasts and cells of at least two other recognizable lineages. Due to their primitive nature, CFU-GEMM tend to produce large colonies of >500 cells.

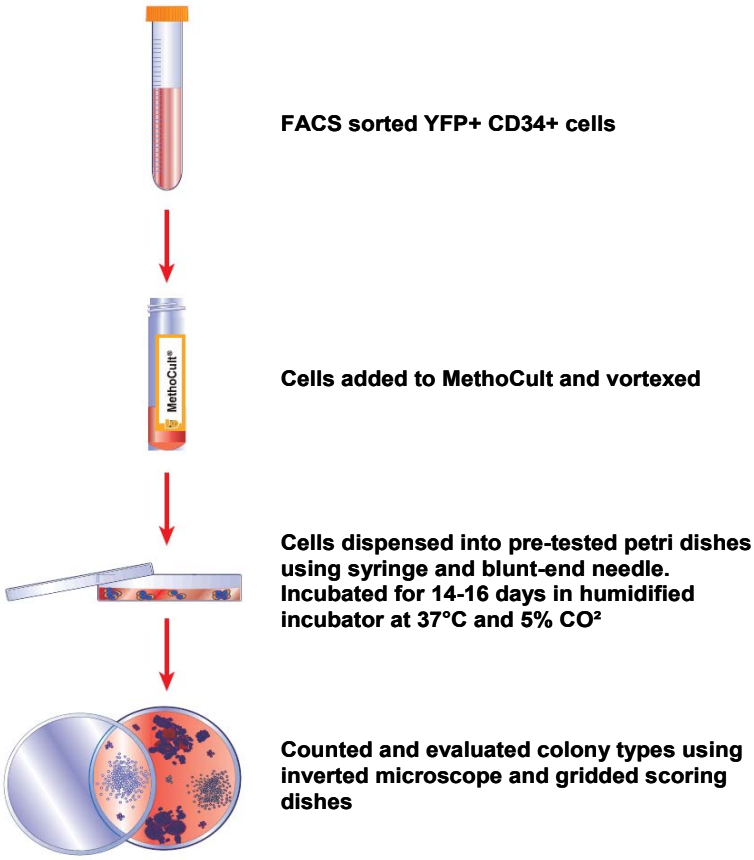

Figure 3.3 CFC assay procedure diagram. Human haematopoietic CFC assay in methylcellulose media detects haematopoietic progenitors (Figure adapted from the Stem Cell Technologies Catalogue).

Table 3.2 Progenitor cells and the corresponding colonies

Colonies	Progenitor cell
Granulocytes	CFU-G
Macrophages	CFU-M
Erythroid	BFU-E
	CFU-E
Megakaryocytes	CFU-Meg
Eosinophils	CFU-Eo
Granulocytes, Macrophages, Erythroid, Megakaryocytes	CFU-GEMM
Mast cells	CFU-mast
Fibroblasts	CFU-F

3.2.9 Liquid expansion assay

To set up liquid suspension cultures, transduced YFP^+ $CD34^+$ cells were placed in the same cytokine-supplemented serum-free medium described above (section

1.2.7) for a period of up to 5 weeks. Weekly half media exchange was performed by removing half of the cells and medium and replacing them with fresh cytokine supplemented SFM. Aliquots of cells were taken from the medium removed and placed in methylcellulose for clonogenic progenitor assay. The colonies were scored after 14 days.

3.2.10 Human Long-Term Culture-Initiating Cell (LTC-IC) Assay

The two engineered murine fibroblast cell lines M2-10B4 G-CSF / IL-3 (expressing human G-CSF and IL-3) and Sl/Sl SF / IL-3 (expressing human SF and IL-3), were grown in selection medium. A 1:1 mixture of the above mentioned cell lines, was irradiated at 8000 cGy, and plated in collagen coated tissue culture dishes (3.0×10^5 cells) for more than 24 hours before the addition of test cells. For adding the test cells, the medium of the dishes was replaced with freshly prepared human long-term culture medium (HLTM; Myelocult H5100), containing the required concentration of test cells ($1x\ 10^4 – 2x10^4$), taking care not to disturb the underlying feeder cells. Hydrocortisone (Solucortef) was added to the HLTM to achieve a final concentration of 10^{-6} M. The dishes were incubated in a CO_2 incubator at 37°C. Weekly half of the HLTM and cells were replaced with freshly prepared HLTM. Following 5-6 weeks of incubation, LTC-IC cultures were harvested, including the adherent cells using Tyrpsin/EDTA. The harvested cells were washed twice, counted and re-plated in MethoCult for CFC assay. The CFC dishes were scored after 14-16 days. The number of LTC-IC present in the initial test cell suspension was calculated by dividing the total number of CFC detected in the culture by the average number of clonogenic progenitors per LTC-IC for the standard conditions used. Values may also be expressed as LTC-IC derived CFC per number of test cells.

3.2.11 LTC-IC Limiting Dilution Analysis

M2-10B4 G-CSF / IL-3 and Sl/Sl SF / IL-3 cells (1:1 mixture) were established in 96-well flat-bottom culture plates at a density of 1.25×10^4 cells (fig 3.4). On the day of assay, HLTM was removed using multi-channel pipettor with sterile tips and discarded. The test cells were added to the wells in 0.2 ml of HLTM with solucortef. The number of cells seeded per well is indicated in table 3.3. LTC-IC cultures were incubated at 37°C in humidified incubator (>95%) with 5% CO2 in air for six weeks. For weekly half media exchanges, one half of the medium and cells were removed and replaced with HLTM for five weeks.

Table 3.3 Initial test cell concentrations taken for the LTC-IC limiting dilution analysis.

Cell number per well	Number of wells
1	20
10	20
50	20
200	20
800	16
3200	12
12800	10

To harvest the LTC-IC, the HLTM and non-adherent cells were removed from wells and place into individual 12x75 mm sterile tubes using a pipette and sterile tips. Single wells were harvested at a time to avoid cross contamination of samples. Wells were rinsed once with 0.2 ml PBS and added to tube. 0.1 ml Trypsin-EDTA was added to each well and incubated for 3 to 5 minutes and examined for detached cells. Once the adherent cells are detached, the wells are washed with more PBS and the medium collected in the appropriated tube. The wells are finally washed with 0.2 ml IMDM containing 2% FBS and transferred to the appropriate tube. The tubes were centrifuged at 1200 rpm for 10 minutes and the supernatant removed without disturbing cell pellet. Approximately 0.1 ml of medium was left along with the cell pellet and vortexed. To this 1 ml of Methocult (H4435) methylcellulose medium was added and vortexed again. Each tube (contents of one well) was plated individually into 35 mm petri dish with 1 ml syringe (without needles attached). Several dishes (6-8) were placed in a 15 cm petri-dish along with an additional 60 mm open dish containing 5 ml sterile water to maintain humidity. The dishes are incubated at 37°C in humidified incubator (>95%) with 5% CO2 in air for 12 to 16 days. Colonies were counted and a well scored as positive if one or more BFU-E, CFU-GM or CFU-GEMM were detected or scored as negative if no colonies were present. The LTC-IC frequency in the test cell population was calculated from the proportion of negative wells (no CFC present) and the method of maximum likelihood. Statistical analysis was performed using L-Calc™ software for limiting dilution analyses.

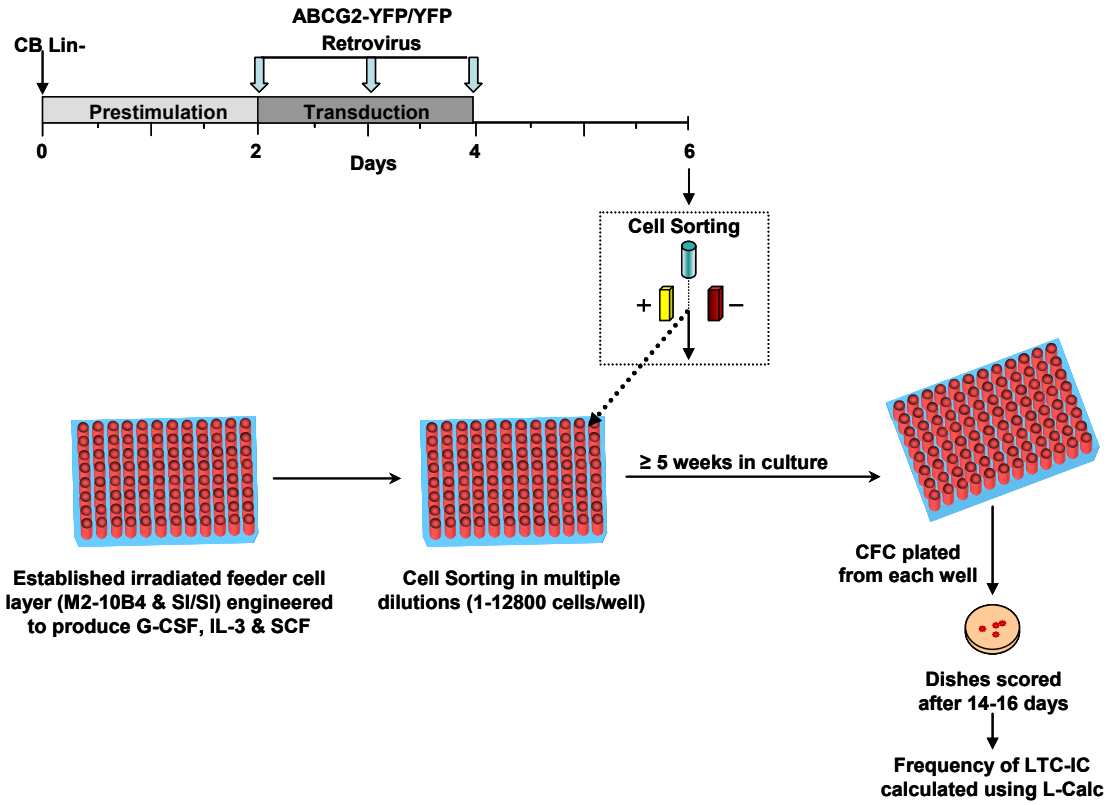

Figure 3.4: Experimental plan for setting up limiting dilution analysis.

3.2.12 B-cell progenitor assay

B-cell progenitor activity was assessed by plating 2 x 10^4 YFP^+CD34^+ cells on murine MS-5 cells in RPMI 1640 with 10% fetal calf serum (FCS) and 5% human AB serum with either 50 ng/mL SF, 10 ng/mL IL-2, and 10 ng/mL IL-15 or with these same factors plus 10 ng/mL IL-7, 100 ng/mL FL, and 50 ng/mL thrombopoietin, conditions permissive for B-lymphoid development in addition to varying degrees of myeloid cell development. After 3 or 6 weeks, both adherent and nonadherent cells were collected and analyzed by FACS for the expression of CD34, CD38, myeloid (CD15, CD33), lymphoid (CD3, CD19, CD20), erythroid (glycophorin A [GlyA], CD71), and megakaryocytic (CD41) and natural killer (NK) cell (CD56) markers.

3.2.13 Transplantation into NOD/SCID mice

Seven to eight week old NOD/SCID mice were sublethally irradiated at 275 cGy one day before injection. ^{137}Cs γ- irradiator was used. For transplantation, transduced cells were washed, counted, resuspended in PBS and injected into lateral tail vein of irradiated mice (300-400µL/mouse). Engraftments of transduced cells into mice were either studied by observing bone marrow aspirates at 3, 6 and 9 weeks or by sacrificing the mice at week 8.

Supra-vital BM aspirations:

BM aspirations were done at weeks 3, 6 and 9. Mice were anesthetized by intra-peritoneal injection of Avertin (0.4-0.75 mg/g in mice) and the medullary cavity of the femur was entered with a 25 gauge needle. The marrow was then aspirated by the syringe attached to the needle and subsequently transferred to a tube containing IMDM with 3% FCS and 0.2mM EDTA. This was followed by red cell removal & FACS staining.

BM analysis:

Mice were sacrificed according to the institutional guidelines. Hind limbs were removed from the animal and cleared of adhering muscles and other connective tissues. BM from the femurs and tibiae were flushed out using IMDM containing 10% FCS and 0.2mM EDTA. This was followed by red cell removal & FACS staining.

FACS staining:

The BM resuspended in IMDM (with 3% FCS, 0.2mM EDTA), was treated with 1% ammonium chloride solution (20 minutes on ice) for selective removal of red cells. The cells were washed, passed through a cell strainer (60 µ pore-size), and the viability was determined by trypan blue exclusion. Approximately 10^6 cells were resuspended in PBS (with 3% FBS) containing FcR blocking reagent and then divided into smaller aliquots for individual / batch staining with monoclonal antibodies indicated in table 3.1.

3.2.14 Intracellular staining with ABCG2 antibody

1x 10^6 cells were resuspended in 200 µl Cytofix to fix the cells, incubated for 20 minutes at 4°C and washed twice with 1x Permwash buffer. The cells were centrifuged at 280 x g for 5 minutes. The pellet was resuspended in 50 µl of PBS and the primary antibody was added and incubated for 15 minutes at 4°C. After incubation, the cells were washed twice with 1x Permwash buffer, resuspended once again in 50 µl PBS, and stained with fluorochrome (PE) conjugated secondary antibody for 15 minutes at 4°C in dark. After staining, the cells were washed twice with 1x Permwash buffer and resuspended in 500 µl PBS and analysed using FACS Calibur.

3.2.15 RNA and genomic DNA isolation and cDNA preparation

The Trizol reagent method described by the manufacturer was used to extract RNA with the addition of 1 ml Trizol solution per million cells. Equal amounts of RNA as

quantified by a spectrophotometer were added to each reaction (in a set) used for cDNA preparation for the semiquantitative PCRs. Each sample was treated with DNaseI for prevention of genomic DNA contamination in cDNA samples. This was performed for each sample prior to cDNA preparation according to the manufacturer's instructions.

Genomic DNA was isolated from a minimum of 1x 10^6 BM cells for retroviral integration detection using the DNAZOL reagent and the protocol for the same according to the manufacturer. Genomic DNA was resuspended in sterile water and quantified using a spectrophotometer after proper dissolution.

cDNA was prepared from DNaseI treated RNA. First-strand cDNA synthesis was done with ThermoScript kit. In a 20 µl reaction volume, 1 µg RNA and 1 µg of oligo (dT) were mixed to a final volume of 11 µl and incubated 10 minutes at 70°C. Then, 4 µl of 5 X first-strand buffer, 2 µl of DTT 0.1 mol/L, 1 µl of 10 mmol/L deoxynucleoside triphosphate mix, and 2 µl of ThermoScript reverse transcriptase were added. The sample was incubated 1 hour at 42°C and used for PCRs.

3.2.16 LM-PCR for detection of retroviral integration site

For the linker-mediated PCR (LM-PCR), integrated long-terminal repeats (LTRs) and flanking genomic sequences were amplified and then isolated using a modification of the bubble LM-PCR strategy (42, 43). Aliquots of the cell lysates from leukemic mice were digested with *Pst*I or *Ase* (New England Biolabs Inc.), and the fragments were ligated overnight at room temperature to a double-stranded bubble linker(5′-CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTCCTTG-3′ and 5′-ANTCAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG- 3′). Next, a first PCR (PCR-A) was performed on 10 µl (one-tenth) of the ligation product using a linkerspecific Vectorette primer (5′-CGAATCGTAACCGTTCGTACGAGAATCGCT- 3′) (Invitrogen Corp.)and an LTR-specific primer (LTR-A: 5′-CAACACACACATTGAAGCACTCAAGGCAAG-3′) under the following conditions: 1 cycle of 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds and 65°C for 1 minute, and 1 cycle of 72°C for 2 minutes. The bubble linker contains a 30-nucleotide nonhomologous sequence in the middle region that prevents binding of the linker primer in the absence of the minus strand generated by the LTR-specific primer. A 1-µl aliquot of the PCR-A reaction (one fifteenth) was then used as a template for a second nested PCR (PCR-B) using an internal LTR-specific

primer (LTR-B: 5′-GAGAGCTCCCAGGCTCAGATCTGGTCTAAC-3′) and the same linker-specific Vectorette primer as was used in PCR-A, with the following conditions: 1 cycle of 94°C for 2 minutes, 30 cycles of 94°C for 60 seconds and 72°C for 1 minute, and 1 cycle of 72°C for 2 minutes. Ten microliters (one-half) of the final PCR-B product was electrophoresed using 2% agarose tris-acetate- EDTA gel. Individual bands were excised and purified using the QIAEX II Gel Extraction Kit (QIAGEN) and then cloned into PCR2.1 (Invitrogen Corp.) before sequencing of the integration site of the retrovirus.

4 Results

4.1 Isolation of human UCB CD133$^+$ cells

Human umbilical cord blood is a relevant source of CD133$^+$ haematopoietic cells. CD133$^+$ cells (>95%) co-express the CD34 antigen, where as an average of 85 percent of CD34$^+$ cells co-expressed CD133 in UCB samples (Bonanno et al., 2004). UCB samples were collected and processed to deplete RBCs and subsequently immunoselected using microbead-conjugated anti-CD133 MoAb (Miltenyi Biotech). The mean volume of the initial UCB samples collected was 13.5 ml / donor (range, 5-35 ml). Isolation using MACS procedures yielded an absolute amount of 2.0 x 10^5 CD133$^+$ cells (range, 0.9-22). The enriched samples had an average CD133$^+$ cell frequency of 92.04 percent (range, 73-100). Enriched samples were also analysed for the expression of CD34 antigen. More than 95% of the CD133+ isolated cells coexpressed the CD34 antigen.

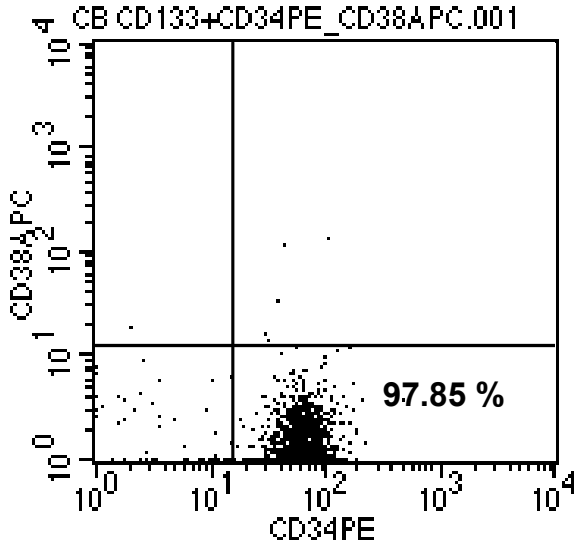

Figure 4.1 Representative dot plot analysis of the CD34 and CD38 markers on MACS-isolated CD133$^+$ cells.

4.2 Efficient retroviral transduction of *ABCG2* in human haematopoietic cells

In order to track the retrovirally transduced cells by FACS, the bi-cistronic vector containing *ABCG2* upstream of an IRES element and an YFP reporter gene was constructed in the MSCV viral backbone (*ABCG2*-YFP) as described in the methods section. The analogous vector containing YFP alone was used as a control (fig 4.2). Gene delivery efficiencies were determined by measuring the EYFP fluorescence 48 hours after the final transduction round. Twenty three percent (± 5%) and 54% (± 4%) of Lin^- cord blood cells exposed to *ABCG2*-YFP VCM or the YFP VCM, respectively, were found to be YFP^+ when examined by FACS, demonstrating high efficiency of gene transfer by both vectors. The CB cells were also analysed for the expression of CD34 marker after transduction. The overall percentage of CD34 expressing cells in the transduced and non transduced compartments did not show any difference between *ABCG2*-YFP and YFP transduced cells. However, in the *ABCG2*-YFP transduced CB cells the proportion of YFP^+ $CD34^+$ cells were 2.3 fold less than the proportion of YFP^+ $CD34^+$ cells in the control vector transduced cells (n= 19; mean *ABCG2*-YFP^+ $CD34^+$ = 6.5%, mean YFP^+ $CD34^+$ = 15%). This difference is due to the 2.3 fold lower transduction efficiency of the *ABCG2*-YFP virus.

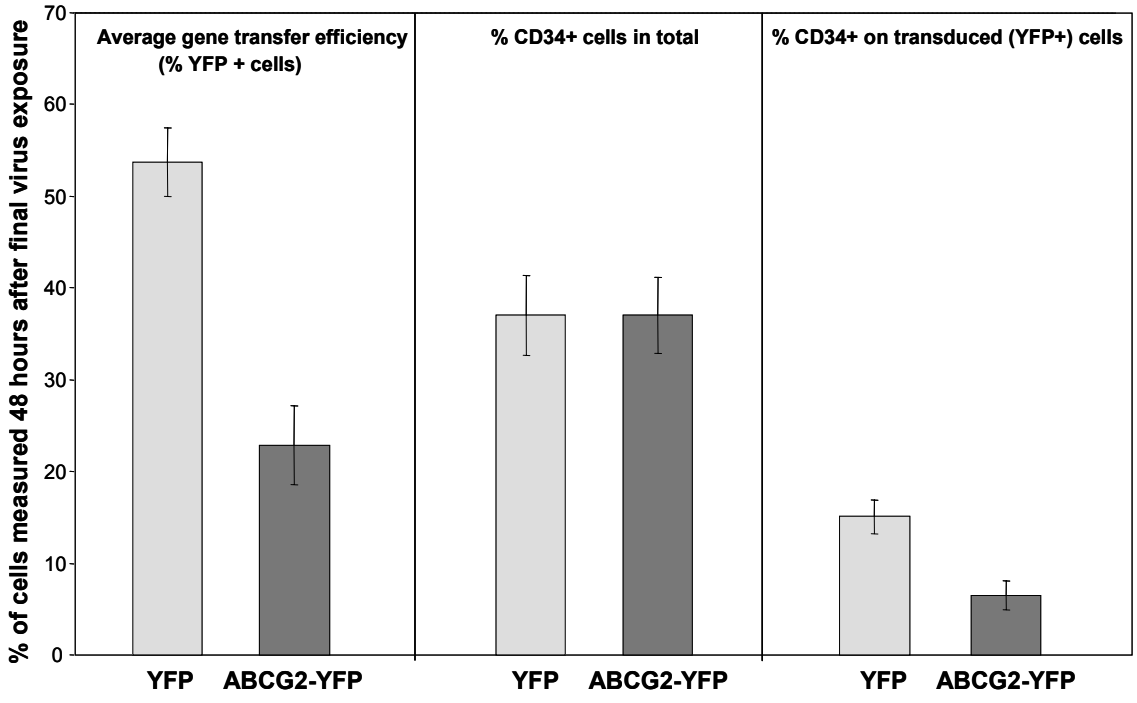

Figure 4.2 Gene transfer efficiencies. *ABCG2*-YFP and YFP (control) transduced $CD133^+$ cells were analysed 48 hours after final transduction for the expression of YFP using FACS Calibur in order

to determine the gene transfer efficiency. Expression of CD34 marker was also analysed and used for sorting out $CD34^+$ YFP^+ cells that were used subsequently in different *in vitro* assays. Although the proportion of total $CD34^+$ cells 2 days after transduction did not differ between the cells transduced with either vectors, the number of transduced $CD34^+$ cells expressing *ABCG2*-YFP was 2.4 fold lower than those expressing YFP vector. Values represent the mean (± s.e.m.) from 19 independent experiments.

4.3 Assessment of ABCG2 overexpression in retrovirally transduced cells

To determine whether the transduced cells properly expressed ABCG2, immunodetection was performed using flow cytometry. K562 cells transduced with *ABCG2* (K562-*ABCG2*) and with YFP vector control (K562-YFP) were stained using anti-ABCG2 antibody BXP-21 (fig 4.3). PE conjugated goat anti-mouse antibody was used to secondarily label the BXP-21 antibody. ABCG2 protein was clearly recognized in K562 cells transduced with ABCG2-virus but not in those transduced with YFP control vector.

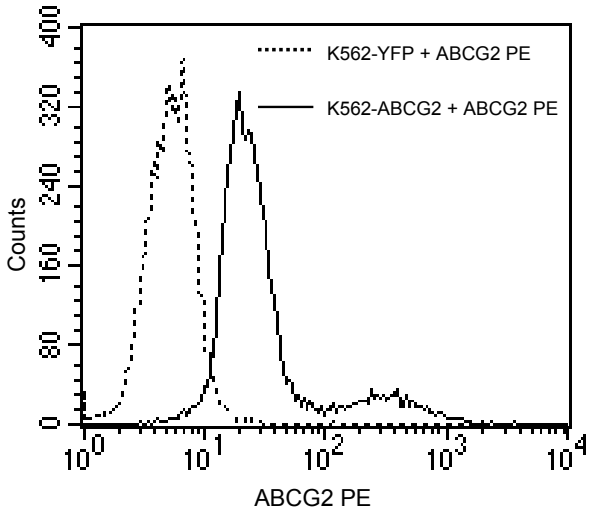

Figure 4.3 ABCG2 protein expression analysis. K562 cells were transduced with *ABCG2*-YFP virus (K562-*ABCG2*) or YFP control virus (K562-YFP) in the same manner as the UCB Lin^- cells. After 48 hours, transduced cells were fixed and permeabilized and stained using anti-ABCG2 antibody. A PE labelled secondary antibody (Goat anti-mouse Ab) was used to detect ABCG2 positive cells by FACS Calibur.

4.4 Constitutive expression of *ABCG2* increases the production of CFC *in vitro*

Clonogenic haematopoietic assays detect progenitor cells committed to a specific lineage by seeding candidate populations into semi-solid methylcellulose media. Progenitors identified by this assay are retrospectively classified as colony forming cells (CFC) and can be quantitatively subdivided into lineage restricted subtypes by examining the composition of the resulting progeny (described in the methods section). UCB cells transduced with *ABCG2*-YFP or YFP vectors were sorted 48 hours after transduction for $CD34^+$ YFP^+ cells and placed in standard methylcellulose assays for evaluation of clonogenic haematopoietic progenitor cells. Primary CFC assay was readout after 14 days. Both YFP infected cells as well as *ABCG2*-YFP infected cells formed normal colonies in the CFC assays and did not show abnormal blasts (fig 4.4 shows the formation of normal colonies by *ABCG2*-YFP infected cells). However, there was a 1.6 fold increase in the mean absolute number of colonies generated by *ABCG2*-YFP infected cells as compared to YFP infected cells (fig 4.5). This increase in the total number of CFC generated was due to an increase in the colony formation by CFU-GEMM, CFU-GM and BFU-E. *ABCG2*-YFP transduced cells showed a modest 2.1-fold higher number of CFU-GEMM colonies than in the YFP controls (n = 12; $P < 0.002$; *ABCG2*-YFP mean = 46.04, YFP mean = 21.93). The number of CFU-GM colonies generated by the *ABCG2*-YFP cells was 1.5-fold higher than in the YFP controls (*ABCG2*-YFP mean = 50.5, YFP mean = 32.75). The erythroid burst-forming units (BFU-E) were 1.7 fold higher in the *ABCG2*-YFP cells as compared to the YFP controls (*ABCG2* mean =50.83, YFP mean = 29.25). No difference was observed in the number of CFU-M colonies between the *ABCG2*-YFP and control vector transduced cells.

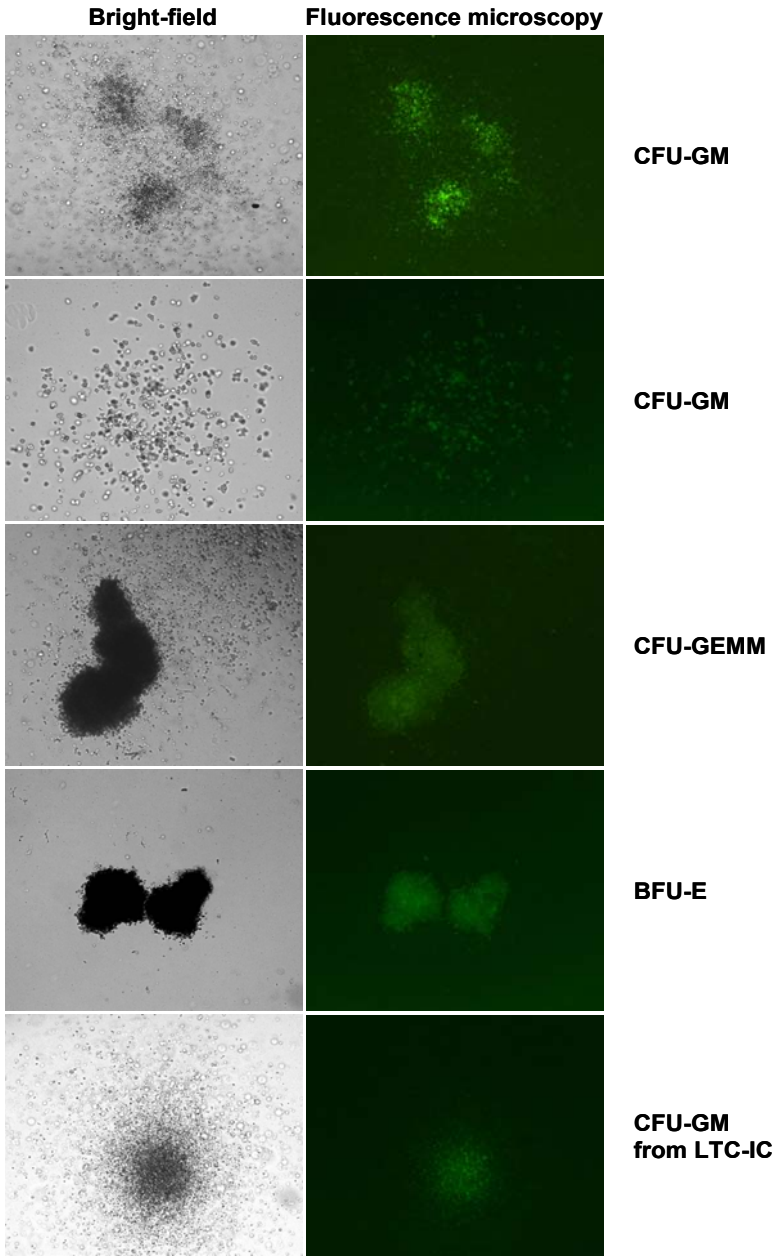

Figure 4.4 Representative photomicrographs of colonies generated by *ABCG2* transduced Lin$^-$ cells. *ABCG2* infected UCB Lin$^-$ cells generated normal colonies from CFU-GM, CFU-M, CFU-GEMM and BFU-E. Methylcellulose dishes were observed under light and fluorescent microscopes: On the left panel are shown the light micrographs of colonies and the right panel depict fluorescence micrographs of the same colonies showing the expression of YFP (50 X magnification).

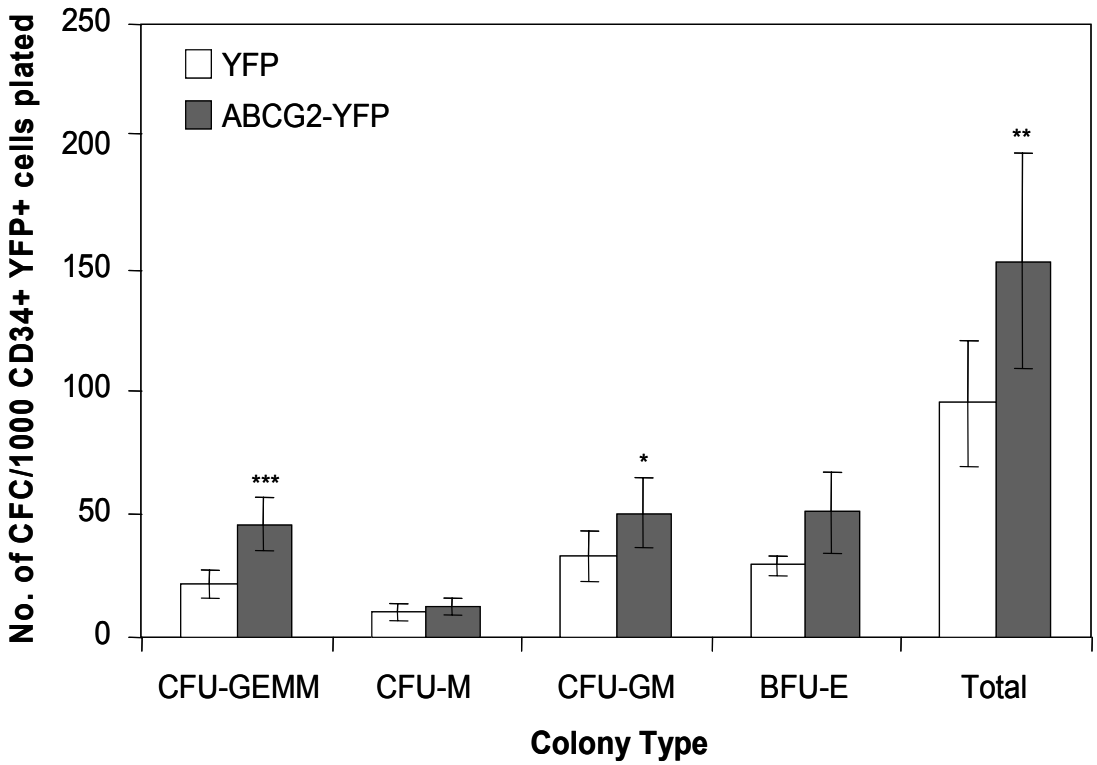

Figure 4.5 Colonies derived from *ABCG2*–YFP- and YFP-transduced progenitors. Umbilical cord blood derived $CD34^+$ cells were transduced with *ABCG2*-YFP or YFP vector and plated in methylcellulose CFC assays. Results are shown as the frequency and distribution of colonies scored on day 14. Bars represent mean (± s.e.m.) frequencies from 12 independent experiments performed with several different umbilical cord blood samples. Significance was determined by a paired Student's t-test and is indicated above the respective bars. **P* < 0.05, ***P* < 0.02, ****P* < 0.002 indicate statistically significant differences from the control.

4.5 *ABCG2* overexpression increases the replating potential of the CFC *in vitro*

In order to detect the clonogenic potential of cells generated by the primary colonies, secondary CFC assays were performed. The methylcellulose dishes containing primary colonies were harvested, replated into fresh methylcellulose dishes and scored after 12-14 days (fig 4.6).

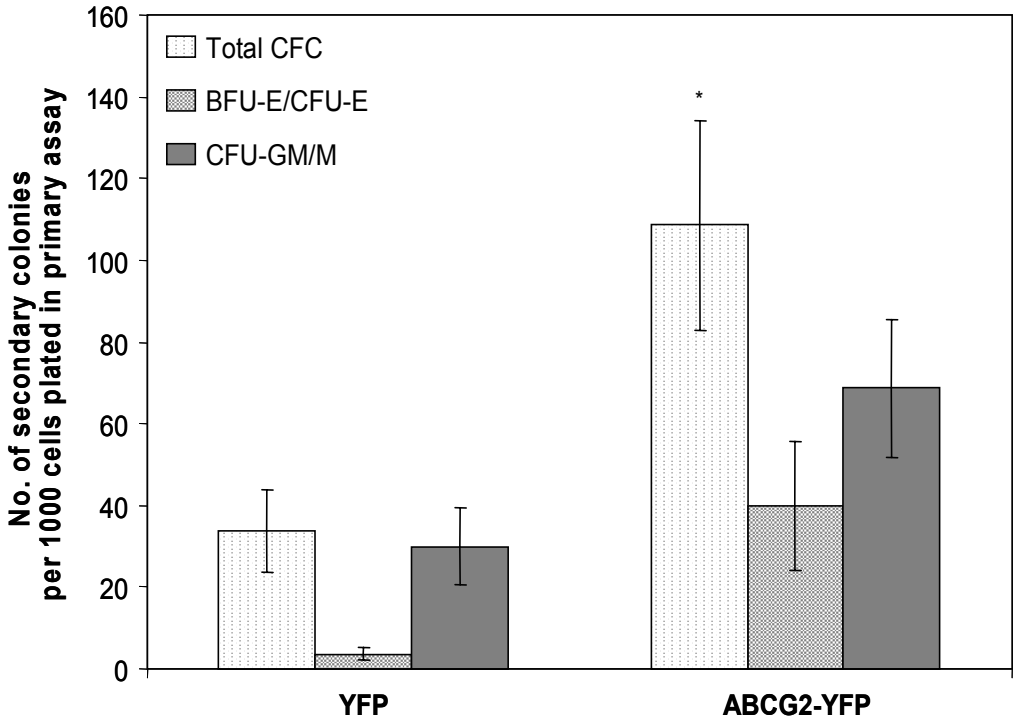

Figure 4.6 Secondary colony formation. Methylcellulose dishes of primary CFC cultures derived from *ABCG2*-YFP-and YFP-transduced progenitors were harvested on day 14, and the progenitor cells resuspended in IMDM 2% FBS were replated in secondary methylcellulose cultures. Secondary colony formation was assessed after 12-14 days. Mean values of secondary colony formation of 9 independent experiments are indicated by bars(± s.e.m.). *P < 0.01 indicates statistically significant difference from the control.

In the secondary CFC assays, *ABCG2*-YFP transduced cells generated 3 fold more total colonies as compared to the YFP transduced cells (n = 9; P < 0.01; *ABCG2*-YFP mean = 111 colonies, YFP mean = 35 colonies / 1000 cells plated in the primary CFC assay).The *ABCG2*-YFP transduced progenitors generated 12 - fold more secondary erythroid colonies as compared to the YFP control (*ABCG2*-YFP mean = 36.5 colonies, YFP mean = 3.17 colonies / 1000 cells plated in the primary CFC assay). Moreover, the number of granulocyte and macrophage secondary colonies was found to be 2.4 fold higher in the *ABCG2*-YFP transduced cells as compared to the YFP control (n = 9; P < 0.03; *ABCG2*-YFP mean = 74.5, YFP mean = 32.0).

4.6 Constitutive expression of *ABCG2* does not change the expansion kinetics of CD34$^+$ cells

In order to study the effects of constitutive expression of *ABCG2* on the proliferative potential of progenitor cells, *in vitro* liquid expansion experiments were performed. YFP$^+$ CD34$^+$ cells were sorted from *ABCG2*-YFP and YFP transduced CD133$^+$ cells and grown in SFM as described in methods section. Both *ABCG2*-YFP and YFP transduced cells showed similar growth kinetics and there was no proliferative advantage demonstrated by the *ABCG2* transduced cells over the control arm (fig 4.7). In order to check the relative numbers of clonogenic progenitors throughout the liquid expansion, we performed CFC assays from cells in liquid cultures at different time points (fig 4.8). Typical expansions generated an approximate 1000-fold increase in the content of progenitors by third week and did not differ significantly between *ABCG2*-YFP and YFP transduced cells.

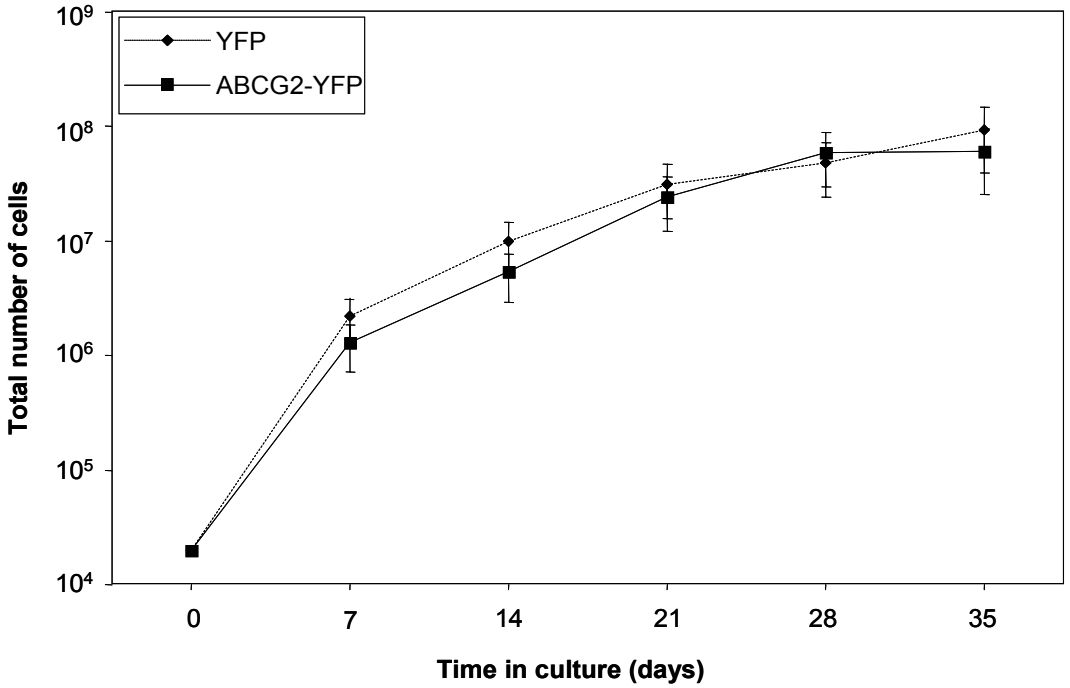

Figure 4.7 Growth kinetics of *ABCG2*-YFP and YFP transduced CD34$^+$ cells. *In vitro* expansion of UCB derived cells transduced with *ABCG2*-YFP and control (YFP) vectors and sorted for YFP$^+$ CD34$^+$ cells. Twenty thousand sorted cells were cultured for upto a period of 5 weeks in SFM supplemented with 5-cytokine combination (rh FLT-3 ligand, SCF, IL-3, IL6 and G-CSF), with weekly half media change. Mean total cell counts (± SEM) performed weekly are shown here for the *ABCG2*-YFP and YFP transduced cells from 5 independent experiments. No significant difference in cell expansion was noted between these two arms.

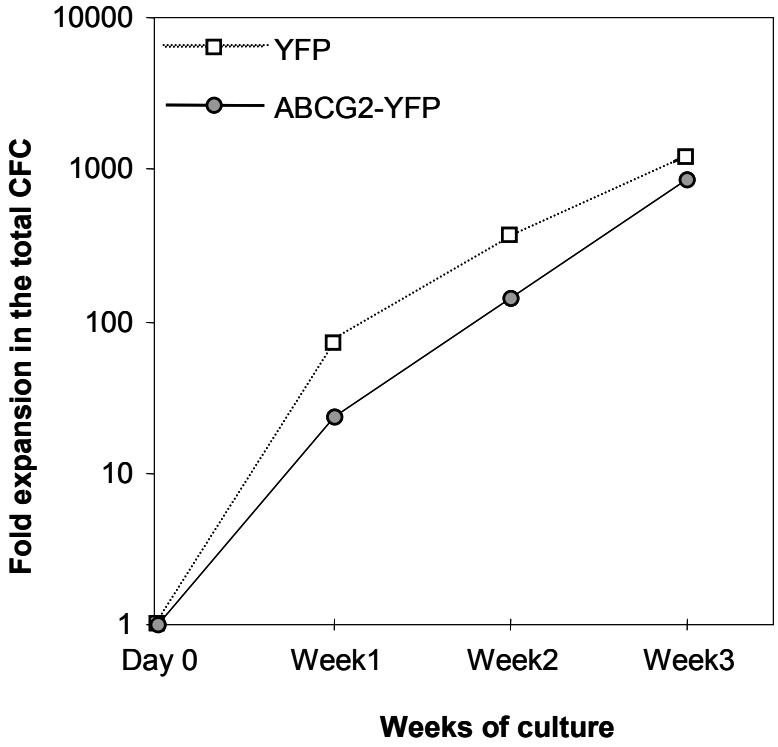

Figure 4.8. Expansion kinetics for progenitors in liquid expansion experiments. *ABCG2*-YFP and YFP transduced CD34$^+$ cells were maintained in liquid suspension cultures as described in methods section. Cells were removed during the weekly half media change for clonogenic progenitor assays in methylcellulose. The progenitor population was found to expand at similar rates in cells with *ABCG2*-YFP and YFP expression. Mean fold expansion observed in 5 independent liquid culture experiments are represented here.

4.7 Bulk LTC-IC assays demonstrate no increase in the output of the LTC-IC by overexpression of *ABCG2*

In order to detect long-term culture-initiating cells (LTC-IC), 5 week long term cultures were performed with sorted *ABCG2*-YFP or YFP transduced UCB CD34$^+$ cells. The CFC readout at the end of this assay was used for calculation of LTC-IC present at the time of seeding of cells. The data obtained from this assay is represented as the mean number of LTC-IC per 10,000 cells. The expression of *ABCG2* did not increase the number of LTC-IC as compared to the control vector (data not shown). To ascertain whether the constitutive expression of *ABCG2* produced any change in the frequency of LTC-IC, another set of experiments called the LTC-IC limiting dilution analysis (LTC-IC LDA) were performed.

4.8 LTC-IC limiting dilution analysis

The LTC-IC limiting dilution analyses (LTC-IC LDA) were performed as described in the methods section. The CFC assay performed at the end of supported culture was scored after 14-16 days. Individual dishes were quantified as either positive or negative for the presence of colonies (table 4.1). Additionally, in one of the two independent LTC-IC LDA experiments, each positive dish was analysed in detail and colonies were classified. The frequencies of LTC-IC obtained in two independent experiments are indicated in table 4.2. The expression of *ABCG2* did not change the frequencies of LTC-IC as compared to the control arm. In order to investigate the clonogenic potential of the LTC-IC, the detailed observation of the kind of colonies generated was performed (fig 4.10).

Table 4.1 Results from two LTC-IC LDA: In each experiment several dilutions of transduced CB cells were plated (doses). Each dilution had several iterations as indicated here. The percentage negative wells observed are indicated in the table.

Dose (Cells/well)	Iteration	% negative wells in experiment no. 1		% negative wells in experiment no. 2	
		YFP	*ABCG2*-YFP	YFP	*ABCG2*-YFP
1	20	95	95	100	100
10	20	95	100	100	100
50	20	75	80	90	90
200	20	55	60	78.95	89.47
800	16	50	18.75	68.75	50
3200	12	25	60	87.50	57.14
12800	10	16.67	14.29	50	62.5

Table 4.2 LTC-IC frequencies obtained from LTC-IC LDA.

	LTC-IC frequency	
	Exp no. 1	Exp no. 2
ABCG2-YFP	1 in 1595	1 in 6227
YFP	1 in 1366	1 in 5197

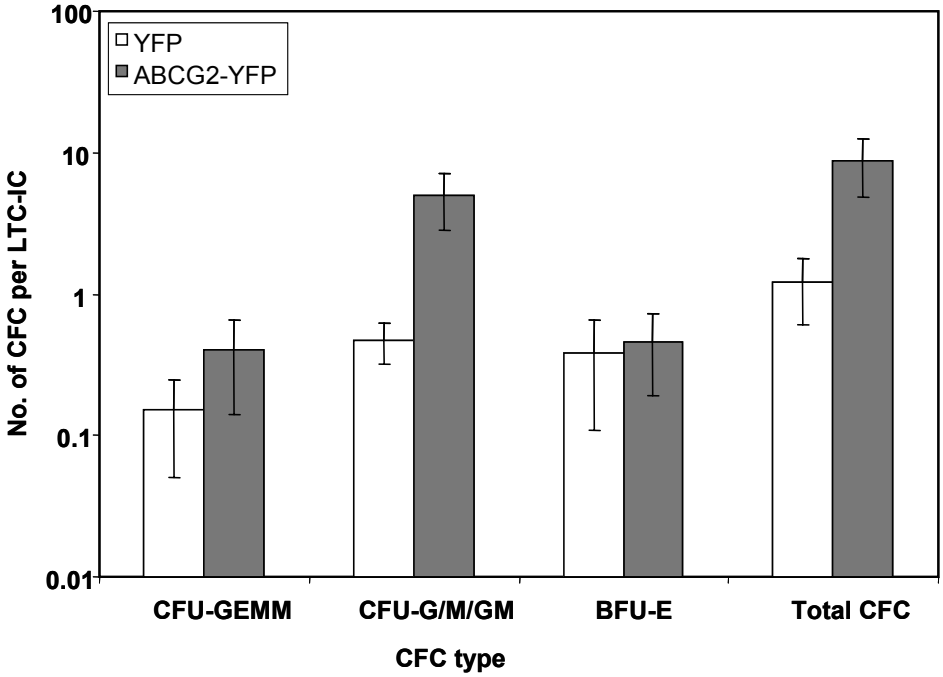

Figure 4.9 LTC-IC derived CFC. Colonies derived from LTC-IC limiting dilution analyses classified according to their morphology. In one of the LTC-IC assay, detail analysis of the types of CFC generated from the LTC-IC was performed.

There was an increase in the number of colonies derived from CFU-GEMM by 2.6 folds in the *ABCG2*-YFP arm as compared to the YFP arm (*ABCG2*-YFP mean = 0.4, YFP mean = 0.15 CFC per LTC-IC). An increase of 10.6 fold in the colonies derived from CFU-G, GM and M together was observed in the *ABCG2*-YFP arm as compared to the YFP arm (*ABCG2*-YFP mean = 5, YFP mean = 0.47 CFC per LTC-IC). There was only a meagre increase of 1.2 fold in the BFU-E colonies formed in the *ABCG2*-YFP arm as compared to the YFP control. The total CFC number that comprises of all kinds of colonies observed was found to be 7 fold higher in the *ABCG2*-YFP arm as compared to the YFP arm (*ABCG2*-YFP mean = 8.7, YFP mean = 1.2). All these data suggest that even though transduction of *ABCG2* did not change the frequency of LTC-IC, it was able to positively influence the colony formation ability of the LTC-IC. In order to further investigate the effects of constitutive expression of *ABCG2* on haematopoietic stem cells, transplantation studies in the NOD/SCID model was performed.

4.9 Multilineage differentiation of *ABCG2*-transduced human CB cells in NOD/SCID mice

In order to evaluate the effects of ABCG2 on the growth potential of human haematopoietic stem cells in the NOD/SCID mice, xeno-transplantations were performed. Irradiated NOD/SCID mice were transplanted with the unselected progeny of 3-5 x 10^5 $CD133^+$ cells 4-6 hours after their final exposure to *ABCG2*-YFP or YFP (control) VCM. *In vitro* analyses for gene transfer and CD34 expression indicated that 6.5 ± 2 % and 15 ± 6% of the CD34 expressing cells have been transduced with *ABCG2*-YFP or YFP VCM respectively, (i.e. % of YFP^+ cells 2 days post-transplantation that were $CD34^+$). The difference in these values is consistent with the 2.3 fold difference in the titres of *ABCG2*-YFP and the YFP VCM used in these experiments. The mice were analysed for engraftment of human SCID repopulating cells (SRC) 8 weeks post transplantation. In order to detect SRC, multilineage differentiation of the transplanted $CD34^+$ YFP^+ cells was examined in the NOD/SCID mice (table 4.3) Multilineage cells were detected in human $CD45^+$ YFP^+ cells from BM of *ABCG2*-YFP-SRC and YFP-SRC transplanted mice. These findings revealed that *ABCG2* transduced CB cells contain normal SRC that are able to repopulate the NOD/SCID mice.

Table 4.3 Multilineage engraftment generated by *ABCG2*-transduced human CB cells transplanted into immunodeficient mice.

NOD/SCID mice 8 weeks post-transplantation	Multilineage engraftment			
	Lymphoid	Myeloid		
	% CD19	% CD33	% CD15	% CD11b
YFP (n = 4)	50 ± 4	51 ±5	25 ± 4	38 ± 4
ABCG2-YFP (n = 4)	31 ± 7	71 ± 9	43 ± 10	62 ± 10

4.10 Detailed phenotypic analysis of human CB cells in NOD/SCID mice

To gain insight into the differentiation pattern of the *ABCG2*-transduced CB cells in immunodeficient mice, a detailed analysis of the human cells generated in the mice was performed. A panel of anti-human antibodies targeting different haematopoietic

cells, described in the material section, was used in the study. All the engrafted NOD/SCID BM were analysed using this antibody panel and the results obtained are indicated in figure 4.11.

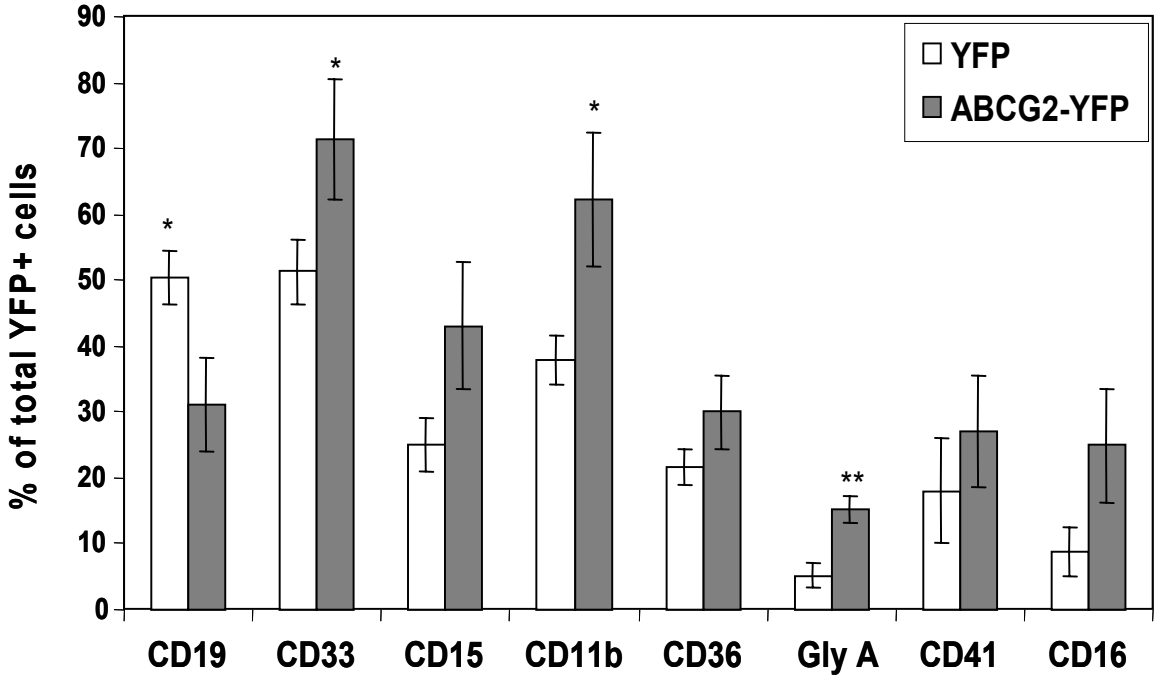

Figure 4.10 Output of human cells produced in the NOD/SCID mice transplanted with *ABCG2*-transduced CB cells. Detail analyses of the type of human cells produced in the NOD/SCID BM was performed 8 weeks post-transplantation. The data is represented in this figure as the mean (± SEM) percentage of total transduced human cells found in the BM of mice. *$P < 0.05$, **$P < 0.01$ indicate statistically significant differences from the control.

A clear reduction in the proportion of $CD19^+$(B-lymphoid) cells was observed in the *ABCG2*-transduced CB cells as compared to the YFP control (*ABCG2*-YFP mean = 31%, YFP mean = 50% $P < 0.05$). The *ABCG2*-YFP transduced CB cells showed a 1.4 fold increase in the proportion of CD 33^+ (myeloid) cells as compared to the YFP control (*ABCG2*-YFP mean = 71.5%, YFP mean = 51%, $P < 0.05$). The production of differentiated myeloid cells was further analysed using markers for all three major myeloid lineages. The proportion of granulocytic cells ($CD15^+$) was found to be increased in the *ABCG2*-YFP transduced CB cells as compared to the YFP-transduced cells (*ABCG2*-YFP mean = 43%, YFP mean = 25%). The proportion of another type of late myeloid cells ($CD11b^+$ cells) was also found to be increased on the expression of *ABCG2*-YFP in CB cells as compared to the YFP control (*ABCG2*-YFP mean = 62%, YFP mean = 38%, $P < 0.05$). The generation of megakaryopoietic ($CD41^+$) lineage showed no significant changes in *ABCG2*-YFP transduced cells as compared to the YFP transduced cells. The proportion of CD36 (found on platelets

and their precursors, monocytes, but absent on adult erythroid cells) expressing cells also did not change significantly upon constitutive expression of *ABCG2* in CB cells. The percentage output of $CD16^+$ (Natural Killer) cells by the *ABCG2*-YFP transduced cells showed a 2.8 fold increase as compared to the output in the vector control cells (*ABCG2*-YFP mean = 25%, YFP mean = 8.8%). The most dramatic effect of *ABCG2* constitutive expression was notable in the output of erythroid cells. The production of glycophorin A^+ (Gly A^+) erythroid cells from *ABCG2*-YFP transduced CB cells showed a 3 fold increase over those produced from YFP transduced cells (*ABCG2*-YFP mean = 15.3%, YFP mean = 5.1%, $P < 0.006$). This result is consistent with the increase in erythroid colonies in primary and secondary CFC assays demonstrated upon constitutive expression of *ABCG2* in CB cells. Overall these results demonstrate that constitutive expression of *ABCG2* increases most of the differentiated myeloid progeny of transduced CB cells in the NOD/SCID mice but there is a decrease in production of B-lymphoid progeny. This skewed haematopoietic engraftment pattern is described in the following section.

4.11 Inversion of the lymphoid-myeloid ratio

The production of mature progeny of transplanted CB lin^- cells in the NOD/SCID mice is normally biased towards the lymphoid lineage and more B-lymphoid cells appear than myeloid cells (Glimm et al., 2001). Thus, the lymphoid-myeloid ratio is more than 1. In the recipients of *ABCG2*-YFP transduced CB cells, however, the number of $CD19^+$ B-lymphoid cells generated was found to be 2.3 fold lower than those generated in the control mice (*ABCG2*-YFP mean = 5.6×10^5, YFP mean = 1.3×10^6 cells, $P < 0.05$) (fig 4.12).

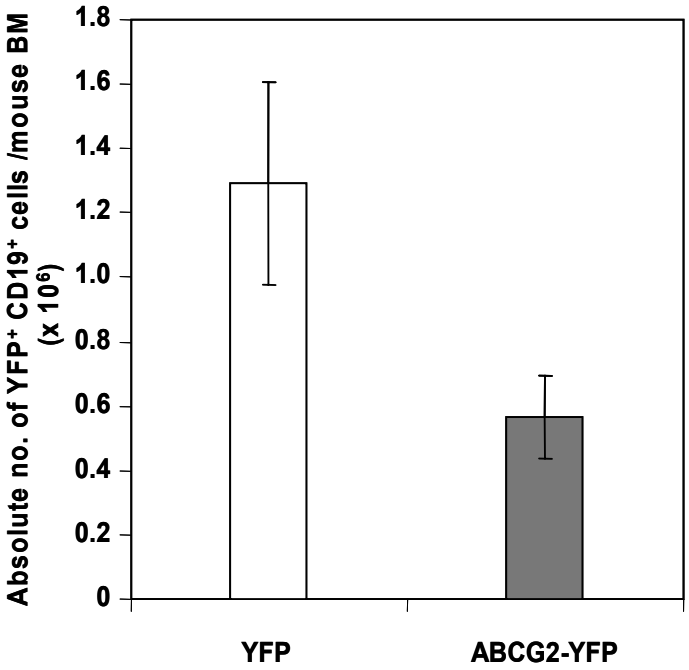

Figure 4.11 Comparison of absolute number of YFP^+ $CD19^+$ cells present in the BM of NOD/SCID 8 weeks after transplantation with *ABCG2*-YFP and YFP (control) transduced CB cells.

The significantly lowered number of $CD19^+$ cells and the increase in myeloid cell numbers in the mice transplanted with *ABCG2*-YFP transduced CB cells led to an inversion of the lymphoid-myeloid ratio (fig 4.13) defined as the ratio between $CD19^+$ and $CD15^+$ cells (median ratios of *ABCG2*-YFP = 0.75, YFP = 2.22, $P < 0.05$). The lymphoid-myeloid ratio observed in the non-transduced cells of the same mice that contained *ABCG2*-YFP transduced cells was found to be normal (fig 4.13).

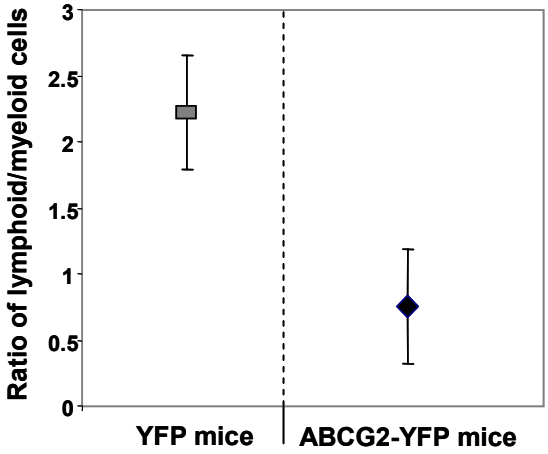

Figure 4.12 Inversion of lymphoid-myeloid ratio in the *ABCG2*-YFP transduced CB cells. Eight weeks post transplantation, the NOD/SCID mice BM were analysed for the presence of YFP^+ $CD19^+$ and YFP^+ $CD15^+$ cells. The median ratios of the total lymphoid and myeloid cells generated are shown in the figure (n = 5 in each arm).

4.12 Effect on HSC

Our data suggest that overexpression of *ABCG2* resulted in increased expansion or proliferation of SRC. In order to investigate this further, we analysed the engrafted NOD/SCID mice for the presence of primitive human haematopoietic cells. The NOD/SCID mice BM cells were stained with CD34 and CD38, and cKIT antibodies (fig 4.14). YFP^+ cells expressing CD34 antigen were detected in both YFP and *ABCG2*-YFP transduced cells, however the proportion of $CD34^+$ cells in the *ABCG2* mice was significantly higher than in the vector control (1.5 fold, *ABCG2*-YFP mean = 21.6%, YFP mean = 14.6%, $P < 0.05$). The CD34 cells were further analysed for the presence of early differentiation marker CD38. The proportion of cells expressing both CD34 and CD38 ($CD34^+$ $CD38^+$ cells) was also found to be increased in the mice transplanted with *ABCG2*-YFP transduced cells as compared to the vector control (1.5 fold, *ABCG2*-YFP mean = 18.8%, YFP mean = 12.6%). The percentage of $cKIT^+$ cells (haematopoietic progenitors) showed a slight increase in the ABCG2 mice (fold increase = 1.5, *ABCG2*-YFP mean = 9.3%, YFP mean = 6.2%). However, no difference was observed in the proportion of most primitive haematopoietic cells ($CD34^+$ $CD38^-$), comprising 0.81 and 0.83% of human cells in the BM of *ABCG2*-YFP and YFP mice respectively. These observations indicate that expression of *ABCG2* in CB Lin^- cells does not increase the number of human $CD34^+$ $CD38^-$ most primitive cells in the NOD/SCID mice, but of more differentiated progenitors known to comprise the fraction of clonogenic progenitors

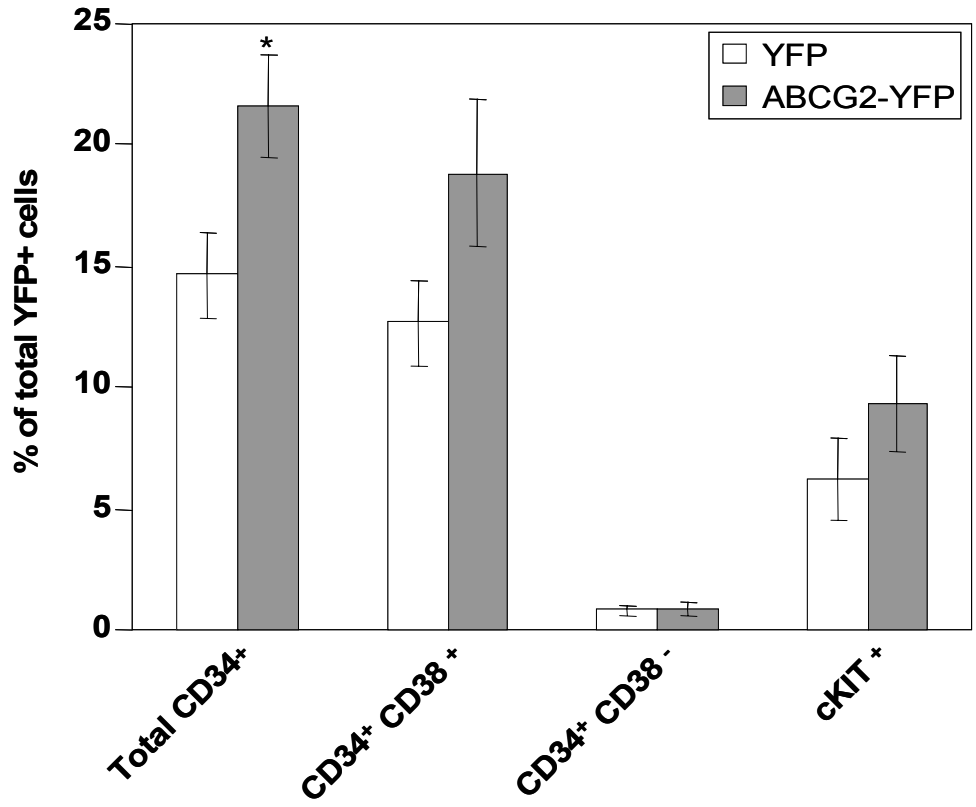

Figure 4.13 Comparative analysis of the expression of haematopoietic stem and progenitor cell markers in the YFP^+ human cells derived from the NOD/SCID mice transplanted with *ABCG2*-YFP and YFP transduced CB cells. Cells derived from the NOD/SCID BM 8 weeks post transplantation was stained with CD34 and CD38 markers simultaneously and separately for cKIT. The mean percentage (± SEM) of YFP^+ $CD34^+$, $CD34^+$ and $CD38^+$, $CD34^+$ and $CD38^-$ cells, and $cKIT^+$ cells are represented here. * $P < 0.05$ indicates statistically significant differences from the controls.

4.13 Constitutive expression of *ABCG2* does not change the SRC frequency

To determine if *ABCG2* affects the frequency of SRC, limiting dilution analysis in NOD/SCID mice were performed. CB derived $CD133^+$ cells were transduced with *ABCG2*-YFP and YFP vectors and transplanted into sublethally irradiated NOD/SCID mice in limited numbers without any pre-sorting. The *ABCG2*-YFP transduction generates one third the amount of YFP^+ $CD34^+$ cells, therefore 3 fold more total cells were injected into mice transplanted for *ABCG2*-arm. The exact numbers of transplanted $CD34^+YFP^+$ cells were determined 48 hours after the final transduction and used later for calculation of the frequencies of SRC. The mice were sacrificed after a period of 6 weeks and the BM analysed for the presence of multilineage cells.

Table 4.4 Limiting dilution assay of CB cells transduced with *ABCG2*-YFP and YFP vectors.

Phenotype	No. of YFP^+ $CD34^+$ cells transplanted	No. of positive mice/total no.	SRC frequency (upper & lower limits)
YFP	133000	3/3	1/80 000 (1/47 705 -1/132 724)
	32000	0/4	
	8000	1/6	
ABCG2-YFP	114000	3/3	1/47 000 (1/28 572 -1/75 783)
	28500	2/5	
	7130	0/2	

As shown in table 4.4, all of the mice (3/3) transplanted with highest dilution of cells (1.14- and 1.33 x 10^5 of *ABCG2* and YFP-transduced $CD34^+$cells respectively), were found to be multilineage engrafted. In the middle dilution (injected 2.85- and 3.2 x 10^4 $CD34^+$ YFP^+ of *ABCG2* and YFP-transduced $CD34^+$cells respectively per mouse) showed no engrafted mice in the cohort of YFP mice. In contrast, 2 mice (2/5) transplanted with *ABCG2* transduced cells were found to be multilineage engrafted. In the lowest dilution of cells injected (1.12 x10^4 of *ABCG2*-YFP and YFP transduced $CD34^+$ cells), none of the mice transplanted with *ABCG2* cells were found to be engrafted. However one mouse (1/6) transplanted with YFP transduced cells showed multilineage engraftment. Based on these limiting dilution results, the

frequencies of SRC in the transduced CB cells (calculated using L-calc. software), were estimated to be 1/47 000 *ABCG2*-YFP transduced $CD34^+$ cells and 1/80 000 YFP transduced $CD34^+$ cells. Taken together, these results suggest that *ABCG2* does not drastically increase the SRC frequency in the NOD/SCID mice.

5 Discussion

A unique property of HSC is the ability to efflux fluorescent dyes such as Hoechst 33342 and Rhodamine 123 and this property has been attributed to the ABC drug transporters ABCG2 and MDR1, respectively. *ABCG2*, a member of the G-subfamily of ABC transporters, is highly expressed in haematopoietic stem cells but is turned off in most committed progenitors and differentiated cells, suggesting a role in early haematopoietic cells (Scharenberg et al., 2002). A distinct population of BM cells expelling the Hoechst dye can be defined by flow cytometry and this population (termed side population) is enriched in HSC in mice (Goodell et al., 1996) as well as in humans (Feuring-Buske and Hogge, 2001; Uchida et al., 2001). Targeted gene ablation studies in mice have revealed that SP cell phenotype in haematopoietic cells is determined by *Abcg2*. Another ABC drug transporter that is overexpressed in HSC is MDR1. Several lines of evidence suggest that MDR1 expression is conserved in HSC. Previous studies have demonstrated that retroviral vectors expressing MDR1 in murine haematopoietic progenitors resulted in an expansion of murine BM cells. A myeloproliferative disorder developed in mice transplanted with such expanded cells (Bunting et al., 1998). On the other hand, the expression of human *ABCG2* in mouse BM cells significantly blocked haematopoietic development leading to speculation that *ABCG2* expression might play a role in early stem cell self-renewal by blocking differentiation. Studies in a non-human primate model demonstrated that forced expression of *ABCG2* in bone marrow stem cells of rhesus macaques does not interfere with haematopoietic stem cell maturation *in vivo* (Ueda et al., 2005). However, till date there has been no study analyzing the role of *ABCG2* in primitive human hematopoiesis.

The establishment of a retroviral transduction model to constitutively express *ABCG2* in human $CD133^+$ cells has allowed us to investigate its role in primary human haematopoietic stem and progenitor cells. In this gene transfer model, CB derived $CD133^+$ cells were activated into cell cycle by a combination of stimulatory cytokines, and subsequently transduced with retroviral supernatant from producer cells. CD133, the novel marker present on primitive haematopoietic cells, was used for isolation of HSC using MACS technique. Isolation procedures yielded highly pure $CD133^+$ cells. CD133 is a good substitute of the more commonly used marker CD34 as it is

expressed on most of the $CD34^+$ cells, and additionally, it comprises a small subset of very early haematopoietic $CD133^+$ $CD34^-$ stem cells that are believed to be even more primitive than the $CD34^+$ cells (Gallacher et al., 2000). Transduced $CD133^+$ cells were purified by FACS and plated in different *in vitro* assays representing different stages of primitive haematopoietic stem and progenitor cell development.

Our *in vitro* results indicate that the constitutive expression of *ABCG2* enhances the formation of clonogenic progenitors with an increase of all clonogenic progenitor cells. This effect was mostly pronounced in the most immature clonogenic progenitor cells, the CFU-GEMM. In addition to the increase of the number of clonogenic progenitors we also observed a higher proliferative capacity as compared to the empty vector control with a higher number of secondary colonies formed by those cells constitutively expressing *ABCG2.* It is important to note that the constitutive expression of *ABCG2* did not have any positive effect on the cell expansion in liquid culture as well as the LTC-ICs. However the differentiation of LTC-IC into clonogenic progenitors after 6 weeks in culture was again found to be enhanced by the expression of *ABCG2*. This indicates that *ABCG2* was able to positively influence the colony formation ability of the LTC-IC. These observations suggest that *ABCG2* plays a strong role in the differentiation and proliferation of clonogenic progenitors developing from primitive haematopoietic stem cells. In order to characterize the role of *ABCG2* in the most primitive haematopoietic stem cells represented as long-term reconstituting stem cells in immunodeficient mice we transplanted sublethally irradiated NOD/SCID mice as previously described (Buske et al., 2002; Dick et al., 1997; Glimm et al., 2001). Cells capable of multilineage repopulation of transplanted NOD/SCID mice, termed as SCID repopulating cells (SRC) represent the most primitive haematopoietic stem cells identifiable *in vivo*. In our experiments, sub-lethally irradiated NOD/SCID mice were injected with *ABCG2* and control vector transduced cells. Transduced cells could be easily followed due to YFP labelling and multilineage engraftment was studied 8 weeks post transplantation. *ABCG2* expression induced a clear reduction in the development of B-lymphoid cells and an increase in the number and proportion of myeloid cells engrafted in NOD/SCID mice, leading to an inversion of the lympho-myeloid ratio. A similar inversion of the lympho-myeloid ratio has been observed in SRC transduced with *HOXA10* (Buske et al., 2001), due to an impairment of B-cell development. However, this is the first study to demonstrate that the constitutive expression of an ABC transporter in primitive progenitors can lead to such a drastic effect on the lympho-myeloid ratio of cells

generated in NOD/SCID mice. This observation suggests that *ABCG2* either alters the cell fate decisions of multipotent cells with myeloid and lymphoid differentiation capacity, or influences a subset of committed myeloid or lymphoid progeny to produce altered frequencies of mature cells. The observation that differentiated myeloid progeny are generated from *ABCG2* transduced SRC leads us to conclude that myeloid differentiation is not perturbed and that the increase in myeloid cells observed is not due to an accumulation of undifferentiated myeloid cells. Since the NOD/SCID assay for human repopulating cells does not readily detect differentiation into the T-cell lineage, use of alternative recipients capable of supporting human T lymphopoiesis will be required to determine whether *ABCG2* expression affects lymphoid T-cell development.

In this study we also analyzed the impact of *ABCG2* expression on the primitive human haematopoietic cells in the NOD/SCID mice. Our results indicate that the constitutive expression of *ABCG2* does not affect the number of most primitive human $CD34^+$ $CD38^-$ cells in the NOD/SCID mice, but increases the number of the more differentiated $CD34^+$ $CD38^+$ cells known to comprise the fraction of clonogenic progenitors. This result is consistent with our *in vitro* results that demonstrate an enhancement of clonogenic progenitors. In order to find out the frequencies of SRC in $CD34^+$ cells transduced with *ABCG2*, we performed the CRU assay. Transduction of cells with *ABCG2* showed only a meagre increase of the CRU frequency in the NOD/SCID mouse model as compared to the empty vector control. This result leads us to conclude that *ABCG2* overexpression does not affect the primitive haematopoietic stem cell with SCID repopulating properties in a positive manner and perhaps only regulates the generation of clonogenic progeny from primitive haematopoietic cells.

Our data characterize *ABCG2* as a previously unknown positive regulator of human hematopoiesis. There is no previous indication from the literature that an ABC transporter can regulate hematopoiesis. Our hypothesis that *ABCG2* plays an important role in haematopoietic stem cell regulation is supported by reports that *ABCG2* expression is restricted to early haematopoietic cells with a decrease of expression levels during cell differentiation (Zhou et al., 2001). The mechanism by which *ABCG2* achieves this effect on haematopoietic progenitors remains to be elucidated and these efforts will be facilitated by preliminary microarray data that identify regulated genes in response to *ABCG2* overexpression as well as by identification of substrates that might mediate such effects on HSC.

6 Summary

ABCG2 is a transporter protein that has the ability to efflux many drugs and fluorescent dyes. Primitive haematopoietic stem cells highly express *ABCG2* and the expression level decreases as these cells differentiate indicating a possible role of this transporter in HSC. In the present study, we have analyzed the role of *ABCG2* in early haematopoietic stem cells by constitutively expressing *ABCG2* in human CB derived $CD133^+$ cells. This constitutive expression of *ABCG2* demonstrated an enhancement of primary CFCs *in vitro*, including the most primitive clonogenic cells the CFU-GEMM ($n=12$, $p<0.002$). *ABCG2* enhances the replating capacity of primary colonies with a mean 3.0 fold increase in the number of 2^{nd} colonies ($n=9$, $p<0.01$), indicating a substantial enhancement of the proliferative potential of clonogenic progenitors by constitutive *ABCG2* expression. Overexpression of *ABCG2* did not have any positive effect on cell expansion in liquid culture as well as the frequency of LTC-IC, however, the production of CFC per LTC-IC was found to be enhanced, again supporting the fact that *ABCG2* might play an important role in the differentiation and proliferation of clonogenic progenitors.

Using the NOD/SCID mouse model, we were able to demonstrate that enforced expression of *ABCG2* in human primitive haematopoietic cells leads to inversion of lymphoid-myeloid ratio, suggesting that *ABCG2* perhaps alters the cell fate decisions of multipotent cells with myeloid and lymphoid differentiation capacity. An enhanced production of differentiated myeloid cells was observed on *ABCG2* overexpression. In order to analyze the effect of *ABCG2* on early haematopoietic cells, NOD/SCID mice transplanted with CB cells either expressing *ABCG2* or the empty viral vector, were analyzed for the presence of HSC. Although the number of human $CD34^+$ $CD38^-$ cells did not show any difference, the number of $CD34^+$ $CD38^+$ progenitor cells was significantly increased ($n=5$, $p<0.05$), indicating that *ABCG2* plays a role in the differentiation of clonogenic progenitors. CRU assays were performed to detect the effect of *ABCG2* expression on the frequency of SRC and did not show any significant increase in the frequency of SRC.

Taken together, these results indicate that *ABCG2* is a potent positive regulator of human hematopoiesis at the level of early haematopoietic development.

7 Zusammenfassung

ABCG2 gehört zur Gruppe der ATP-bindenden Transportproteine, die die Fähigkeit besitzen, viele Wirkstoffe, wie z.B. Zytostatika und fluoreszierende Farbstoffe aus der Zelle zu befördern. ABCG2 weist ein stammzelltypisches Expressionsprofil auf, mit einer hohen Expression in den frühesten hämatopoetischen Stammzellen (HSZ) und einer Abnahme bis zum Verlust der Expression in reifen Blutzellen. Dieses Stammzelltypische Expressionsprofil weist auf eine mögliche Rolle von ABCG2 in der Stammzellentwicklung und –differenzierung hin. In der vorliegenden Studie war es unser Anliegen, durch die konstitutive Expression von ABCG2 in humanen, aus Nabelschnurblut stammenden CD133 - positiven Zellen, die Rolle von ABCG2 in frühen hämatopoetischen Stammzellen zu untersuchen. Die konstitutive Expression von ABCG2 führte *in vitro* zu einem Anstieg klonogener Progenitorzellen, einschließlich der primitivsten klonogenen Progenitorzellen, der CFU-GEMM ($n=12$, $p<0{,}002$). Die konstitutive Expression von ABCG2 führte weiterhin zu einem Anstieg des proliferativen Potentials klonogener Progenitorzellen um den Faktor 3,0, ablesbar an der Anzahl sekundärer Kolonien ($n=9$, $p<0{,}01$). Um die Rolle von ABCG2 auf der Ebene der hämatopoetischen Stammzelle zu untersuchen, führten wir stromazell-abhängige und serumfreie Langzeitkulturen durch, und transplantierten NOD/SCID Mäuse mit Nabelschnurblutzellen, die konstitutiv ABCG2 exprimierten. Wir konnten zeigen, dass die konstitutive Expression von ABCG2 die Frequenz HSZ sowohl *in vitro* als auch *in vivo* nicht ändert, die Produktion klonogener Progenitorzellen pro LTC-IC aber steigert. Auch im NOD/SCID Mausmodell konnten wir zeigen, dass die CRU-Frequenz zwar nicht gesteigert wurde, und auch die Anzahl an humanen $CD34^+$ $CD38^-$ Zellen in Mäusen, die mit Nabelschnurblutzellen transplantiert wurden, die retrovirales ABCG2 exprimierten, nicht verändert, die Anzahl reiferer $CD34^+$ $CD38^+$ Progenitorzellen aber signifikant erhöht war ($n=5$, $p<0{,}05$). Um zu überprüfen, ob das Differenzierungsmuster repopulierender Stammzellen durch ABCG2 verändert wird, haben wir das immunphänotypische Profil mittel Durchflusszytometrie überprüft und konnten zeigen, dass die konstitutive Expression von ABCG2 zu einer Umkehr des lympho-myeloischen Engraftments mit einem Überwiegen der myeloischen Repopulation führte. Die Tatsache, dass trotzdem ein multilineäres Engraftment zu beobachten war, weist daraufhin, dass ABCG2 einen Einfluss auf der Ebene der multipotenten hämatopoetischen Progenitorzellen hat. Sowohl *in vitro* als auch *in vivo* induzierte ABCG2 keine maligne Transformation hämatopoetischer Zellen. Zusammengefasst lässt sich somit sagen, dass ABCG2 ein wirksamer positiver Regulator der humanen Hämatopoese, vor allem im frühen Stadium ist.

8 References

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