SHORT AND LONG-TERM REPOPULATING HEMATOPOIETIC STEM CELLS IN THE MOUSE

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Loo, Johannes Christiaan Maria van der

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SHORT AND LONG-TERM REPOPULATING HEMATOPOIETIC STEM CELLS IN THE MOUSE

KORTE EN LANGE TERMIJN REPOPULERENDE HEMATOPOIETISCHE STAMCELLEN BIJ DE MUIS

PROEFSCHRIFT

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Hematopoietic stem cells

The formation and development of blood cells, or hematopoiesis, normally takes place in the bone marrow, which serves as the major hematopoietic organ during adult life. A small population of bone marrow cells (BMC), designated as hematopoietic stem cells, underlies the process of blood cell formation [1]. This population is defined by its extensive self-renewal capacity and ability to contribute to blood cell formation for extended periods of time. It has been shown by retransplantation studies that single and uniquely marked murine hematopoietic stem cells are able to provide blood cells for a period of time that even approximates the lifespan of a mouse [2-4]. Most of the stem cells in the bone marrow do not actively participate in blood cell formation but remain in a quiescent state, which makes stem cells relatively resistant to the effects of irradiation or chemotherapy. The frequency of stem cells in the bone marrow is very low and has been estimated in the mouse at 1-2 per 100,000 BMC [5]. Therefore, study of stem cell biology *in vitro* and *in vivo* requires the purification of this extremely rare cell. Over the years, the methods used for purification have gradually changed, influenced by the changing functional definition of the stem cell and understanding of its biology [6]. The development of assays to identify primitive hematopoietic cells have been an integral part of the search for the stem cell. In this chapter some of these assays will be introduced and the methods used for the purification of murine hematopoietic stem cells will be discussed.

The dissection of the hematopoietic stem cell compartment and isolation of its different stem cell subsets serves multiple goals. It enables researchers to gather basic information on the phenotype and growth factor requirements of the different stem cell subsets and on their repopulating abilities *in vitro* and *in vivo*. Further, purified subsets are being used to search for conditions that enable manipulation of hematopoietic cells *ex vivo*, without them losing their ability to self-renew and repopulate a host upon transplantation. These manipulations include the purification of stem cells and purging of tumor cells from leukemic bone marrow, the use of stem cells as a vehicle for somatic cell gene therapy, and the collection and storage of hematopoietic cells. Furthermore, isolated stem cell subsets are used to develop and validate hematological assays, like the long-term bone marrow culture (LTBMC) and cobblestone area-forming cell (CAFC) assay, that can aid the clinical hematologist in estimating the functional ability of a bone marrow graft and predicting the effect of a treatment or manipulation of the graft based on quantitative studies *in vitro*.

1.2 Assays for primitive hematopoietic cells

The different hematopoietic stem and progenitor cell subsets in the bone marrow are identified and categorized by functional characteristics, such as their ability to form a cluster or colony of cells *in vitro* or to repopulate the hematopoietic system of an irradiated host for a shorter or longer period of time. However, the various functional definitions may partly overlap as some cells may show up in multiple assays. Hematopoietic stem and progenitor cells can be globally ordered on the basis of their primitivity as is shown in Figure 1.1. Since the description of the spleen colony-forming unit (CFU-S) by Till and McCulloch in 1961 [7], which was then considered as the hematopoietic stem cell [8], the definition of the stem cell has gradually changed [9]. Now, most investigators consider the CFU-S to be a relatively primitive cell that is capable of *in vivo* repopulation, but only for a limited period of time. It has been suggested that it is the combination of CFU-S and long-term repopulating cells that is responsible for the radioprotecting effect when they are transplanted in lethally irradiated animals. Therefore, radioprotection is no longer considered to be a characteristic of the most primitive stem cell but merely reflects the activity of multiple stem and progenitor cell populations [10]. At this time, a stem cell is defined as a cell that is capable of extensive self-renewal and confers multilineage long-term repopulation upon infusion into an irradiated animal for at least 4 months.

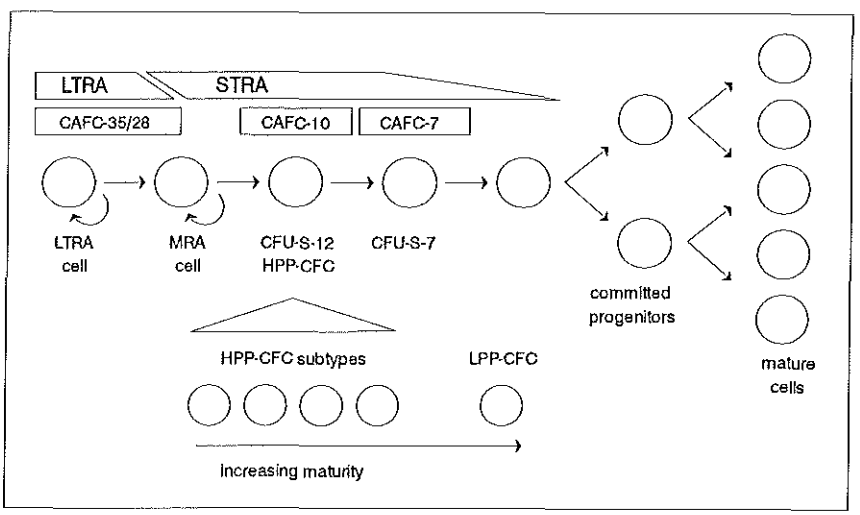

Figure 1.1 Schematic diagram of hematopoietic differentiation in bone marrow of the mature mouse. Functionally defined hematopoietic stem and progenitor cell subsets are ordered on the basis of their primitivity, which decreases from left to right. Possible overlap between assays has not been taken into account. LTRA = long-term repopulating ability, STRA = short-term repopulating ability, MRA = marrow repopulating ability, CAFC = cobblestone area forming cell, CFU-S = spleen colony-forming unit, HPP-CFC = high proliferative potential colony-forming cell, LPP-CFC = low proliferative potential colony-forming cell.

To determine the *in vivo* repopulating ability of a hematopoietic cells different transplantation models have been developed. In addition, several *in vitro* assays have been designed that enable researchers to measure relatively primitive hematopoietic cells and give an estimation of the frequency of the *in vivo* repopulating stem cells in a given sample. In this chapter a brief summary will be given of the *in vivo* and *in vitro* assays that have been used most commonly to determine the frequency of subsets of primitive hematopoietic cells.

1.2.1 Spleen colony formation

The CFU-S, which is measured by the macroscopic spleen colony assay [7], has long been thought to represent the hematopoietic stem cell [8,11]. Extensive research on the nature of this stem cell revealed that the CFU-S in fact represents a heterogeneous population of precursors, differing in self-renewal capacity and in time of appearance in the spleen after intravenous injection [12-16]. Nodules, or colonies, appearing at the surface of the spleen at 12 days after injection were derived from a subset of cells designated as CFU-S-12, which were more primitive than the day-8 CFU-S who give rise to spleen colonies after 8 days. The concept of the CFU-S being the stem cell was challenged by experiments using intravenously injected 5-fluorouracil, bromodeoxyuridine, hydroxyurea and melphalan [17-21]. The marrow of mice treated with these cytostatic agents contained only few primary CFU-S, but was enriched for cells with marrow-repopulating ability (MRA), determined as day-12 CFU-S (CFU-S-12) in the marrow 13 days after injection [22]. This suggested the existence of a class of more primitive quiescent hematopoietic stem cells, tentatively designated as pre-CFU-S [17]. In 1988 this was supported by the physical sorting of BMC into a population enriched for MRA, which was severely depleted of CFU-S activity, and a population enriched for CFU-S that contained low MRA [23,24]. Using counterflow elutriation, a subset of cells could be isolated that contained no detectable CFU-S but was capable of fully reconstituting the hematopoietic system of the irradiated recipient upon intravenous transfer [25]. Although later studies would show that this subpopulation contained only a subset of all cells with long-term *in vivo* repopulating ability (LTRA) [26] and that the study did not prove the inability of CFU-S-12 to induce long-term repopulation [27], it supported the existence of a non-CFU-S, or pre-CFU-S, hematopoietic stem cell.

The MRA-assay [22] measures a more primitive cell than the CFU-S but has several disadvantages when compared to the long-term repopulation assay (*paragraph 1.2.2*). First of all, MRA measures an intermediate type of stem cell, which can developmentally be placed between the more mature CFU-S-12 and the primitive LTRA-cell. Secondly, the MRA-assay only takes a snapshot of the repopulation in the bone marrow at 13 days after transplantation, while the LTRA-assay measures the dynamics and level of multilineage hematopoietic reconstitution over extended periods of time, which is the hallmark of the hematopoietic stem cell [2-4,28,29]. Finally, the

MRA-assay has the additional disadvantage that it is based on a double transplantation, using much more mice than is needed for the determination of LTRA. *In vitro* systems that determine the frequency of primitive hematopoietic cells, and can serve as alternative for the MRA-assay and LTRA-assay, will be discussed further on in this chapter.

1.2.2 Long-term repopulation

To determine the long-term repopulating ability (LTRA) of a graft several mouse models and techniques have come available that enable a discrimination of donor-derived and recipient-type hematopoietic cells [30]. These models include various congenic mouse strains that display allelic differences in the expression of certain cell surface antigens, like Thy-1.1 and Thy-1.2, or Ly-5.1 and Ly-5.2 [31]. Also used are polymorphisms in hemoglobin [26,32,33] or in electrophoretically distinguishable enzymes, like glucose phosphate isomerase (*GPI*) [33-38] and phosphoglycerate kinase (*PGK*) [39-42]. The most sensitive method, in which signals can be amplified by the polymerase chain reaction (PCR), makes use of differences between donor and recipient at the level of DNA. These methods include the use of chromosomal markers [28,43], sex-mismatched transplantation [25,44-48], and retroviral transfection of the donor stem cells [49-53]. During transfection, the retroviral marker integrates in the host DNA in an almost random fashion. Therefore, hematopoietic stem cell clones and their descendants are individually marked, which makes it possible to track the fate of specific stem cell clones *in vivo* [2-4,54-56]. In a sex-mismatched transplantation model host and donor nucleated cells can be distinguished in blood smears or cytospin preparations by *in situ* hybridization using a Y chromosome-specific probe [57].

In addition to the congenic mouse models also genetically anemic mice, like the heterozygous *W/Wv* and α-thalassemic $Hba^{th/+}$ mice, have been used for transplantation studies. *W/Wv* mice suffer from a stem cell defect due to a mutation at the white spotting locus (*W*) that encodes for the proto-oncogene *c-kit* [58], resulting in a macrocytic anemia. α-Thalassemic mice suffer from a defect in their α-globin genes causing a characteristic hypochromic microcytic anemia [59-61]. It has been shown that the infusion of relatively low numbers of normal BMC (which were derived from their hematologically normal littermates) in unirradiated or sublethally irradiated *W/Wv* mice [34,62,63], or in sublethally irradiated α-thalassemic mice [64,65], could correct their anemia. A disadvantage of the *W/Wv* model when used for long-term repopulation assays is that some recipients lose their cure with time. This phenomenon is not yet fully understood but seems to be caused by a deficiency of the hematopoietic microenvironment which, apparently, is related to the age of the animals [66]. Nevertheless, *W/Wv* mice have often been used in combination with one of the methods mentioned above to distinguish donor from recipient cells after transplantation. A method that has only recently been introduced, and has the advantage of being the

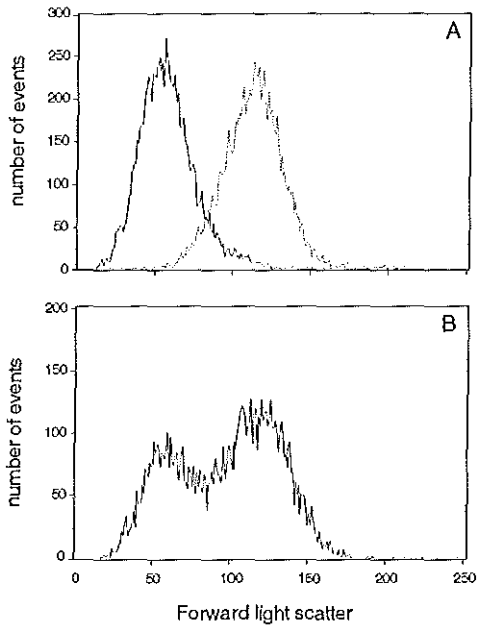

Figure 1.2 Forward light scatter distribution of **(A):** α-thalassemic (—) and normal (...) red blood cells; and **(B):** the red blood cells of a partial chimera containing 55% normal and 45% thalassemic red blood cells.

fastest method to date to routinely determine chimerism in large groups of recipients, makes use of the FACScan [67,68]. Differences in size between α-thalassemic or *W/Wv* red blood cells and healthy (donor-type) red blood cells are detected on the basis of their respective forward light-scatter characteristics (Figure 1.2). The method has successfully been applied in the studies described in *Chapters 3* and *4* of this thesis. To determine chimerism in the other lineages in these experiments we included a sex-difference between donor and recipient and used Y-chromosome specific probe. A common feature of the genetically anemic α-thalassemic or *W/Wv* mice is that chimerism, after transplantation with normal bone marrow, is always higher in the red blood cell lineage than in the other lineages [68,69]. However, the results in *Chapter 3* show that red blood cell chimerism can be used as a reliable index for chimerism at the level of the hematopoietic stem cell. At two months post-transplant and at later times red and nucleated blood cell chimerism were shown to correlate highly.

To enable relatively low numbers of intravenously injected hematopoietic cells to enter the bone marrow, mice receive a lethal or sublethal dose of total body irradiation. Untreated recipients can also be stably engrafted, but only after infusion of large

numbers of cells [70,71]. Theoretically, this indicates that niches for primitive hematopoietic stem cells are available under steady state conditions. In a clinical setting, however, such high numbers of cells could never be used for BMT, which makes it a less suitable model for transplantation. Recent studies have shown that irradiation or treatment with cytostatic drugs facilitates the entry of intravenously injected cells into the bone marrow by disrupting the marrow sinus endothelial barrier [72-74]. The final homing and binding of stem cells to their appropriate microenvironment is a complex process that involves stromal cells as well as elements of the extracellular matrix [75-79]. A study on the seeding of various stem cell subsets to bone marrow and spleen in lethally irradiated recipients is presented in *Chapter 7*.

To evaluate the capacity of a few hematopoietic stem cells to compete against the recipients' remaining hematopoietic stem cells, competitive repopulation may be the most appropriate assay [44,80]. Limiting numbers of donor-type cells (type A) are intravenously injected together with a constant numbers of cells (type B) that are distinguishable from donor as well as from recipient-type cells. The frequency of the cell responsible for a chosen level of chimerism is then calculated based on the statistical variation between the contribution of type A and type B using a binomial formula. Details on the mathematical model have been given by Harrison [6]. To prevent mice from radiation inflicted death, recipient-type BMC (that are depleted of LTRA cells by three successive rounds of retransplantation) are normally co-transplanted. The benefit of this assay compared to 'normal' repopulation (in which cells compete against the host's remaining stem cells) is that repopulation in different samples is estimated based on competition with a fixed number of co-injected stem cells within each recipient, which eliminates much of the variation present when samples have to be compared between animals. In the experiments presented in this thesis we did not use competitive repopulation since a mice strain that allowed discrimination of donor-type, recipient-type and co-transplanted cells in a syngeneic transplantation setting was not available. Instead, we used sublethally irradiated female animals that were transplanted with male BMC or used genetically anemic mice as explained above.

Retroviral tagging of hematopoietic stem cells revealed that only few hematopoietic stem cell clones actively contribute to hematopoiesis at one time in a stabilized hematopoietic system, of which the longest clone continues for 2½ years [2-4,51,55]. Although self-renewal exists at the level of the "stem cell compartment", it seems not to exist at the level of the individual stem cell as no clone was found to be immortal [30,56]. Based on these studies it could be argued that not only the level of chimerism but also the duration of chimerism after bone marrow transplantation is determined by the number of stem cells injected. However, in bone marrow transplantation this does not have to play an important role considering the enormous capacity of hematopoietic stem cell clones to produce progeny and the fact that normally enough clones can be transplanted to provide for a lifelong chimerism. The technique of retroviral marking

has the disadvantage that only infected clones can be studied. Theoretically, these clones do not necessarily represent the whole stem cell population. The cells that got infected might even have lost some of their self-renewal capacity when they went through the cell cycle, which is an obligatory event to become infected [81]. Therefore, the conclusion that immortal self-renewing hematopoietic stem cells do not exist [30,56] might be based on an artifact. Recently, a contradicting conclusion was reached by Brecher *et al.* [82], who demonstrated an extensive self-renewal capacity of LTRA cells in unperturbed bone marrow by transplantation and retransplantation of only low numbers of cells. Confusing the issue even more, Harrison showed that the number and repopulating ability of stem cells is reduced by the transplantation and retransplantation technique itself [35]. Therefore, using the present techniques, it might be difficult to answer the question whether infinite self-renewal does exist at the hematopoietic stem cell level. For now, the LTRA cells is functionally characterized by its 'extended' ability to self-renew.

Transplantation studies with individually tagged stem cells have shown that the first 4 to 6 months after transplantation are characterized by frequent fluctuations in the contribution of different clones [2-4,51,54,56,83,84]. In addition, the study presented in *Chapter 3* of this thesis shows that a population which is highly enriched for CFU-S-12 contributed to hematopoiesis for 4 to 5 months after transplantation [69]. This demonstrates that CFU-S-12 are by definition short-term *in vivo* repopulating cells (STRA cells). Consequently, the LTRA of a graft can be determined only after a period of minimally 4 months. At that time a stabilized hematopoietic system has been reached and most short-term clones have disappeared [38,42,85-87]. Some LTRA cells, however, can also contribute to hematopoiesis within 2 months after transplantation [2,88]. The delayed activation of LTRA cells, as observed in most studies, does not seem to be an obligatory consequence of their primitiveness, but merely reflects the time at which their recruitment is required. In other words, a system that is depleted of hematopoietic cells might recruit its stem cells at an earlier time than a system that is not as severely depleted. Therefore, by definition, LTRA cells can have STRA, but STRA cells do not possess LTRA.

1.2.3 High proliferative potential colony-forming cell assays

The high proliferative potential colony-forming cells (HPP-CFC) [89] are among the most primitive quiescent hematopoietic cells identified *in vitro* that are stroma-independent. They give rise to large (> 0.5 mm in diameter) colonies in semi-solid cultures (containing at least 50,000 cells) and are strictly dependent on a specific (combination of) hematopoietic growth factors [90-92]. The classical HPP-CFC, grown from 2-day post-5-fluorouracil bone marrow, depends on the combined stimulus of interleukin 1α (IL-1α) plus IL-3 plus colony-stimulating factor-1 (CSF-1) [90]. The Pro-HPP-CFC-1, HPP-CFC-1, HPP-CFC-2, HPP-CFC-3 and the low proliferative potential colony-forming cell (LPP-CFC) represent a series of progressively maturing

progenitor cell populations that respond to specific combinations of 4, 3, 2 or single growth factors, respectively, as was shown in a recent review of Kriegler *et al.* [93] (Figure 1.1). Although the most primitive HPP-CFC resemble primitive hematopoietic stem cells in phenotype, resistance to 5-fluorouracil and multipotentiality, they do not represent LTRA cells since the outcome of *in vivo* transplantation could not be predicted by the HPP-CFC assay [94]. Two other primitive multipotential hematopoietic cells of high proliferative potential that need to be mentioned are the blast colony-forming unit (CFU-Bl) [95], which is stimulated by combinations of hematopoietic growth factors such as IL-3 + IL-6 (IL-1), and the colony-forming unit (type A) in agar (CFU-A), which is stimulated by AF1-19T (rat kidney cell line) and L929 (fibroblast cell line) conditioned media [96,97]. The conditioned medium of the AF1-19T contains multiple cytokines whereas medium from the L929 contains primarily macrophage colony-stimulating factor (M-CSF). As discussed by Bertoncello [92], both the CFU-Bl and CFU-A are closely related to the IL-1α + IL-3 + CSF-1 responsive HPP-CFC, if not equivalent. Finally, a cell of high proliferative potential that is grown *in vivo*, using intraperitoneally implanted diffusion chambers (diffusion chamber colony-forming unit, or CFU-D) was also shown to be a primitive cell [98], capable of giving rise to CFU-S-12 [99], multilineage CFU-C [99,100], HPP-CFC-1 and HPP-CFC-2 [101]. *In vivo*, the CFU-D had radioprotecting short-term repopulating abilities but lacked LTRA [102]. In summary, HPP-CFC, CFU-Bl, CFU-A and CFU-D constitute primitive progenitors that are valuable tools in the analysis of relatively early events in hematopoiesis, but do not belong to the most immature population of hematopoietic stem cells.

1.2.4 Cobblestone area-forming cell assay

The technique of murine long-term bone marrow culture (LTBMC) has been developed by Dexter *et al.* [103,104] in 1976, who described the conditions needed to grow a hematologically active bone marrow microenvironment *in vitro* in tissue culture flasks. Ultrastructural studies of the stroma revealed the presence of an intricate network of multilayered stromal cells, including adipocytes, fibroblasts, fibroblast-related barrier cells and reticulum cells, macrophages and endothelial cells that support the hematopoietic cells [105-108]. Primitive hematopoietic cells and groups of actively proliferating cells, characterized by their typical appearance as "cobblestone areas" (CA), are located within the adherent layer covered by endothelial cells [108]. With increasing maturity the hematopoietic cells migrate to the surface of the layer to either stay there, or to disappear into the medium [109]. Therefore, the presence of a CA, but not necessarily the presence of a mature cluster on top of the stroma (which has been used as an endpoint by others [110,111]) reflects hematopoietic activity. Ploemacher *et al.* used this culture method to design a limiting dilution assay for murine

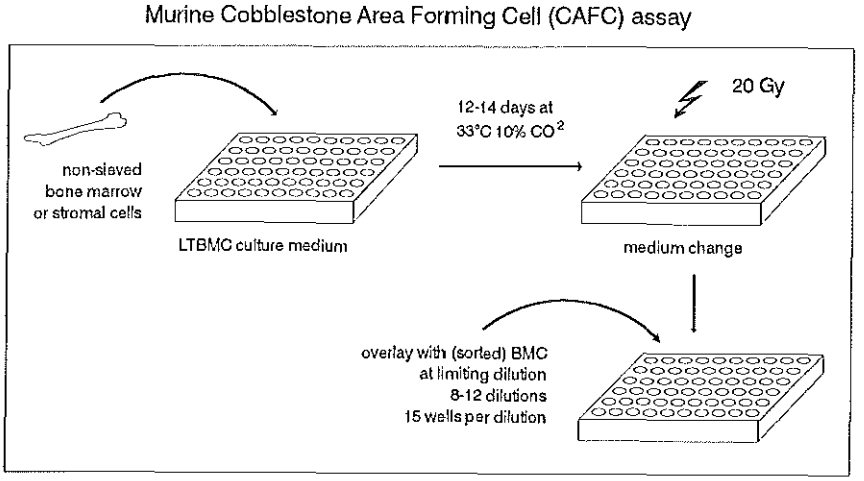

Figure 1.3 Schematic representation of the setup of the murine cobblestone area-forming cell (CAFC) assay. Stroma is grown in α-modified DMEM supplemented with 10% FCS and 5%HS. In addition, the medium contains penicillin, streptomycin, L-glutamine, β-mercapto-ethanol and hydrocortisone. After irradiation medium with 20% HS was used. Between 5 days and 4-5 weeks after inoculation the individual wells are checked for presence of a cobblestone area (CA) using a phase-contrast inverted microscope. The frequency of CA can be calculated using Poisson statistics.

hematopoietic cells using 96-well plates [112,113]. A stromal layer was grown in 12-14 days in 96-well plates from fresh murine BMC, as is schematically shown in Figure 1.3. The stromas are irradiated at confluency to stop all endogenous hematopoietic activity and to serve as a supportive microenvironment. It has been shown that irradiation did not affect the ability of the stroma to support hematopoiesis [114].

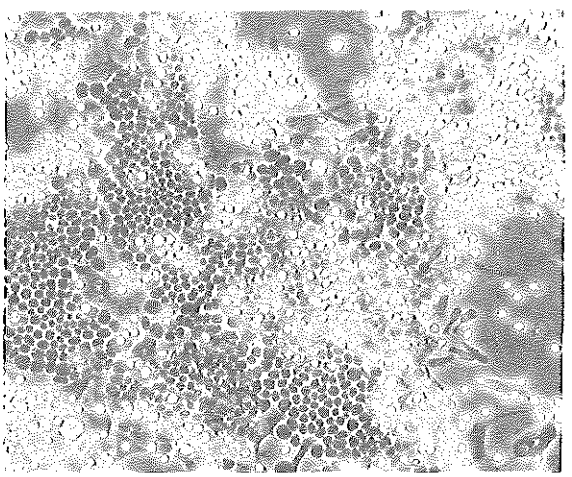

Figure 1.4 Picture of a murine CAFC culture. The cobblestone areas appear dark in phase contrast and are surrounded by stromal cells of the adherent layer (original magnification 250x). Maturing hematopoietic cells are more round and therefore reflect more light.

Whole or sorted BMC were then inoculated at limiting dilution using 15-20 wells per dilution. CA, appearing as flat dark hematopoietic clones of 5 cells or more located under the stroma (Figure 1.4), were scored by phase contrast microscopy at different time points after inoculation. The frequency of the cobblestone area-forming cell (CAFC) is calculated based on the proportion of negative wells, i.e. wells without CA, using Poisson statistics [115,116].

Previously, time course studies on colony-forming cells, *in vitro* as well as *in vivo*, had revealed that the primitiveness of a particular subset within the hierarchy of the stem cell compartment was inversely related to the length of the interval required for their clonal expansion [13,117-119]. Similarly, using post-5FU and different sorted progenitor and stem cell subsets, the time of appearance of a CA after inoculation was inversely related to the primitiveness of the CAFC [112]. Most importantly, extensive correlation studies have shown that CAFC measured at day 10 after inoculation (CAFC-10) correlate highly with the number of CFU-S-12, while enrichments for CAFC measured after 4-5 weeks (i.e. CAFC-28 to CAFC-35) correlate highly with the MRA and LTRA of a graft (when compared at a 40% to 50% chimerism level) [38,48,112,120,121]. This is schematically shown in Figure 1.5. Furthermore, the absolute number of CAFC-35 in normal bone marrow (0.5 to 2 per 100,000 cells inoculated) agrees with previous estimates of the frequency of LTRA cells determined in different mouse models *in vivo* [5,29,41,122].

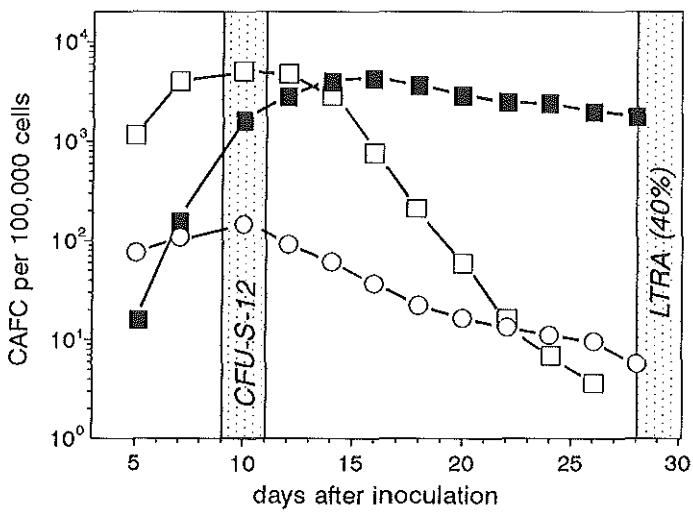

Figure 1.5 Frequency analysis of cobblestone area-forming cells (CAFC) in unseparated murine bone marrow (○); in a $Rh123^{bright}$ fraction of low-density bone marrow (□); and in a $Rh123^{dull}$ fraction of low-density bone marrow (■). The shaded areas indicate the days at which high correlations were found between (1) day-10 CAFC and CFU-S-12 (n=55, r=0.96), and (2) day-28 CAFC and LTRA cells responsible for a 40% level of donor-type repopulation (n=23, r=0.97).

In conclusion, the CAFC assay represents a novel method that can be used to determine the frequency of hematopoietic stem cells *in vitro*. In addition, it offers an insight into the frequency distribution of the different hematopoietic subsets within the stem cell hierarchy, ranging from the most mature progenitors as detected by CAFC in the first week after inoculation to the most primitive cells with LTRA that are represented by CAFC at week 4-5 [123]. The assay has recently been simplified by the use of the cloned murine stromal cell line GBl/6 instead of fresh bone marrow, showing similar correlations between CAFC and *in vivo* stem cell subsets [33]. In the mean time, a similar assay was developed in our laboratory that allows frequency analysis of human stem and progenitor cells [124]. The assay was set up using the murine stromal cell line FBMD-1, which was kindly provided by Dr. S. Neben (Genetics Institute, Boston). To stimulate the growth of human CA the culture medium was supplemented with recombinant human IL-3 and G-CSF. Frequencies of primitive and more mature human hematopoietic stem cell subsets can be determined without the need for extensive replating studies, such as those used in the original long-term culture initiating cell (LTC-IC) assay [125,126]. The results show that immature human hematopoietic cells give rise to CAFC between 5 and 8 weeks after inoculation while progenitor cell frequencies can be measured within the first 4 weeks [124]. CAFC frequencies derived from transplant material are currently being compared with the *in vivo* reconstituting ability of the same cells after transplantation. As is indicated in the *General discussion*, the CAFC assay will be a useful tool for the clinician as it can be applied to predict the survival and proliferative ability of the LTRA cells in a transplant *in vitro* prior to the actual transplantation.

1.3 Purification of murine hematopoietic stem cell subsets

Murine hematopoietic stem cells can be purified using a variety of different techniques [8,9,127-129]. The first purification step often includes a separation of hematopoietic cells from the vast bulk of mature bone marrow or peripheral blood cells, using differences in cell size and density. Density gradients, for instance, allow large numbers of cells to be processed relatively quickly prior to the more delicate and time-consuming method of fluorescence-activated cell sorting (FACS). Alternatively, BMC can be purified using panning or magnetic separation techniques. The FACS offers the opportunity to separate cells on the basis of small differences in cell surface molecule expression, such as proteins or sugar moieties that can be recognized by antibodies or lectines. Cells can also be separated on the basis of differences in the number and activity of cell organelles, such as mitochondria, or on the basis of DNA content using specific fluorescent dyes. In the following paragraphs the methods most commonly used for the purification of murine hematopoietic stem and progenitor cells are described. In particular, the methods will be discussed with respect to the ability to separate mature and primitive hematopoietic stem cell subsets. The paragraphs in this chapter are arranged in a way that every paragraph includes the information needed to read the following sections. Successively are described: density separation, the expression of Thy-1, Sca-1 and lineage-specific cell surface antigens, the expression of the growth factor receptor *c-kit*, rhodamine-123 fluorescence, Hoechst 33342 fluorescence, and the cell surface expression of sialic acid residues as detected by wheat germ agglutinin binding. The phenotype of CFU-S-12 and LTRA cells is schematically summarized in Figure 1.6, located at the end of this chapter.

1.3.1 Density separation

The first separation of BMC on the basis of differences in density dates back to 1969 when Worton *et al.* used velocity sedimentation to separate CFU-S from a subpopulation of smaller cells that had the capacity to produce CFU-S as well as secondary colonies after retransplantation [16]. These finding were extended by others and led to the use of several equilibrium density centrifugation protocols and the development of counterflow centrifugal elutriation, as was reviewed by Visser and Van Bekkum [9]. Using discontinuous gradients of either bovine serum albumin (BSA) [11,130], metrizamide [131], Percoll [132] or Ficoll-400 [120,133], primitive hematopoietic cells and CFU-S-12 were found to have a relatively low density, ranging from 1.055 to 1.078 g/mL. Quiescent cells, as has been shown for CFU-S, have a slightly lower density than cycling cells [134]. Using counterflow elutriation, CFU-S-12 could be partly separated from the more primitive cells with marrow repopulating ability (MRA) [135]. Quantitative analysis of the different fractions of murine bone marrow has shown that cells with long-term repopulating ability (LTRA) are heterogeneously distributed over the different elutriation fractions [26]. LTRA cells

were distributed over fractions with varying flow rates, from 25 up to 35 mL/min, with the highest concentration in the 29-30 mL/min fraction. CFU-S-12, on the other hand, were found exclusively in the 29-35 mL/min fractions [26]. Consequently, it could be demonstrated that the 25 mL/min fraction contained a subset of LTRA cells and no CFU-S-12, adding evidence to the distinct nature of these subsets [25].

1.3.2 Cell surface phenotype of murine hematopoietic stem cells

The first step in the definition of selection markers for murine hematopoietic stem cells was the demonstration of Thy-1 antigen on early hematopoietic progenitors in the rat [136,137]. Thy-1 is a glycoprotein that is encoded by a member of the immunoglobulin gene superfamily [138]. Using rat bone marrow anti-Thy-1 antibodies have successfully been used for the purification of CFU-S, cells with radioprotective ability and hematopoietic stem cells [139-141]. Mouse bone marrow, on the other hand, also expresses Thy-1 but contains less Thy-1 positive cells than rat bone marrow, which is beneficial for its use as selection marker [142]. In the mouse, Thy-1 was shown to be highly expressed on *in vitro* clonable progenitors, like high proliferative potential colony-forming cells (HPP-CFC), erythroid burst-forming units (BFU-E) and granulocyte-macrophage colony-forming units in culture (CFU-GM), whereas expression was gradually lost upon maturation and differentiation [143-146]. In addition, lethally irradiated animals could be rescued and completely repopulated by intravenously injected Thy-1-positive (Thy-1$^+$) BMC, indicating that Thy-1 is also expressed by hematopoietic stem cells [147]. Also human hematopoietic stem cells probably express the Thy-1 antigen as is indicated by the presence of Thy-1$^+$ long-term culture initiating cells (LTC-IC) and cells capable of engrafting mice with a severe combined immuno-deficiency (SCID mice) [148-150]. In mice, two allelic variants of the Thy-1 antigen have been described: Thy-1.1 and Thy-1.2. It has been found that the expression and distribution of Thy-1 on murine hematopoietic stem cells and CFU-S-12 differs between the two variants [151]. In mouse strains expressing Thy-1.1 approximately 99% of the hematopoietic stem cells were found in the Thy-1.1-low-positive (Thy-1.1^{lo}) population of bone marrow. In contrast, only 19% of the stem cells could be found in the Thy-1.2^{lo} population in Thy-1.2 genotype mice, while the remaining 81% were recovered from the Thy-1.2^{neg} subset. Based on these data the seemingly contradicting results with respect to the distribution of the Thy-1 antigen on hematopoietic cells in mice could finally be explained. It remains to be tested whether similar differences in distribution of the Thy-1 antigen exist in other species.

Mouse hematopoietic stem cells and immature progenitors lack a high expression of lineage markers that are specific for mature B cells (B220), mature T cells (CD4 or CD8), granulocytes (Gr-1) and myelomonocytic cells (Mac-1) [147]. To achieve a population of lineage marker-negative (Lin$^-$) BMC, magnetic separation may be the technique of choice. It has the advantage over sorting by FACS that many cells can be processed within a relatively short time. For magnetic separation BMC are labeled with

the appropriate antibodies and coupled to magnetic beads using second step anti-immunoglobulin antibodies [142,147,152,153]. The antigen-bearing cells are separated from the bone marrow by placing the tube with labeled cells in a strong magnetic field. The supernate that is collected contains the Lin^- cells while Lin^+ cells remain behind. It has recently been shown that hematopoietic stem cells express low levels of B220, CD4, Gr-1, and an erythroid specific antigen recognized by monoclonal antibody TER-119, but only at a low level [88,154-157]. With magnetic separation only strongly antigen-expressing cells are depleted. When Lin^- cells are collected by FACS, however, one should be aware that there is a risk of depleting part of the hematopoietic stem cells when the sorting windows are not set to include the dimly stained populations. It has been demonstrated that hematopoietic stem cells can be significantly enriched by combining lineage depletion with sorting for Thy-1^+ BMC [147]. The population of Lin^- Thy-1.1^{lo} cells comprises about 0.2% of the BMC and includes all the hematopoietic stem cells .

The Lin^- Thy-1.1^{lo} population could be further enriched for hematopoietic stem cells by selecting cells that expressed stem cell antigen-1 (Sca-1) [31]. Sca-1 is a member of the Ly-6 antigen family [158] which exists in two allelic forms that are primarily expressed by lymphocytes and to a lesser extent by other hematopoietic and non-hematopoietic cells [158,159]. The Ly-6E.1 specificity of the Ly-6 antigen is expressed in Ly-6^a haplotype mice (e.g. BALB/c, C3H/J, CBA/J, and A/J) whereas the Ly-6A.2 molecule is only expressed in Ly-6^b haplotype mice (e.g. C57BL, SJL/J, DBA/J, and AKR/J). In addition, Ly-6A.2 was found to be expressed constitutively in lymphocytes, while Ly-6E.1 was expressed weakly in non-stimulated, and strongly in mitogen-stimulated T cells [158,160]. Differences have also been found in the expression of the Ly-6A/E molecules on hematopoietic stem cells and progenitors. On average, 4.6% of all BMC (or 3.7% of Lin^- BMC) expressed Ly-6A/E in Ly-6^b mice whereas only 2.1% of the BMC (or 0.5% of Lin^- BMC) expressed the antigen in Ly-6^a mice [161]. Furthermore, about 99% of all MRA in Ly-6^b haplotype mice could be recovered in the population of Sca-1^+ BMC whereas the same population in Ly-6^a haplotype mice contained only 24% of the total MRA [161]. These findings demonstrate that between inbred mouse strains and, therefore, probably also in outbred populations, differences exist in the phenotype of hematopoietic stem cells.

The Thy-1.1^{lo} Lin^- Sca-1^+ subpopulation in Ly-6^b mice comprised 0.05% of all nucleated BMC and was shown to be virtually pure in CFU-S-12 [31,162-164]. Compared to unseparated BMC, this subset was also highly enriched (1000-fold) for thymus-repopulating cells as determined by intrathymic transfer. In addition, only 30 to 100 cells were needed to protect 50% of lethally irradiated mice for at least 30 days and to induce a multilineage repopulation [31,162-164]. The Sca-1^- subpopulation, on the other hand, contained primarily day-8 CFU-S (CFU-S-8) and more differentiated progenitors. This subset contained a low number of CFU-S-12 and was unable to save mice from lethal irradiation. Sca-1^- cells were capable of inducing a short-term

repopulation when injected into sublethally irradiated mice which gave rise to only myelo-erythroid lineages but not to lymphocytes. Also, CFU-S-12 colonies from the Sca-1$^-$ subset were significantly smaller, as compared to the Sca-1$^+$ subset [151], indicating that the Sca-1$^-$ subset contained more mature progenitors . *In vitro*, Sca-1$^+$ (but not Sca-1$^-$ cells) required multiple growth factors in methylcellulose cultures [165], which supports this notion. Furthermore, a subset of the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells also formed delayed colonies in methylcellulose [165]. In conclusion, the data indicate that Sca-1$^-$ hematopoietic cells in Ly-6^b haplotype mice have all the characteristics of more committed myelo-erythroid progenitors, while the Sca-1$^+$ subpopulation contains more immature precursor cells and long-term repopulating hematopoietic stem cells.

The Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population was initially thought to be pure in hematopoietic stem cells [31]. However, repopulation studies *in vivo* showed that only 1 in every 39 Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ cells was able to induce more than 1% donor-type repopulation [162]. Furthermore, chimerism was only observed in the first few months after transplantation and could only be extended into 1 of 280 secondary recipients by retransplantation [162]. In addition, a separate study had shown that long-term repopulation could be induced by a subpopulation of low density BMC, isolated by counterflow elutriation, that completely lacked CFU-S-12 [25]. This was in apparent contrast with the findings of Spangrude *et al* [31] and generated some controversy as to whether or not the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ population was homogeneous for both CFU-S-12 and long-term repopulating stem cells. It was also not clear whether or not this subpopulation contained all stem cells in the bone marrow or just a subset [166-171]. Subsequent experiments showed that all pluripotent hematopoietic stem cells in Ly-6^b mice were confined to the Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ subpopulation [163]. It was also shown that the population of Thy-1^{lo} Lin$^-$ Sca-1$^+$ was heterogeneous and contained in vitro CFC, day-12 CFU-S, and long-term repopulating cells for both the myeloid and lymphoid lineages [164]. It is now clear that Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ subpopulation is not the "end of the road in the purification of hematopoietic stem cells" [166].

Reassessment of the purification procedure by Spangrude *et al.* showed that the selection for Thy-1^{lo} could be left out without affecting the functional characteristics of the sorted population [10]. Enrichment levels of progenitors and stem cells in the Lin$^-$ Sca-1$^+$ subset decreased about 2-fold as compared to the Thy-1^{lo} Lin$^-$ Sca-1$^+$ subpopulation . The CFU-S-12 activity in the Lin$^-$ Sca-1$^+$ subset was almost completely confined to the 40% of the cells that expressed low levels of Thy-1, which is in agreement with the other data. Furthermore, it was demonstrated that this population could protect lethally irradiated animals for at least 30 days in the absence of a long-term hematopoietic reconstitution [10]. This demonstrates that the ability to rescue lethally irradiated animals from hematopoietic failure is not an adequate indicator of the long-term repopulating ability of a graft and, thus, is not a characteristic of the most primitive engrafting hematopoietic stem cell. Phenotypic analysis of the Lin$^-$ Sca-1$^+$ population revealed that it expressed intermediate levels of the polymorphic

glycoprotein-1 (Pgp-1), low levels of heat-stable antigen (HSA), and high levels of the class I major histocompatibility antigen (H-2K/D) [10]. Except for Thy-1 expression, the population was shown to be heterogeneous for wheat germ agglutinin (WGA) affinity as well as for rhodamine-123 (Rh123) retention [10]. Further subdivision of both the Thy-1^{lo} Lin^- Sca-1^+ and Lin^- Sca-1^+ cells into a $Rh123^{hi}$ (or $Rh123^{med+hi}$) and $Rh123^{dull}$ subset revealed a partial separation of CFU-S-12 and long-term repopulating stem cells [172,173]. Both subpopulations contained CFU-S activity, whereas only the $Rh123^{dull}$ subpopulation was able to support a multilineage hematopoietic reconstitution of secondary recipients. This again confirms that the Lin^- Sca-1^+ and Thy-1^{lo} Lin^- Sca-1^+ populations in the bone marrow are quite heterogeneous. In summary, the LTRA cell can be defined as a cell that expresses low levels of Thy-1.1 and lineage markers, that is positive for Sca-1 in Ly-6^b haplotype mice and that has a low retention of Rh123. The CFU-S-12, on the other hand, shares this phenotype with the exception that the majority of CFU-S-12 retain high levels of Rh123. Details on the retention of Rh123 will be given in *paragraph 1.3.4.*

1.3.3 The expression of the c-kit receptor

The proto-oncogene *c-kit*, which maps to the white-spotting (*W*) locus [174-176], encodes a transmembrane tyrosine kinase receptor for *kit* ligand (KL) [177], also called stem cell factor (SCF) [178,179], steel factor (SF) or mast cell growth factor (MGF) [180]. SCF is a hematopoietic growth factor with a wide range of biological activities. Mutations at the *W* locus affect melanogenesis, gametogenesis and also hematopoiesis during fetal development and in adult life [58,181,182]. Phenotypic analysis of murine bone marrow showed that *c-kit* was highly expressed in 5% to 10% of all BMC, and in 70% to 80% of the Thy-1.1^{lo} Lin^- Sca-1^+ BMC [183-185]. Upon maturation from Lin^- into Lin^+ BMC, *c-kit* expression gradually decreased. Of the mature cells, only a small number of granulocytic and monocytic cells (Gr-1^+ and Mac-1^+ cells) were found to express *c-kit*, and only at a low level. When ACK2, an antagonistic anti-*c-kit* mAb, was added to a long-term bone marrow culture, hematopoiesis could be completely inhibited [183,186]. Apparently, ACK2 blocks a functional epitope on *c-kit* that prevents the interaction of the receptor with its ligand. Also, injection of ACK2 *in vivo* resulted in a complete depletion of myeloid and erythroid cell lineages in the bone marrow. Furthermore, when given after irradiation and bone marrow transplantation, ACK2 completely suppressed CFU-S-8 colony formation and dramatically decreased the number of CFU-S-12 colonies [183,185]. This indicates that the *c-kit* receptor is expressed in all *in vitro* clonable progenitors (except IL-7 responsive B-lineage progenitors), in all day-8 and day-12 CFU-S, in thymus-repopulating cells [187], and in long-term repopulating stem cells [157,184,185,188,189]. The *c-kit*$^-$ subpopulation contained neither CFU-S-12 nor LTRA cells.

To investigate whether *c-kit* could be used in combination with Sca-1 to improve the purification of hematopoietic stem cells, Lin^- *c-kit*$^+$ cells were fractionated into a Sca-1^+ and Sca-1^- population [189]. The Lin^- *c-kit*$^+$ Sca-1^+ subpopulation contained 0.08% of all BMC. This subpopulation did not contain any CFU-S-8, but consisted for 80% of CFU-S-12 (based on a spleen seeding efficiency of 10%). It was shown that 100 cells could rescue lethally irradiated mice and fully reconstitute hematopoiesis [189]. The functional characteristics of the Lin^- *c-kit*$^+$ Sca-1^+ subpopulation are remarkably similar to those of the Thy-1^{lo} Lin^- Sca-1^+ subpopulation (*see paragraph 1.3.2*). As *c-kit* is found on both primitive hematopoietic stem cells and progenitors, sorting on the basis of *c-kit* expression in itself offers no advantage over the use of Sca-1 with respect to the separation of the different stem cell subsets from unseparated bone marrow. However, combined with counterflow centrifugal elutriation (CCE) (*paragraph 1.3.1*) this method has several advantages over other methods. Firstly, hematopoietic stem cells can be 500 to 1000-fold enriched while including only few contaminating CFU-S-12 (one per 253 cells) by sorting Lin^- *c-kit*^{bright} cells from CCE low density fraction FR25 [157]. This subset contained less than 2.7% of all CFU-S-12 in Lin^- *c-kit*^{bright} bone marrow. About 100-200 FR25 Lin^- *c-kit*^{bright} cells were sufficient to repopulate W/Wv recipients [157], which is comparable to the repopulating ability of the Thy-1^{lo} Lin^- Sca-1^+ subpopulation. Secondly, *c-kit* is cross-species distributed and can be used in all strains of mice. In contrast, the expression of the Thy-1 and Sca-1 (Ly-6A/E) antigens are haplotype restricted and are only expressed together in few mouse strains [151,161]. In man, *c-kit* appears to be differently expressed as long-term culture initiating cells were predominantly found to express low levels of *c-kit*, while most progenitors expressed *c-kit* highly [148,190].

1.3.4 Rhodamine-123 retention

In the past, rhodamine-123 (Rh123) retention has been used extensively for the purification of primitive murine hematopoietic cells [22-24,172,173,191-194]. Rh123 is a supravital fluorescent dye that binds to the mitochondrial membrane of living cells and is retained much more in cycling than in quiescent cells [195-197]. As Rh123 can be excited by light with a wavelength of 488 nm it can be applied directly for cell sorting without the need for antibodies and second step incubations, using a cell sorter equipped with a single standard argon-ion laser. CFU-S-8, CFU-S-12 and cells with MRA can be ordered on the basis of their decreasing Rh123-retention, respectively [22-24,198]. The partial separation of these subsets confirmed earlier data on the existence of a more primitive pre-CFU-S that was shown to be associated with MRA and was resistant to the cytostatic effects of 5-fluorouracil or bromodeoxyuridine [17,20]. Using irradiated stromal layers of long-term bone marrow cultures, it was shown that Rh123^{bright} cells were unable to maintain hematopoiesis for more than a few weeks. In contrast, the Rh123^{dull} fraction, which was depleted of CFU-S-8 and contained low numbers of CFU-S-12, was able to maintain hematopoiesis for a

prolonged period of time [191,199]. Sorting on the basis of Rh123 retention has been combined with many of the methods that are described in this chapter, such as sorting on the basis of *c-kit* expression [185,188] (*paragraph 1.3.3*), wheat germ agglutinin (WGA) affinity [191,192] (*paragraph 1.3.6*), or Thy-1, linage marker and Sca-1 expression [172,173] (*paragraph 1.3.2*). However, no combination of Rh123 with other markers did result in a better separation of CFU-S-12 and LTRA cells . This might be caused by the fact that the retention of Rh123 in cells is determined by multiple parameters, as will be discussed in the following.

Experiments on the nature of Rh123 fluorescence showed that the retention of Rh123 by CFU-S could not be explained solely by CFU-S cell cycle activity. The assumption that there would be a linear relationship between cell cycle and Rh123 retention was based on the fact that cycling cells have more, and more active, mitochondria. In normal bone marrow, however, 100% of the CFU-S-8 and 60% of the CFU-S-12 stain brightly with Rh123 [198] while only 10% of both populations are cycling, as determined by S-phase-specific killing [200,201]. Likewise, the difference in size between the mitochondria of $Rh123^{dull}$ and $Rh123^{bright}$ BMC was also insufficient to explain the difference in fluorescence intensities [46]. Recently, it has been shown that the retention of Rh123, in both human and murine BMC, is inversely related to the expression of the multidrug resistance (MDR) P-glycoprotein [202,203]. P-glycoprotein, the product of the MDR1 gene, is a transmembrane efflux pump that is able to decrease the intracellular concentrations of various lipophilic cytotoxic drugs as well as the accumulation of fluorescent dyes like Rh123 [204]. In both mouse [22-24,172,173,191-194] and human [148,149,205] the most primitive hematopoietic cells in the bone marrow are characterized by a high P-glycoprotein expression and, consequently, a low Rh123 retention.

For cell sorting, however, Rh123 cannot be used under all circumstances as its retention is directly influenced by the cycling and metabolic status of the hematopoietic cells, which is reflected by the activity, size and number of mitochondria per cell [46,88,195,196,198]. Treatments and conditions that activate hematopoiesis, and thus change the cycling behavior of the hematopoietic cells, change the retention of Rh123 in the bone marrow. As treatment of the donor with cytostatic agents can change the expression and activity of P-glycoprotein in BMC, this can also influence Rh123 retention. Also the concentration of glucose in the cell culture medium in which the cells are kept after harvest has been shown to influences the level of Rh123 fluorescence [206]. In addition, it has been shown that BMC from genetically different mouse strains even intrinsically differ in their Rh123 retention [203]. Therefore, Rh123 retention is difficult to standardize and can only be used as a relative selection criterion in cell sorting.

1.3.5 Hoechst 33342 fluorescence

The fluorescent bisbenzimide derivative Hoechst 33342 is a supravital DNA stain [207] that distinguishes cells primarily on the basis of their DNA content and secondly on the basis of differences in membrane permeability, accessibility of their DNA to bind the dye, and probably also by differences in their RNA content [207-209]. Sorting bone marrow on the basis of light-scatter characteristics and a low Hoechst fluorescence enabled relatively high enrichments of CFU-S and committed progenitors [210,211]. CFU-S-12 and HPP-CFC were found to bind less Hoechst than the more mature CFU-S-8 and CFU-GM [212]. In addition, the subpopulation with a low Hoechst fluorescence intensity was shown to contain cells capable of radioprotection, short-term and long-term repopulation [42]. Long-term repopulating stem cells were 150-fold enriched when sorted on the basis of Hoechst alone [42], and 200-fold when sorted on the basis of Hoechst using WGA-positive cells [213]. The most successful purification reported to date, however, was based on the selection of lineage-depleted gradient-enriched marrow cells that bound low levels of Hoechst 33342 and retained low amounts of Rh123 [194]. When as little as 20 male donor-cells were transplanted into lethally irradiated female recipients, the cells completely repopulated the lymphoid as well as the myeloid compartments of all recipients for at least 10 months after transplantation. Donor cells were transplanted along with "compromised" (three times retransplanted) marrow as described by Szilvassy *et al.* [214], to provide temporary support during the first 3 critical weeks following irradiation. Assuming a frequency of 1-2 hematopoietic stem cells per 100,000 normal BMC [5], this purification procedure resulted in a 2,500 to 5,000-fold enrichment for multilineage long-term repopulating stem cells. In these, nor in other experiments [212,215], were any toxic effects of Hoechst on stem cells or progenitors found. However, Hoechst 33342 was reported to exert cytotoxic effects and induce DNA damage in Chinese hamster cells [216]. Although Hoechst 33342 has successfully been used for sorting human BMC at non-toxic concentrations [217], the danger of toxicity still represents a major drawback for application of intracellular dyes in the purification of human hematopoietic stem cells.

1.3.6 Wheat Germ Agglutinin affinity

The lectin wheat germ agglutinin (WGA) has been used since 1981 to positively identify hematopoietic progenitors and stem cells from bone marrow and spleen, and has been integrated in many purification procedures [46,130,131,191,192,218-221]. WGA has an affinity for sialic acid residues of glycoproteins or glycolipids on the cell membrane of hematopoietic cells. Sialic acids play a role in the lodging and homing of hematopoietic cells to their respective microenvironments in that they prevent sequestration in the liver by a mechanism involving asialoglycoprotein receptors with specificity for galactosyl moieties [222-225]. Removal of sialic acid residues from intravenously infused BMC by neuraminidase treatment resulted in a sequestration of

the CFU-S in the liver [222-224]. Also, infusion of WGA-labeled cells gave reduced spleen-colony counts indicating that WGA may interfere with a function of the negatively charged sialic acid residues in preventing sequestration (R.E. Ploemacher, unpublished observation). This can be counteracted by incubation of WGA-positive cells with the competitive sugar N-acetyl-*D*-glucosamine, which efficiently removes most of the surface-bound WGA with a short incubation [130]. An effect of WGA on

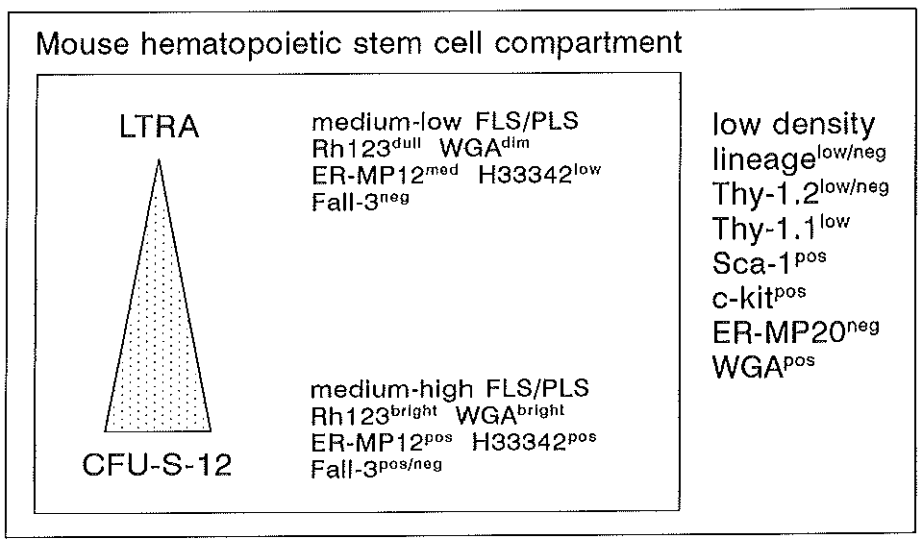

Figure 1.6 Schematic representation of the mouse hematopoietic stem cell compartment with the CFU-S-12 and LTRA cell located at opposite ends. The phenotype and cell surface markers characteristic for both cell types are indicated outside the box, whereas specific markers are shown inside the box. Most of the markers shown are described in this chapter. ER-MP12 and ER-MP20 are studied and described in *Chapters 4,5 and 6*, and Wheat germ agglutinin (WGA) and rhodamine-123 (Rh123) are discussed in *Chapters 2,3* and *6*.

the homing of hematopoietic stem cells to the bone marrow, or on hematopoietic activity *in vitro* has never been observed. The isolation of cells with a medium or high affinity for WGA, sorted from lipopolysaccharide (LPS)-stimulated murine spleen and bone marrow, revealed different recoveries for CFU-S-12 and cells associated with MRA[CFU-S-12] [220,226]. The highest incidence of CFU-S-12 and cells capable of protecting lethally irradiated animals were found among the WGA^{bright} cells, while cells with MRA bound less of the lectin [220,226]. This observation suggested that functionally different hematopoietic cell subsets could be separated on the basis of differences in sialic acid expression. As presented in *Chapter 2* of this thesis, we determined the recovery of different hematopoietic stem and progenitor cell subsets with respect to their affinity for WGA [121]. Sialic acid residues have been demonstrated on hematopoietic cells in several species, including rhesus monkey and

man [227,228]. The specificity of WGA affinity in mouse, and the ease of labeling without the extra physical manipulation of the cells (as required when labeling cells with monoclonal antibodies) makes WGA an excellent candidate for the purification of human hematopoietic stem cells.

1.4 Introduction to the experimental work

The murine hematopoietic stem cell has been extensively studied using different purification methods and various *in vitro* and *in vivo* assays, as has been pointed out in the previous paragraphs. However, over time it became clear that functional definitions that had been used for years had to be re-evaluated. For instance, a report on the purification of mouse hematopoietic stem cells, published in Science in 1988 [31], created a lot of confusion as the authors used day-12 spleen colony formation to define stem cells (see *paragraph 1.3.2*). In contrast, other investigators found evidence of a more primitive stem cell subset in the bone marrow and emphasized the heterogeneity of the stem cell compartment [17,18,20,21,23-25]. In summary, these studies raised a number of questions with respect to the identity of the hematopoietic stem cell and the role of the different stem cell subsets in bone marrow transplantation. Some of the issues are addressed in the experimental work presented in this thesis.

The experiments described in ***Chapter 2*** were designed to test whether or not wheat germ agglutinin (WGA) affinity could be used as a parameter to separate CFU-S-12 from cells with marrow and long-term repopulating ability (MRA and LTRA, respectively). The study was based on the observation that BMC with a high affinity for WGA were highly enriched for CFU-S-12 and relatively depleted for MRA [135,226]. The present study provides a detailed map of the different hematopoietic stem cell subsets and mature cells in the bone marrow with respect to their affinity for WGA, which is specific for cell surface sialic acid residues (see *paragraph 1.3.6*). Subsets were determined *in vitro* using the CAFC assay and *in vivo* using the CFU-S, MRA and LTRA assays. The results show that primitive hematopoietic stem cell subsets express sialic acid at a low level while the more mature subsets, such as the CFU-S, express it at a much higher level. BMC could be highly enriched for LTRA, but not for CFU-S-12, by sorting cells on the basis of a low affinity for WGA.

The experiments in ***Chapter 3*** were designed to answer the question whether CFU-S-12 and LTRA cells constitute different stem cell subsets or broadly overlap in their functional definition. Previously, Jones *et al.* have shown that mice could be fully repopulated with a low density fraction of bone marrow that contained no CFU-S-12 [25], indicating that at least part of the LTRA cells were not able to form spleen colonies. The study was commented on by Weissman *et al.*[170] who postulated that the CFU-S-12 and LTRA cell are the same at high enrichment levels. To address this issue we highly enriched BMC for either CFU-S-12 or LTRA cells by sorting on the basis of rhodamine-123 retention (see *paragraph 1.3.4*), or using WGA affinity as described in *Chapter 2*. Repopulation *in vivo* was determined using a recently developed α-thalassemic chimeric mouse model [65,67,68,229]. In short, we found that CFU-S-12 and LTRA cells could be extensively separated, which supports the findings of Jones.

In ***Chapter 4*** we evaluated the expression of a new antigen on hematopoietic stem and progenitor cell subsets in mouse bone marrow. The antigen is recognized by antibody ER-MP12, which was originally raised against macrophage precursor cells and was found to recognize 'immature' progenitors [230]. Initial experiments indicated that the majority of CFU-S-12 and LTRA cells expressed different levels of antigen. To test whether stem cell subsets could be distinguished on the basis of ER-MP12 antigen expression we determined its expression throughout hematopoietic differentiation, from hematopoietic stem cells to mature BMC, using *in vitro* and *in vivo* assays. The data show that the ER-MP12 antigen was expressed at a low level by LTRA cells and at a higher level by more mature progenitors such as CFU-S and CFU-C. The antigen was absent from mature BMC except from mature lymphocytes. The experiments also included a selection for antigen Ly-6C, which is recognized by antibody ER-MP20. Two-color immunofluorescence analysis with ER-MP12 and ER-MP20 revealed six subpopulations of BMC. Ly-6C was intermediately expressed by mature granulocytes and highly by monocytes and their committed progenitors, but was absent from CFU-S-12 and LTRA cells. In conclusion, the two antibodies provided a basis for a further separation of the subsets in the hematopoietic stem cell compartment which is shown in *Chapter 6*.

The experiments described in ***Chapter 5*** are closely linked to the work described in the previous chapter. They were designed to investigate whether committed bone marrow pro-thymocytes could be identified on the basis of a differential expression of the ER-MP12 and ER-MP20 antigens. Subsets of BMC were tested for their thymus-homing and -repopulating ability using intravenous and intrathymic injection. The data show that these antigens could be used to sort BMC that were enriched for T-cell precursors. However, no distinction could be made between T-cell-restricted and non-restricted progenitors.

In ***Chapter 6*** we compared the separation of hematopoietic stem cell subsets using the methods described in *Chapter 3*, i.e. separation on the basis of Rh123 retention and WGA affinity, with the method described in *Chapter 4*, i.e. separation on the basis of ER-MP12 antigen expression. We concluded that the best separation of LTRA cells and CFU-S-12 could be achieved by sorting cells on the basis of WGA affinity. We also found that mature BMC could be simultaneously excluded by selecting cells with an intermediate expression of the ER-MP12 antigen. Furthermore, both WGA and ER-MP12 could be used as positive selection markers for hematopoietic stem cells using bone marrow derived from 5-fluorouracil-treated mice.

Comparison of the numbers of CFU-S-12 and LTRA cells in different sorted populations, as described in *Chapter 3*, raised the question whether short and long-term repopulating stem cell subsets would differ in their bone marrow and spleen seeding efficiency. In the study described in ***Chapter 7*** we addressed this question by assessing the *in vivo* seeding of short and long-term repopulating stem cell subsets, in the bone

marrow as well as in the spleen, using the CAFC assay. It had been postulated that the seeding efficiency of hematopoietic stem cells could be increased by a short pre-incubation of the graft with hematopoietic growth factors [78,231,232]. However, these investigators had not determined seeding efficiencies. Therefore, we also tested the effect of growth factor pre-incubation on the seeding and final engraftment *in vivo*. Briefly, we found no difference in the seeding of different hematopoietic stem cell subsets and failed to observe a beneficial effect of growth factor pre-incubation. In contrast, the seeding of mouse hematopoietic stem cells and progenitors was significantly reduced.

In summary, the work presented in this thesis provides further evidence on the distinct nature of CFU-S and LTRA cells. We described the phenotype of the different murine hematopoietic stem cell subsets and investigated their clonal expansion *in vitro* and post-transplant engraftment *in vivo*. Furthermore, the study presents and compares different methods that allow the purification of murine hematopoietic stem cells subsets. The experimental work includes the validation of a new mouse model that allows a rapid measurement of the LTRA of a graft and contributes to the validation of the CAFC assay as an *in vitro* assay that is able to predict the *in vivo* repopulating ability of a graft.

1.5 References

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CHAPTER 2

WHEAT GERM AGGLUTININ AFFINITY OF MURINE HEMOPOIETIC STEM CELL SUBPOPULATIONS IS AN INVERSE FUNCTION OF THEIR LONG-TERM REPOPULATING ABILITY *IN VITRO* AND *IN VIVO*

Rob E. Ploemacher[1], Johannes C.M. van der Loo[1],
Carin A.J. van Beurden[2], and Miranda R.M. Baert[2]

1. Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
2. Department of Immunology, Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

Hemopoietic stem cells show extensive heterogeneity with respect to their proliferative potential and activity. We have recently reported that the accepted technique for sorting stem cells on the basis of high affinity for the lectin wheat germ agglutinin (WGA) did not select for cells initiating long term production of new stem cells on a stromal layer *in vitro*. We have therefore revisited the expression of cell surface sialic acid residues in the hemopoietic stem cell compartment by sorting murine bone marrow cells on the basis of affinity for WGA. Frequency analysis of long-term bone marrow culture initiating stem cells was done using the cobblestone area forming cell (CAFC) assay with limiting dilution set-up. *In vivo* stem cell quality was determined by spleen colony formation, marrow-repopulating ability (MRA) and long-term repopulating ability (LTRA) using sex-mismatched hemopoietic chimerism. The data indicate that MRA and LTRA *in vivo* and *in vitro* are among the most WGA^{dim} cells. In contrast, the enrichment factors for day-12 spleen colony-forming units (CFU-S-12) and transient CAFC increase with increasing WGA affinity. These characteristics allowed us to concentrate LTRA cells 590- to 850-fold over their activity in normal bone marrow without significant enrichment of CFU-S-12. The data reveal that WGA affinity is an inverse function of the primitiveness of murine hemopoietic stem cells and that long-term production of blood cells *in vivo* and *in vitro* is provided for by primitive cells that are physically separable from the vast majority of CFU-S-12. In addition the data reveal that the day-28/35 CAFC frequency of a graft strongly correlates with the number of cells required to induce 40 percent donor-type chimerism at 15 months post-transplantation and thus predicts the *in vivo* LTRA of a graft.

INTRODUCTION

The population of hemopoietic stem cells represents a hierarchy of immature cells with extensive heterogeneity on the basis of decreasing pluripotentiality and proliferative potential, increasing turnover rate [1-3], variation in length of interval required for their clonal expansion [1,4-9], decreasing requirement for multiple growth factor stimulation in order to induce cycling and stimulate clonal amplification [6,10-14], and radiosensitivity [15,16]. For many years clonal techniques (spleen colony-forming units and colony-forming units in culture, CFU-S and CFU-C) and functional endpoints (30-day radioprotective ability; repopulation of marrow cavities, thymus and spleen with new precursor cells) have been used as a measure of primitive hemopoietic stem cell (PHSC) activity, paving the diverging roads leading to the further characterization of such cells [17]. The use of these endpoints has led to the view that PHSC have spleen colony-forming ability and that CFU-S therefore represent a heterogeneous population of stem cells [18-22]. Other studies have cast serious doubt on this view by presenting evidence in favor of the existence of a separate pre-CFU-S compartment [3,23], that may or may not overlap with part of the CFU-S-12 population with respect to its engraftment potential. Indeed, the existing colony assays are not predictive of the marrow-repopulating (MRA) or long-term repopulating ability (LTRA) of a graft both *in vivo* and *in vitro* [24-31]. In addition, the frequency of PHSC with long-term repopulating ability has been estimated at only 2 per 10^5 to 1 per 10^4 [25,32-36], and hence their number is considerably lower than the estimated 1 per 100 to 500 for more differentiated precursors, such as the CFU-S and a variety of *in vitro* clonable progenitor cells. Such PHSC have the ability to give stable multilineage engraftment as demonstrated in recipients of cells with either chromosome markers [37,38], unique retroviral vector integration sites [39-44], or polymorphism for hemoglobin, isozyme or immunoglobulin variants [25,33,45-48]. Detailed tracing of retrovirally tagged stem cells has indicated that the lifespan of some stem cell clones may even approximate that of the mouse [49-51].

In the present work we have reinvestigated the distribution of functionally different HSC subsets on the basis of affinity for wheat germ agglutinin (WGA) using a wide spectrum of stem cell assays *in vivo* and *in vitro*, tuned to assess the potential of a wide variety of stem cell subsets, ranging from day-7 CFU-S (CFU-S-7) to the primitive stem cells that ensure long-term hemopoietic engraftment. This included the use of our recently developed cobblestone area forming cell (CAFC) assay, which is essentially a miniaturized long-term bone marrow culture (LTBMC) allowing the frequency analysis of CFU-S-12 and cells with MRA via limiting dilution analysis *in vitro* as a function of the time of onset and longevity of clonal expansion under identical culture conditions [8,9]. The lectin WGA has affinity for cell surface sialic acid residues that allow, but do not determine, proper homing of infused CFU-S [52-54]. WGA has been successfully used since 1981 to discriminate CFU-S from part of the lymphocytes in

spleen and bone marrow (BM) preparations, thereby assisting in a positive selection strategy for their isolation [55-58]. Recently, we have found evidence that 200-fold concentrated CFU-S-12 had unexpectedly low MRA [27]. We show here that the various stem cells subsets, ranging from the stem cells with LTRA to CFU-S-7, display large differences in WGA-binding and that WGA is an extremely suitable and simple tool in the positive selection and separation of PHSC from CFU-S-12.

MATERIALS AND METHODS

Mice. Male and female (C57BLxCBA)F1 (BCBA) mice, 12 to 30 weeks old, and female C57BL/Ka-Thy-1.1 and male C57BL/6-Ly5.1-pep^{3b}, 12 to 35 weeks old and originally obtained from Dr. I.L. Weissman (Stanford University, Stanford, CA), were bred in the Central Animal Department of the Erasmus University Rotterdam, The Netherlands, and maintained under clean conventional conditions. The drinking water was acidified to pH 2.8. In specific experiments BCBA mice were injected with 150 mg 5-fluorouracil (5FU; Sigma, St Louis, MO) in phosphate-buffered saline (PBS) per kg of body weight in a lateral tail vein.

Irradiation procedure. Two opposing ^{137}Cesium sources (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) were used to irradiate the recipient mice at a dose rate of 1.05 to 1.15 Gy/min. For CFU-S and MRA assays mice received a single dose of 9.3 Gy (BCBA mice) or 9.0 Gy (C57BL mice) whole body irradiation 1 to 4 hours before infusion of cells. In LTRA experiments, BCBA mice received a single sublethal dose of 8.3 Gy one day before grafting. For the 30-day survival studies C57BL mice received a lethal dose of 9.0 Gy 1 to 4 hours before grafting.

Preparation of cells and density centrifugation. Bone marrow cells (BMC) were prepared by cleaning femora and tibiae from muscles and tendons and grinding them in a mortar using PBS. The cell suspensions were sieved over a nylon filter (mesh size 100 μm). For buoyant density centrifugation a modification of a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient designed to isolate thymocyte and BM subpopulations was used [59]. Cells with a density of 1.069-1.075 g/mL (NBM) or 1.069-1.078 g/mL ($FU_{6d}BM$) were collected, washed twice in PBS and 5% fetal bovine serum, and properly diluted.

Magnetic activated cell sorting (MACS). To deplete monocytes and granulocytes from the BMC suspension, density fractionated cells were washed and suspended in cold PBS containing 0.01% sodium azide (PBS-SA) at a concentration of 5-6 x 10^7 cells/mL containing biotinylated monoclonal antibody ER-MP20 [60]. About 65% of the low-density BMC consisted of granulocytes, monocytes and their morphologically recognizable precursors and were therefore positively stained by ER-MP20. After 30 minutes on ice, cells were washed and incubated for 30 minutes with streptavidin-conjugated R-Phycoerythrin (Caltag Laboratories, South San Francisco, CA). The cells were subsequently washed in PBS-SA containing 5 mmol/L EDTA (Titriplex III; Merck, Amsterdam, The Netherlands) and incubated with a 1:100 dilution of biotinylated paramagnetic microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany), at a concentration of 1 to 2 x 10^8 cells/mL (10 min on ice). Cells were washed in PBS-SA containing 5 mmol/L EDTA and 1% (wt/vol) bovine serum albumin (BSA; Fraction V; Sigma) and resuspended in 0.5 mL for separation on the MACS column A2 (Miltenyi Biotec), which was sterilized using ethanol 70%. The flow was regulated by a 27 Gauge x 7/8 inch (0.42 x 22 mm) needle at a rate of 0.08 to 0.10 mL/min and the non-magnetic ER-MP20$^-$ population was collected for further separation.

Fluorescence activated cell sorting (FACS). Previous to FACS sorting, the cells were incubated with 0.25 μg/mL fluoresceinated wheat germ agglutinin (WGA-FITC; Vector,

Burlingame, CA). In some experiments the cells were first incubated with 0.1 μg rhodamine-123 per mL PBS (Rh123; Eastman Kodak, Rochester, NY) for 30 minutes at 37 °C, and than centrifuged and incubated for 15 minutes at 37 °C in PBS containing 5% fetal calf serum to wash out the excess of Rh123. Following sorting on the basis of Rh123 retention, the cells were incubated with WGA-FITC and sorted again. Sorting and part of the analysis of the marrow cells was performed by a FACS II (B-D Systems, Becton Dickinson & Co, Sunnyvale, CA) using a single argon laser at 488 nm tuned at 350 mW output. FITC intensity was measured using a combined 510/515 long-pass and 540 short-pass filter set, and PE using a 580/590 long-pass filter. In order to more clearly distinguish WGA^{dim} cells from WGA^{bright} cells the WGA sorting thresholds were set on a linear instead of a logarithmic scale. After sorting, the WGA-FITC-labeled cell suspensions were added to equal volumes of 0.4 mol/L of the competitive sugar *N*-acetyl-*D*-glucosamine (Sigma). Cytospins were stained with May-Grünwald/Giemsa to allow the morphological identification of sorted or unfractionated cells by light microscopy.

Colony assays. The CFU-S-7 and CFU-S-12 content of cell suspensions was determined by injecting the appropriate dilutions into a lateral tail vein of lethally irradiated mice [61]. Control irradiated mice that did not receive cells were included for all observation days in each experiment. No endogenous spleen colonies were found in the these mice. Seven and 12 days later their spleen were excised, fixed in Telleyesniczky's solution, and the macroscopic surface colonies counted. Quantitation of CFU-C (including macrophage, granulocyte and granulocyte-macrophage colony-forming units, CFU-M, CFU-G and CFU-GM) was performed using a semisolid (0.8% methylcellulose; Methocel MC, Fluka, Buchs, Switzerland) culture medium (α-modification of Dulbecco's modified Eagle Medium, DMEM) in a fully humidified incubator at 37 °C and 5% CO_2. The cultures contained 10% pokeweed mitogen-stimulated mouse spleen-conditioned medium, 20% horse serum and 1% (wt/vol) BSA. Colonies were counted on day 7 of culture with an inverted microscope.

Marrow Repopulating Ability (MRA). The MRA describes the ability of a cell suspension to generate new CFU-C or CFU-S-12 in the BM of a lethally irradiated recipient mouse over a period of 13 days [27]. This period is determined by the survival of fatally irradiated mice (97% at this time point over the last 3 years) that have not been grafted with BMC. In order to measure MRA, five to six lethally irradiated mice per group were infused with sorted or unfractionated BMC, and after 13 days different aliquots of their femoral marrow content were assayed for the presence of CFU-C. MRA was expressed as the number of CFU-C per femur equivalent per 10^5 cells infused. Control irradiated mice were included in each experiment and their endogenous CFU-C (1 to 200 per femur) on day 13 was used to correct experimental data.

Long-term repopulation *in vivo*. LTRA was estimated using a sex-mismatched syngeneic chimeric model in which the grafted stem cells had to compete with surviving host stem cells. Four to five dilutions, three-fold apart, of every cell suspension from male donor mice were infused into six to eight sublethally (8.3 Gy) irradiated syngeneic female BCBA recipient mice. The percentage of donor-type contribution to the peripheral blood leukocytes at 4 months post-transplantation into the hosts was measured with a fluorescent *in situ* hybridization technique using the Y-chromosome-specific probe P17-M34/2 [18,62]. The sublethal irradiation dose was

chosen as to avoid radiation mortality due to insufficient numbers of hemopoietic rescuing units in the suspensions infused. Per blood smear or cytospin preparation 100 nucleated cells were counted per mouse on each time point.

Radioprotection assay. Female C57BL/Ka-Thy-1.1 mice were lethally irradiated with 9.0 Gy and groups of 10 mice were infused with limiting numbers of sorted or control marrow cells from male C57BL/6-Ly-5.1 mice. Survival was monitored over a 30-day period. At day 32 a few drops of peripheral blood was taken from all surviving mice and the extent of mixed chimerism in the white blood cells determined using *in situ* hybridization with a Y chromosome-specific probe.

Cobblestone area forming cell (CAFC) assay. LTBMC were established as previously described [8,9]. Briefly, flat-bottomed 96-well plates were inoculated with 5 to 10 x 10^5 non-sieved BMC in 0.2 mL of LTBMC-medium per well and an adherent stromal layer was grown in 10 to 12 days at 33 °C, 10% CO_2 and 100% humidity. The layers were then irradiated (20 Gy ^{137}Cs gamma) in order to eliminate hematopoietic activity without affecting the ability of the stroma to support hemopoiesis [63]. One day later the medium was changed and various dilutions of freshly sorted or unseparated BMC were overlaid between 1 day and 6 weeks following irradiation to allow limiting dilution analysis of the precursor cells forming hemopoietic clones under the stromal layers. To assay a particular cell suspension we used 8 to 12 dilution steps differing with a factor of 2 to 3, with 15 to 20 wells per dilution. Cultures were fed weekly by changing half of the medium. Between 3 and 28 to 41 days after overlay, all wells were inspected with intervals of one to six days and scored positive if at least one phase-dark hemopoietic clone (cobblestone area, containing 5 to over 10^5 cells) was observed [64]. The frequency of CAFC was then calculated using the maximum likelihood solution [65].

RESULTS

CFU-S and MRA cells in normal BM have different WGA affinity profiles. All nucleated cells in a NBMC suspension were sorted into 4 fractions of about equal numbers differing in WGA-binding (Figure 2.1B), i.e. 2 fractions that were considered to be WGA^{neg}, containing the 0-22 and 23-45 percentile of all nucleated NBMC, and a WGA^{dim} and a WGA^{bright} fraction, containing 27% and 28% of all NBMC, respectively. From Table 2.1 it appears that the cell types recognized showed a rather heterogeneous distribution on the basis of WGA affinity, with immature myeloid cells, unidentifiable blast cells and monocytes more concentrated in the WGA^{dim} window. Virtually all CFU-S-12 and about 92% of all CFU-S-7 were retrieved in the one quarter of NBMC with the highest affinity for WGA (73-100 percentile, Table 2.2). CAFC-34 were remarkably concentrated in the WGA^{dim} fraction. More than half the MRA activity was measured in the WGA^{dim} group and one third among the WGA^{bright} cells, while about 9.8% of MRA was detected in the two WGA^{neg} fractions that represented about one-half of all NBMC.

Table 2.2. Relative distribution of stem cell subsets in NBMC fractions separated on the basis of affinity for WGA.

stem cell subset (0-22)[a]	WGA^{neg} (23-45)	WGA^{neg} (46-72)	WGA^{dim} (73-100)	WGA^{bright}
maturing				
CFU-S-7	1.0	2.0	7.0	90.0
CAFC-7	2.6	1.2	4.0	92.2
intermediate				
CFU-S-12	< 1	< 1	< 1	100.0
CAFC-10	1.2	1.1	3.2	94.5
primitive				
MRA[CFU-C]	7.6	2.2	57.1	33.1
CAFC-28	< 1	< 1	56.8	43.2
CAFC-34	< 1	< 1	82.5	17.5

Data represent percentages of a particular stem cell subset contained in a sorted fraction. Values for unfractionated bone marrow per 10^5 nucleated cells were: CFU-S-7, 34.3; CAFC-6, 91.55; CFU-S-12, 26.2; CAFC-10, 173.9; CAFC-28, 2.12; CAFC-34, 0.75. MRA[CFU-C], 3159 CFU-G/M per femur per 10^5 BMC infused.

[a] Numbers in parentheses denote percentiles of all sorted cells according to the settings as displayed in Figure 2.1B.

Table 2.1. Differential count of normal bone marrow cells sorted on differences in WGA affinity

cell fraction	granuloid early	granuloid late	erythroid	lymphocytes	monocytes	blasts
NBMC	8.8	35.5	13.8	24.0	8.6	7.6
WGA^{neg} (0-22)[a]	0.5	3.0	34.5	55.5	1.5	4.0
WGA^{neg} (23-45)	1.8	54.0	19.2	12.3	6.0	6.7
WGA^{dim} (46-72)	23.7	35.8	4.7	10.3	12.5	12.0
WGA^{bright} (73-100)	5.8	31.7	23.3	25.7	8.3	4.5

Data are expressed as percentage of all nucleated cells differentiated. Two hundred cells were counted in each fraction. The category 'other cells' is not listed. Granuloid cells were differentiated as early (myeloblast to metamyelocyte) and late (band and segmented). Blasts represented unidentifiable (small to medium sized) cells with disperse chromatin and basophilic cytoplasm.
Abbreviations: NBMC, normal bone marrow cells; WGA, wheat germ agglutinin.
[a] Numbers in parentheses denote percentiles of all sorted cells according to the settings as displayed in Figure 2.1B.

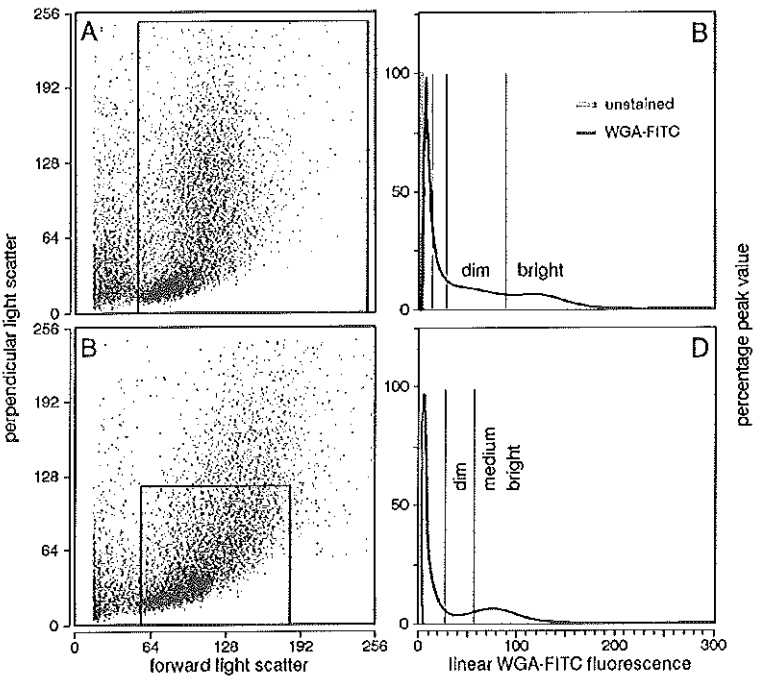

Figure 2.1 Separation of NBMC (in A and B) or LD/FU_{6d}BM cells (in C and D) on the basis of WGA-FITC affinity. **(A)** Light-scatter dot plot of NBMC with sorting window including all nucleated cells; **(B)** gated NBMC from (A), unstained (dotted line) and WGA-FITC stained (solid line). The vertical lines represent the gate settings for collection of the WGA^{neg} (0-22 and 23-45 percentile), WGA^{dim} (46-72) and WGA^{bright} (73-100) subpopulations of cells; **(C)** Light-scatter dot plot of LD/FU_{6d}BM BMC with sorting window; **(D)** WGA-FITC profile of the gated cells in (C). The vertical lines represent the gate settings for collection of the WGA^{dim} (0-33 percentile of all WGA-binding cells), WGA^{med} (34-67) and WGA^{bright} (68-100) subpopulations.

Transient and persistent CAFC in normal BM have different WGA affinity. We have shown previously that early CAFC (CAFC-10) or late CAFC (CAFC-28) correlate well with CFU-S-12 or MRA, respectively [8,9]. Figure 2.2A shows that the WGA^{neg} fractions contained few early hemopoietic clone-forming (CAFC-10) and negligible *in vitro* long-term hemopoietic activity (CAFC-28 to CAFC-34), as compared to the respective CAFC frequencies in NBM. The WGA^{dim} fraction was depleted for early CAFC, however, enriched for late appearing and persistent CAFC (CAFC-34). The most WGA^{bright} fraction (the 73-100 percentile) contained relatively more early CAFC than NBMC. Of all CAFC-5 through CAFC-15, present in NBMC, about 95% could be retrieved in the one quarter of the cells with highest affinity for WGA (Figure 2.2B). In contrast, about 80% of all persistent clone-forming CAFC-34 was detected

in the quarter of the cells that was WGA^{dim}. The observed differences in distribution of the various stem cell subsets on the basis of lectin affinity show that the number of WGA receptors on a stem cell is a function of its primitiveness as judged by the ability to initiate and sustain hemopoietic activity within a stromal layer.

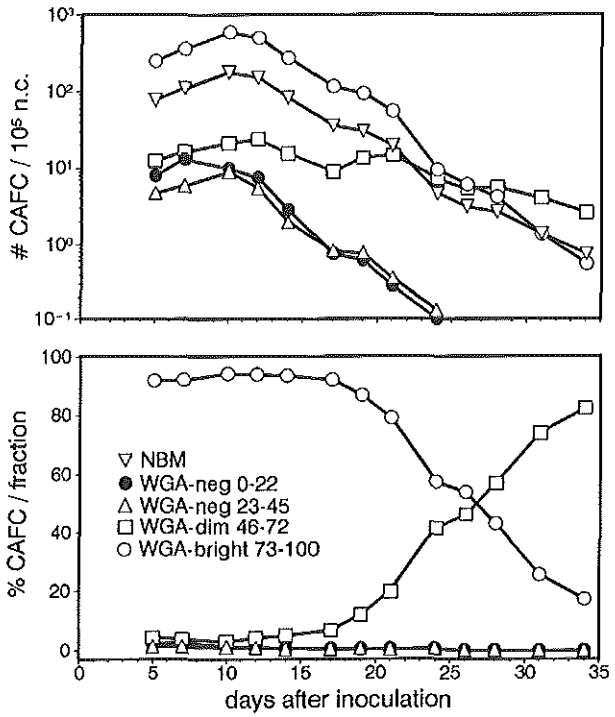

Figure 2.2 Distribution of CAFC subsets in NBMC on the basis of WGA affinity (see Figures 1A and 1B). **(A)** absolute CAFC frequencies. **(B)** relative contribution (%) of indicated fraction to the total CAFC number retrieved. (▽) NBMC; (●) WGA^{neg} (0-22 percentile); (△) WGA^{neg} (23-45); (□) WGA^{dim} (46-72); (○) WGA^{bright} (73-100).

Separation of LTRA from radioprotective ability in normal BM. We have exploited the different WGA profiles of CFU-S-12 and CAFC-10 on one hand, and MRA and *in vitro* LTRA cells (late developing CAFC) on the other, in order to further separate these stem cell subsets. To this end we first depleted the bulk of monocytes and granulocytes from the 1.069-1.075 g/mL low-density fraction of NBM by MACS selection for cells that did not bind the monoclonal antibody ER-MP20. Subsequently these cells were sorted within a forward light-scatter window including one third of the lymphocytes into a WGA^{bright} (67-100 percentile of all WGA-binding cells) and a WGA^{dim} (0-33) fraction. While WGA^{bright} cells were 220-fold enriched for CFU-S-12,

100-fold for CAFC-10 and only 3.8 times for CAFC-35, WGA^{dim} cells contrasted with a 1.6-fold higher CFU-S-12 frequency, 1.1-fold higher CAFC-10 frequency, but with CAFC-35 150 times more concentrated than in NBM.

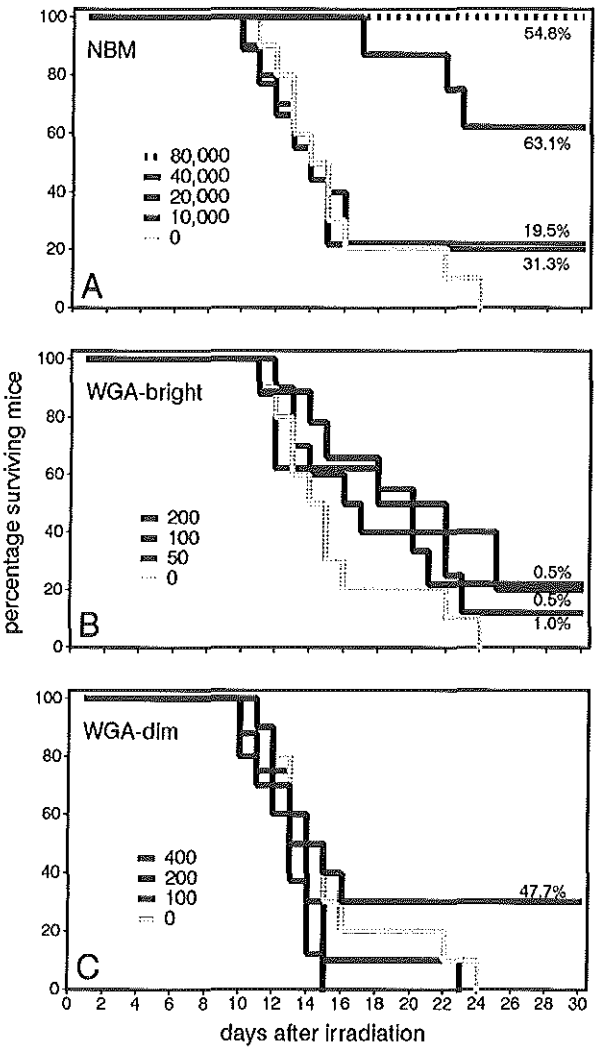

Figure 2.3 Thirty-day survival curves for lethally irradiated female C57BL mice infused with limiting numbers of male **(A)** NBMC, **(B)** WGA^{bright} cells, or **(C)** WGA^{dim} cells. Data in the figures denote the percentage donor-type nucleated blood cells detected in the surviving mice on day 32 after irradiation.

In order to study their LTRA and radioprotective ability *in vivo*, limiting numbers of these sorted or NBMC from male C57BL/6-Ly-5.1 mice were injected into lethally irradiated congenic female C57BL/Ka-Thy-1.1 recipients and the 30-day survival was

scored. The survival curves in Figure 2.3A show that decreasing NBMC numbers decreasingly protected the recipients from radiation-inflicted death, while a significant 19.5% to 63.1% donor-type engraftment in the blood leukocytes at day 32 was established. The three cell doses used for the WGA^{bright} fraction, which was 220-fold enriched for CFU-S-12, gave a prolongation of the recipient's mean survival time, however, survival was poor and donor-type contribution to the hemopoietic recovery was hardly detectable (Figure 2.3B, range 0.5% to 1.0%). In contrast, 400 WGA^{dim} cells could only rescue 3 out of 10 recipients, but an average of 47.7% donor-type repopulation was observed in the surviving mice at day 32 (Figure 2.3C), a percentage similar to the extent of donor-type repopulation in recipients of 4 to 8 x 10^4 NBMC.

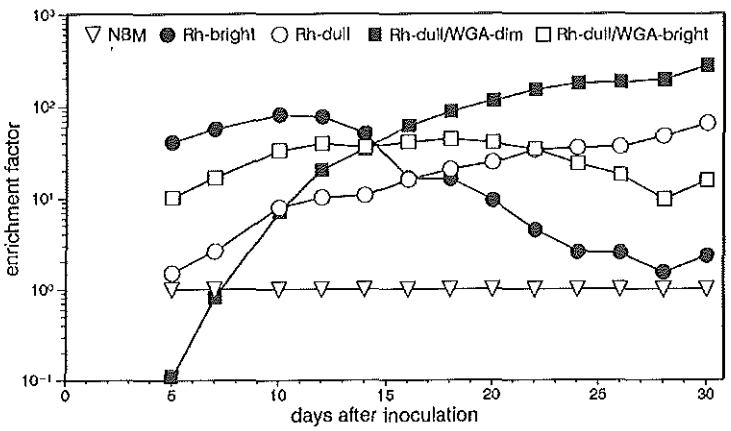

Figure 2.4 Enrichment of CAFC subsets differing in WGA affinity and Rh123 retention in a monocyte and granulocyte-depleted low-density fraction of NBM. Enrichment was related to the NBMC-activity. Symbols are explained in the figure.

We also studied whether $Rh123^{dull}$ stem cells, previously reported to include stem cells with MRA and almost without any spleen colony forming ability [3], were heterogeneous for WGA affinity. To test this, the 10% most $Rh123^{dull}$ cells in the low-density ER-$MP20^-$ fraction of NBM were sorted on the basis of WGA affinity into a WGA^{bright} (51-100 percentile of all WGA-binding cells) and a WGA^{dim} (0-50%) fraction. $Rh123^{dull}$ cells comprised high frequencies of late developing CAFC, but CFU-S-12 and CAFC-10 concentrated in the $Rh123^{bright}$ cell fraction (Figure 2.4). Following sorting of $Rh123^{dull}$ cells in 2 equal fractions on their WGA affinity, 95% of the primitive late developing and persistent CAFC (CAFC-30) was retrieved in the WGA^{dim} fraction and 95% of the early developing and transient CAFC-8 through CAFC-15 concentrated among the WGA^{bright} cells. These data therefore demonstrate that $Rh123^{dull}$ stem cells are heterogeneous in WGA affinity.

Separation of transient and persistent CAFC subsets in $FU_{6d}BM$. To allow further concentration of *in vitro* LTRA cells and separate them from CFU-S-12 and

CAFC subsets, which induce transient stroma-dependent hemopoiesis a powerful pre-enrichment protocol was developed, using WGA as a tool. Following intravenous injection of 150 mg 5FU per kg body weight into BCBA mice a nadir in cellularity (between 5% and 12% of normal) was observed around day 6. Day-6 post-5FU BM ($FU_{6d}BM$) showed an average enrichment of more than 10-fold for late persistent CAFC (Figure 2.5) in 20 experiments, but less for early, transient CAFC. Density separation of $FU_{6d}BM$ led to a further concentration similar for all CAFC subsets, resulting in an average 11-fold higher frequency for CAFC-5 and an average 85-fold higher CAFC-28 frequency than was observed in NBMC. These procedures also depleted most of the granulocytes and erythroblasts in the cell suspension, while enriching for unidentifiable blasts and lymphocytes (Table 2.3).

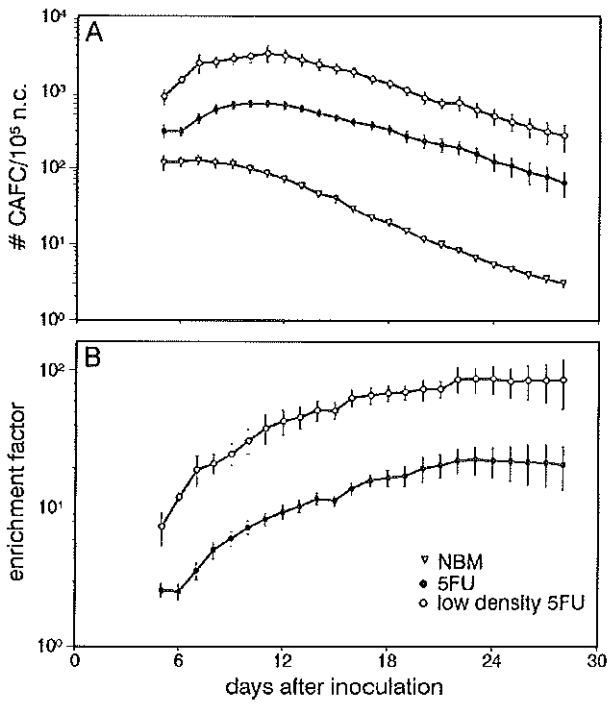

Figure 2.5 Enrichment for late developing and persistent CAFC in $LD/FU_{6d}BM$ cells as compared to NBMC. **(A)** absolute frequencies; **(B)** enrichment over NBMC. Data represent the arithmetic mean of 20 experiments. Bars denote 1 SD. (▽) NBMC; (●) $FU_{6d}BM$; (○) $LD/FU_{6d}BM$.

Subsequently, low-density $FU_{6d}BM$ ($LD/FU_{6d}BM$) was sorted on the basis of differences in WGA affinity in a light-scatter window that included all of the lymphocytic cells, but excluded the larger blast cells on the basis of forward light-scatter, and part of the remaining granulocytes on the basis of perpendicular light-scatter (Figure 2.1C). The WGA-binding cells (12-18% of all $LD/FU_{6d}BM$ cells) were

Table 2.3. Differential count of pre-enriched marrow cells and sorted cell populations differing in WGA affinity.

cell fraction	granuloid early	granuloid late	erythroid	lymphocytes	monocytes	blasts
NBMC	19.0	41.0	11.0	11.5	11.0	5.5
$FU_{6d}BM$	7.5	2.5	8.0	50.5	6.0	23.0
$LD/FU_{6d}BM$	13.4	1.5	0.0	50.8	3.0	31.3
WGA^{dim}	4.0	3.0	2.0	19.5	12.5	59.0
WGA^{bright}	0.5	0.0	2.5	2.5	3.5	91.0

Data are expressed as percentage of all nucleated cells differentiated. Two hundred cells were counted in each fraction. The category 'other cells' is not listed. Granuloid cells and blasts were differentiated as in Table 2.1. WGA^{dim} and WGA^{bright} cells represented the 0-33 and 67-100 percentile, respectively, of all WGA-binding cells in the light-scatter window of $LD/FU_{6d}BM$ (see Figure 2.1D).

sorted into a WGA^{dim} (0-33 percentile) and a WGA^{bright} (67-100) fraction (Figure 2.1D). The differential count of these sorted populations (Table 2.3) indicated that WGA^{bright} cells were predominantly unidentifiable blast cells, while in addition, WGA^{dim} cells had considerable proportions of monocytes and lymphocytic cells. It is apparent from Figure

2.6 that relatively early developing CAFC were highly concentrated among the WGA^{bright} cells, with 200 times higher frequencies for CAFC-10 and more than 600 times higher CAFC-18 frequencies. In sharp contrast with the low concentration of persisting CAFC in the WGA^{bright} fraction, the WGA^{dim} cells contained 850-times as many late developing CAFC (CAFC-41) than were observed in NBMC, while CAFC-10 frequencies were similar to those in NBMC. CFU-S-12 in the WGA^{bright} fraction amounted to 5060 per 10^5 nucleated cells, i.e. 220 times more than there frequency in NBMC (23 per 10^5). CFU-S-12 were not detected in the WGA^{dim} fraction following infusion of 6000 n.c. per recipient (CFU-S data not presented).

Kinetics of clonal growth of LTRA and CFU-S seeded on marrow stroma. While CAFC frequencies are routinely computed by limiting dilution analysis, the CAFC clonogenicity can be calculated using microplate mapping in time allowing frequency analysis using the percentage of wells that had remained negative for cobblestone area formation until that day. It can be seen from Figure 2.7 that with NBMC the first CAFC started detectable clone formation between days 3 and 5. Fifty percent of all CAFC had commenced clonal amplification around day 6, whereas all CAFC had started around day 11. WGA^{bright} cells initiated clone formation around day5, with 50% of all CAFC dividing at day 7 and 100% around day 13. In contrast, WGA^{dim} stem cells showed a significantly longer average lag period before clonal amplification started. On day 16 still 50% of these CAFC were quiescent. Only around day 27 were all CAFC proliferating as detected by visual detection of cell clusters.

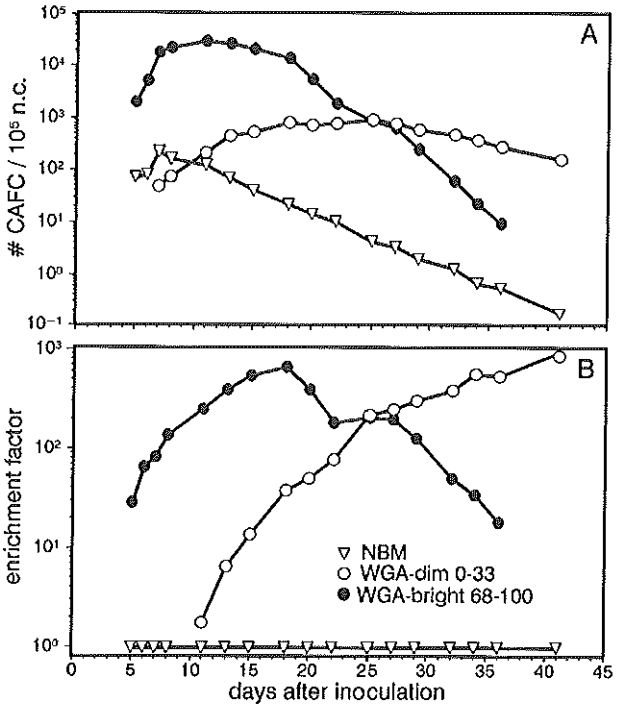

Figure 2.6 Separation of early and late developing CAFC subsets by sorting LD/FU_{6d}BM cells on WGA-FITC binding. Percentiles of all WGA-binding cells are indicated. **(A)** absolute frequencies; **(B)** enrichment over NBMC. (▽) NBMC; (○) WGA^{dim} (0-33 percentile); (●) WGA^{bright} (67-100).

Majority of stem cells with *in vivo* and *in vitro* LTRA has low WGA affinity. To obtain an *in vivo* validation for the CAFC data, LD/FU_{6d}BM cells were sorted within a light-scatter window (as described for previous experiments) on the basis of differences in WGA affinity into 3 fractions, i.e. a WGA^{dim} (0-42 percentile of all WGA-binding cells), a WGA^{med} (43-83) and a WGA^{bright} (84-100) fraction. Subsequently, these sorted cells were assayed for the presence of *in vivo* long-term repopulating stem cells, MRA[CFU-C], and incidence of CFU-S-7 and CFU-S-12. These data were compared with the frequency of the CAFC-5 through CAFC-35. To allow quantitative analysis of the repopulation ability of the different sorted cells *in vivo*, sublethally irradiated female recipient mice were infused with limiting dilutions of unfractionated or sorted male BMC. Lethal irradiation was avoided to allow infusionof cell fractions that do not have radioprotective ability. Four months later, nucleated cells from the recipient's blood leukocytes and BM were studied for the presence of the Y chromosome using *in situ* hybridization, and the percentage of donor-type repopulation was assessed.

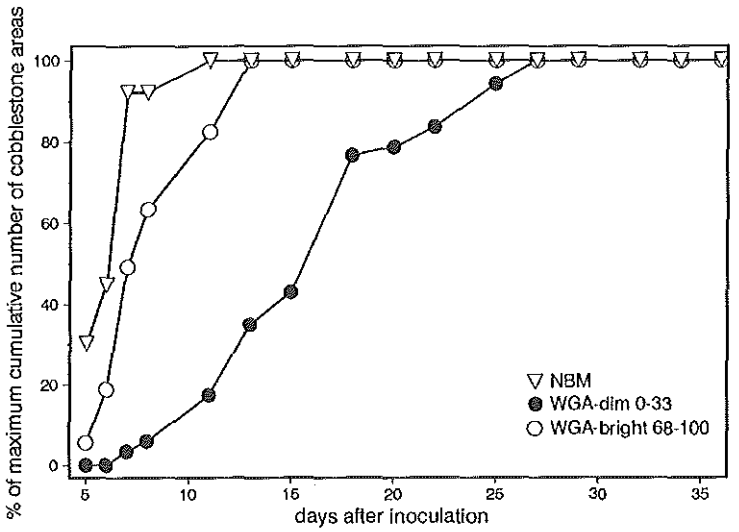

Figure 2.7 Kinetics of clonal growth of LTRA-rich and CFU-S-12-rich BM fractions seeded on marrow stroma *in vitro*. Data represent the percentage of the maximal number of cobblestone areas at various time points as determined by microtiter plate mapping. (▽) NBMC; (●) WGA^{dim} (0-33 percentile); (○) WGA^{bright} (67-100).

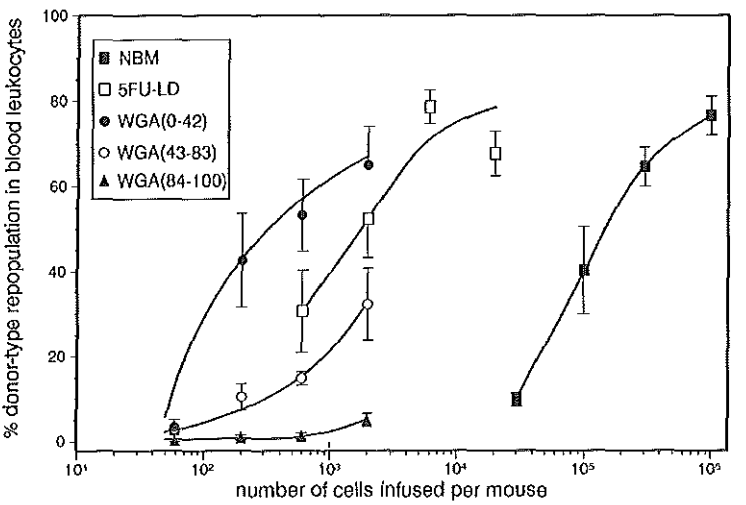

Figure 2.8 Induction of sustained hemopoietic chimerism is inversely correlated with the WGA affinity of stem cell subsets from $LD/FU_{6d}BM$ bone marrow. Data represent the mean percentage (bars denote 1 SEM) of nucleated donor (male) cells in the peripheral blood of six to eight sublethally irradiated syngeneic female recipients per cell dose at 4 months post-transplantation. Percentiles of all WGA-binding cells are indicated. (■) NBMC; (□) $LD/FU_{6d}BM$; (●) WGA^{dim} (0-42 percentile); (○) WGA^{med} (43-83); (▲) WGA^{bright} (84-100).

Figure 2.8 depicts data from such a repopulation study. The lines were fitted to give an approximation of the increasing donor-type repopulation with increasing cell dose infused. The sublethally irradiated recipient mice did not show complete donor-type hemopoiesis because low numbers of infused cells had to compete with surviving recipient stem cells. Comparison at the 40% to 50% repopulation level indicated a 100-fold concentration of LTRA cells in LD/FU_{6d}BM as compared to NBM. The LTRA enrichment of WGA^{bright} cells could not precisely be determined because donor-type chimerism was far below 40%, even when 2000 cells per mouse were infused. From Figure 2.8 it is clear that WGA^{bright} cells from LD/FU_{6d}BM cells actually were severely depleted of their LTRA. WGA^{med} cells contained about 40-fold concentrated LTRA-activity when compared with NBMC at the 40% engraftment level, equalling 6% of all LTRA-activity in the WGA-binding LD/FU_{6d}BM cells. Ninety-three percent of all LTRA in the LD/FU_{6d}BM fraction was concentrated among the WGA^{dim} cells, which had 590-fold higher LTRA than NBMC. Hemopoietic chimerism in BM of individual recipients did not deviate more than 20% from the donor-type percentages that were measured in the peripheral blood. Remarkably, the slopes of the lines that could be fitted to the curves were suggested to become decreasingly smaller with higher WGA affinity, which would indicate qualitative heterogeneity in the LTRA compartment. As can be seen from Table 2.4, both WGA^{med} and WGA^{dim} cells had high frequencies of LTRA units giving at least 5% donor-type engraftment. However, far more WGA^{med} cells than WGA^{dim} cells were needed for the establishment of 50% chimerism.

Table 2.4. Number of cells required for different levels of donor-type engraftment of blood leukocytes.

cell fraction	donor-type engraftment (%)		
	5	25	50
NBMC	23,100	60,000	140,000
LD/FU_{6d}BM	133	460	1,800
WGA^{dim} (0-42)[a]	48	96	315
WGA^{med} (43-83)	165	1,200	12,200
WGA^{bright} (84-100)	2,100	nc	nc

Data were calculated from Figure 2.8 and represent the frequency of LTRA units that initiated a defined percentage of donor-type engraftment in blood leukocytes of sublethally irradiated mice at 4 months post-transplantation.

Abbreviation: nc, not calculated, the low percentage donor-type engraftment did not allow calculation. NBMC, normal bone marrow cells; LD/FU_{6d}BM, low-density day-6 post-5-fluorouracil bone marrow.

[a] Numbers in parentheses denote percentiles of all WGA-binding cells in the light-scatter window of LD/FU_{6d}BM.

Table 2.5 comprises the enrichment factors for a series of stem cell subsets in these sorted fractions. They were tentatively classified into 3 categories according to their contribution to LTRA as a measure of primitiveness, i.e. maturing, intermediate and primitive stem cells. WGA^{dim} cells were depleted for early CAFC and CFU-S-7, but were increasingly more enriched for MRA, primitive CAFC-35 (*in vitro* LTRA), and *in vivo* LTRA cells (see also Figure 2.7). Conversely, WGA^{bright} cells were highly enriched for CFU-S-12 and CAFC-10, less for MRA, while no significant concentration of *in vivo* LTRA cells and CAFC-35 was observed. WGA^{med} cells showed relatively high concentrations of CFU-S-12, CAFC-10 and MRA, yet both transient CFU-S-7, CAFC-7, and the primitive *in vivo* and *in vitro* LTRA cells had lower frequencies. The observations indicate that WGA affinity is an inverse function of the *in vivo* and *in vitro* repopulating ability of stem cell subsets.

Table 2.5. Relative distribution of transiently and long-term repopulating stem cell subsets in $LD/FU_{6d}BM$ compared to the total activity per WGA-binding fraction.

stem cell type	enrichment factor[a]			percentage[b]		
	dim	medium	bright	dim	medium	bright
maturing						
CFU-S-7	0.1	28	44	0.2	49.1	50.8
CAFC-7	1.9	45	109	1.6	37.9	60.5
intermediate						
CFU-S-12	15	117	210	5.7	43.2	51.1
CAFC-10	19	133	200	6.9	46.8	46.4
primitive						
MRA[CFU-C]	199	124	22	59.6	36.2	4.2
CAFC-35	467	25	5	94.4	4.9	0.7
LTRA *in vivo*	590	40	nd	93.1	6.2	0.8

Dim, 0-42 percentile of all WGA-binding cells sorted from $LD/FU_{6d}BM$; medium 43-83; bright, 84-100. Abbreviation: nd, not done; the percentage donor-type repopulation in this group was below 40 percent, therefore, these data could not be determined.

[a] Enrichment over NBMC. Values for NBMC per 10^5 nucleated cells: CFU-S-7, 22.14; CAFC-6, 139; CFU-S-12, 27.9; CAFC-10, 150; CAFC-35, 0.6; MRA[CFU-C], 4050 per femur per 10^5 BMC infused; LTRA *in vivo* was compared at the 40 percent donor-type hemopoiesis level at 4 months post-transplantation (Figure 2.8).

[b] Values are the percentages of stem cell number or activity that the indicated fraction contributed to the 100% activity of all WGA-binding cells in $LD/FU_{6d}BM$.

Correlation of CAFC frequencies and *in vivo* repopulation potential. Because the enrichments for persistent CAFC gave a very good prediction of the *in vivo* repopulation potential of sorted cells we have collected data on *in vivo* chimerism studies in our laboratory that included CAFC measurements. With a similar approach

as described in Figure 2.8 we have determined the number of cells required for 40% donor-type chimerism (being half maximal in these sublethally irradiated recipients) between 12 and 15 months post-grafting using various sorted marrow populations. Figure 2.9 presents an extremely high correlation between the grafts CAFC-28 (r=0.967) or CAFC-35 (r=0.983) frequency and the number of cells required to induce and maintain 40% donor chimerism at a year or more after graft infusion. It can be calculated that 40% donor blood leukocytes is guaranteed by 2.12 ± 0.48 CAFC-28, or 0.89 ± 0.18 CAFC-35. Our observations clearly indicate that the CAFC-28/35 frequencies predict the potential of a graft to give stable chimerism *in vivo*.

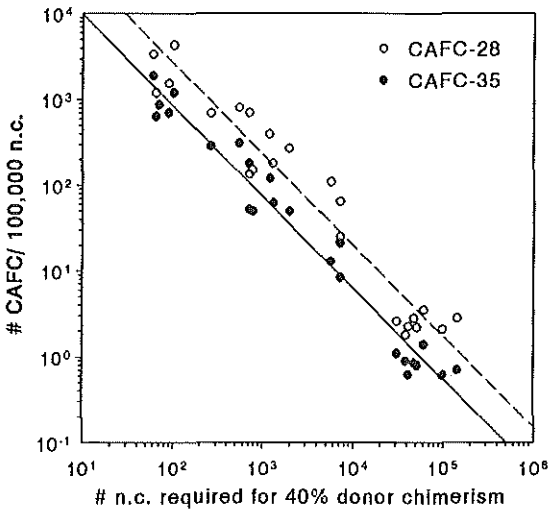

Figure 2.9 Correlation between *in vitro* (CAFC-day 28/35) and *in vivo* (stable blood leukocyte chimerism at 12 to 15 months post-grafting) assays for long-term repopulating stem cells. (o) CAFC-28; (●) CAFC-35.

DISCUSSION.

This study describes the heterogeneity of BM hemopoietic stem cell subsets with respect to their affinity for WGA. All available criteria for functionally discernible stem cell subsets allow the conclusion that the affinity for WGA is an inverse function of the primitiveness of a stem cell subset. Our data indicate that WGA is at least a powerful probe as is Rh123 in the resolution and hierarchical ordering of hemopoietic stem cell populations [3]. By exploiting this characteristic, stem cells with both *in vitro* and *in vivo* LTRA could be concentrated 590- to 850-fold over their frequencies in normal BM, with minimal or no concentration of CFU-S-12 in the same cell suspensions. To our knowledge, this extreme high ratio of LTRA cells over CFU-S-12 and CAFC-10 is the highest reported so far and forms an argument in support for the existence of a pre-CFU-S stem cell subset [23,28,31,66]. In the light of the current uncertainties about the identity of LTRA cells [67], it is relevant to note that the presence of CFU-S-12 activity in our WGA^{dim} fraction with LTRA may have been caused by an insufficient separation technology and establishes, in contrast to other studies [21,36], that the extremely rare stem cell subset expressing LTRA is not associated with high CFU-S-12 activity. We have previously reported on the radioprotective qualities of two BMC fractions sorted on the basis of WGA [68], however, the low WGA-binding fraction was contained in the region that is denoted as WGA^{med} in the present study. Therefore, their characteristics cannot be compared with the WGA^{dim} cells reported here

Our data demonstrate the deficit of CFU-S-7 and the majority of CFU-S-12 to induce stable hemopoietic chimerism of appreciable magnitude and longevity (Figures 2.3 and 2.8). It seems highly unlikely that PHSC with LTRA constitute a substantial part of the CFU-S-12 population and reversely suggest that CFU-S-12 with engrafting potential are part of a more activated [19,20,69], or less primitive stem cell subset in transit between PHSC and CFU-S-12. Apparently, CFU-S-7/8 and CFU-S-12 may only prolong the mean survival time of lethally irradiated recipients by some days, or even display radioprotective abilities [68], but do not contribute to the hemopoietic repopulation of the host to a significant extent (Figure 2.3). This supports our earlier observation that CFU-S rich cell suspensions sorted on the basis of high Rh123 retention, but not $Rh123^{dull}$ cells with MRA, prolonged the survival time of radiation-inflicted mice with 2.8 days [28]. In conclusion, our observations show that the radioprotective and spleen colony-forming performance of a graft is unrelated to its MRA [27] and LTRA, but rather is a measure of its ability to give early but transient post-grafting hemopoietic recovery.

The usefulness and reliability of the CAFC assay as a tool for *in vitro* frequency analysis of a variety of hemopoietic precursors is evident from the good agreement of *in vitro* and *in vivo* data. Not only do the data support the previously described linear

correlation of CAFC-10 and CFU-S-12 on one hand, and those of CAFC-28 and MRA on the other [9], they also indicate that enrichments for CAFC around day-35 highly correlate with the frequencies of *in vivo* LTRA units responsible for initiating 40% donor-type engraftment in sublethally irradiated recipients (Figure 2.8 and Table 2.4). This strongly suggests that the CAFC assay measures the frequency of highly potent LTRA units. Further, our data may point to the existence of a qualitative heterogeneity of LTRA units *in vivo*, which necessitates a more precise definition of LTRA cells. One in 48 WGA^{dim} cells, and 1 in 165 WGA^{med} cells, could be classified as a LTRA unit giving at least 5% repopulation, which compares favorable with the highest LTRA cell frequency reported so far, i.e. the 1 in 39 incidence of Thy-1^{lo} Lin^- Sca-1^+ cells producing at least 1% donor-type hemopoiesis at 9 weeks post-grafting [36]. Yet, a far lower frequency was observed for potent LTRA units responsible for 50% chimerism, i.e. 1 in 315 WGA^{dim} cells (compare CAFC-35, 1 in 357) and only 1 in 12,200 WGA^{med} cells (CAFC-35, 1 in 6667). It follows from this comparison between WGA^{dim} and WGA^{med} cells that sorted cell fractions may greatly differ in their respective contents of low and high capacity LTRA units (i.e. the number of cells initiating either 5% or 50% donor-type engraftment *in vivo*). In NBM we observed an 1 in 23,100 frequency of *in vivo* LTRA units giving 5% donor-type repopulation, but only 1 in 140,000 (CAFC-35, 1 in 167,000) gave an average 50% chimerism, which compares well with previously published LTRA cell frequencies [25,32-36].

Comparison of our data further strengthen the view that both in the LTBMC and *in vivo*, the longevity of clonal activity is a measure of the primitiveness of hemopoietic stem cells. MRA cells, but not the majority of CFU-S-12 [30,70], have earlier been shown to be closely related to cells initiating long-term engraftment of an irradiated stromal layer in flask cultures [30]. The enrichments for primitive stem cells with LTRA or MRA, both in the present study (see Table 2.5) and as previously published [9], are not fully comparable. This suggests that MRA cells represent a transitory subset between LTRA cells and CFU-S-12. Such a conclusion may not be surprising because the endpoints of these assays differ greatly.

The varying and relatively late onset of the clonal amplification in the LTRA-rich WGA^{dim} stem cell population, as compared to the more early clone formation of the CFU-S-12-rich WGA^{bright} population (Figure 2.6), is reminiscent of the behavior of blast-CFC in semi-solid media as described by Ikebuchi *et al.* [6]. It will be of interest to study whether added cytokines, that have been reported to activate quiescent precursor cells with a high proliferative potential [6,11-14,70-72] have similar effects in the LTBMC.

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CHAPTER 3

STABLE MULTILINEAGE HEMATOPOIETIC CHIMERISM IN α-THALASSEMIC MICE INDUCED BY A BONE MARROW SUBPOPULATION THAT EXCLUDES THE MAJORITY OF DAY-12 SPLEEN COLONY-FORMING UNITS

Johannes C.M. van der Loo[1], Cor van den Bos[1], Miranda R.M. Baert[2], Gerard Wagemaker[1] and Rob E. Ploemacher[1]

1. Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
2. Department of Immunology, Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

We have investigated the contribution of highly purified day-12 spleen colony forming units (CFU-S-12) as well as more primitive cells to sustained blood cell production using *in vivo* and *in vitro* assays that allow frequency analysis. Normal or day-6 post-5-fluorouracil light-density bone marrow (BM) was sorted on the basis of differences in rhodamine-123 (Rh123) retention or wheat germ agglutinin (WGA) affinity and tested *in vivo* using a recently developed α-thalassemic chimeric mouse model. In addition, short-term and long-term clonal activity was assessed *in vitro* using a limiting dilution-type long-term BM culture, the cobblestone area forming cell (CAFC) assay. When sublethally irradiated α-thalassemic mice were transplanted with as many as 281 purified WGA^{bright} CFU-S-12, derived from a fraction containing 95% of all CFU-S-12 from day-6 post-5-fluorouracil light-density BM of wild-type mice, detectable chimerism was not observed at 6 months post-transplantation. In contrast, only 3 CFU-S-12 were included in the $Rh123^{dull}$ and WGA^{dim} subpopulations that induced 29 to 58% and 21 to 31% stable multilineage donor-type chimerism of erythrocytes and leukocytes, respectively. The $Rh123^{dull}$ and WGA^{dim} cells were up to 240-fold enriched for long-term repopulating ability (LTRA) as compared with unseparated BM. A comparable level of chimerism was found in the different hematopoietic organs and at the level of BM CFU-S-12. The frequency of the LTRA unit capable of inducing a 10% sustained level of donor-type erythrocytes was calculated to be 1 to 2 per 10^5 BM cells. Several reports have suggested that LTRA and spleen colony formation could be capacities of the same stem cell subset. However, the present results show that the majority of CFU-S-12 have only short-term repopulating ability and are physically separable from more primitive stem cells with long-term multilineage reconstituting capacities.

INTRODUCTION

Data from a variety of physical sorting experiments have initiated a debate whether cells with *in vivo* long-term repopulating ability (LTRA) and day-12 spleen colony-forming units (CFU-S-12) belong to the same stem cell population or represent distinct and separable subsets [1-6]. LTRA and spleen colony-forming ability have been reported to copurify at high enrichment levels in the population bearing the lineage (Lin) negative (CD4, CD8, Gr-1, Mac-1, TER-119)$^-$ Sca-1$^+$ (Ly-6A/E$^+$) Thy-1.1^{lo} phenotype, implicating that they belong to the same subset [1,7,8]. In contrast, BM populations purified by counterflow elutriation have been shown to induce long-term repopulation *in vivo* while being largely depleted of CFU-S activity [9]. Also, flow sorting on the basis of wheat germ agglutinin (WGA) affinity or rhodamine-123 (Rh123) retention has proved to be a powerful tool to separate pre-CFU-S from CFU-S-12 quality [10-12]. Cells with marrow repopulating ability (MRA[CFU-S-13]) or LTRA have been found to have a low affinity for WGA, while CFU-S-12 bind high levels of lectin [10]. Using the supravital dye Rh123, CFU-S-8, CFU-S-12 and cells with MRA[CFU-S-13] could subsequently be ordered on the basis of their decreasing mitochondrial activity [13-15]. It has been demonstrated that almost all CFU-S-12 stain brightly with Rh123, whereas 90% of the pre-CFU-S activity (determined as MRA[CFU-S-13]) is contained in the 25%-most-dull population [15,16]. These reports all show heterogeneity within the stem cell compartment and indicate that CFU-S activity and long-term repopulating ability are separable.

The present study was designed to separate and highly purify CFU-S-12 and primitive stem cells, and study their contribution to short and long-term repopulation using *in vivo* and *in vitro* assays. Light-density BM was sorted using the differences in either Rh123 retention or WGA affinity. Also, BM from mice treated with 5-fluorouracil was used because it was reported to result in high enrichments for stem cells when combined with sorting on the basis of WGA [10]. However, using this BM, Rh123 was not able to discriminate the stem cell populations as well as it did in untreated BM and therefore, was not included. The repopulating ability of purified CFU-S-12 and a population highly enriched for primitive stem cells were analyzed *in vivo* using an α-thalassemic sex-mismatched chimeric mouse model [17-19], and *in vitro* using a limiting dilution type long-term BM culture, the CAFC assay [20-22]. The transplantation model was evaluated by comparison of the enrichment for donor-type erythrocytes determined by FACScan analysis and nucleated blood cells determined by fluorescent *in situ* hybridization (FISH) using a murine Y chromosome-specific probe. In addition, lineage expression of the grafted LTRA cells was studied at 12 months after transplantation. Chimerism at the level of BM CFU-S-12 was further assessed by secondary transplantation.

MATERIALS AND METHODS

Animals. Male and female inbred BALB/cAnCrlRij mice, and female heterozygous α-thalassemic mice ($Hba^{th/+}$), age 12 to 25 weeks, were bred under specific pathogen free conditions at the Former Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands, and received acidified water and food pellets ad libitum. The $Hba^{th/+}$ mice from stock 352HB were originally obtained from Dr. R.A. Popp (Oak Ridge National Laboratory, Oak Ridge, TN) and inbred into BALB/c mice by repeated back crossing [18,23]. In specific experiments, male BALB/c mice were injected intravenously with 150 mg 5-fluorouracil (Sigma, St Louis, MO) in phosphate-buffered saline (PBS) per kg body weight and subsequently killed by carbon dioxide inhalation at day 6 after injection.

Irradiation. One day prior to transplantation, a single dose of 3, 4 or 5 Gy total body irradiation (TBI) was given to α-thalassemic recipient mice at a dose rate of 0.73 Gy/min using two opposing ^{137}Cesium sources. For CFU-S determination, female BALB/c mice received a single dose of 8 Gy at a dose rate of 1.04-1.06 Gy/min, 2 hours before grafting. Stromal layers were irradiated at confluency with a total dose of 20 Gy.

BM cell suspensions. Preparation of the BMC and buoyant density centrifugation using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient, was performed as previously described [10,21]. Cells with a density of 1.069 to 1.075 g/mL or 1.069 to 1.078 g/mL, from untreated or 5-fluorouracil-treated mice, respectively, were collected from the interphases, washed in PBS containing 5% fetal calf serum (FCS) and maintained on ice throughout the staining and purification procedure.

Purification procedure. Light-density BM cells were depleted of monocytes and granulocytes by magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) using monoclonal antibody ER-MP20 (rat IgG_{2a}) [24]. For magnetic sorting, cells were incubated for 30 minutes with biotinylated ER-MP20 followed by a streptavidin-conjugated phycoerythrin (SAV-PE; Caltag, South San Francisco, CA) diluted in PBS containing 0.01% sodium azide (PBS-SA). Antibodies and conjugates were titrated for optimal staining of mouse BM. The cells were then washed in PBS-SA containing 5 mmol/L EDTA (Titriplex III; Merck, Darmstadt, Germany) and incubated for 15 minutes with a 1:100 dilution of biotinylated paramagnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), at a concentration of 1 to 2 x 10^8 cells/mL. After washing the cells in PBS-SA containing 5 mmol/L EDTA and 1% (wt/vol) bovine serum albumin (BSA; Fraction V; Sigma), they were separated on the MACS column A2 (Miltenyi Biotec). The flow-rate was set at 0.08 to 0.10 mL/min using a 27 Gauge x 7/8 inch (0.42 x 22 mm) needle. The non-magnetic ER-MP20$^-$ population was collected in PBS containing 5% FCS and maintained on ice. For separation by FACS, light-density BM cells were stained for 30 minutes with biotinylated ER-MP20 followed by sAV-PE in PBS-SA containing 5% BSA. The ER-MP20$^-$ cells, obtained by either FACS or MACS, were further separated by FACS on the basis of Rh123 retention or affinity for WGA. Light-density BM cells from 5-fluorouracil-treated mice ($LD/FU_{6d}BM$) were not separated using ER-MP20, but were directly labeled with WGA for further sorting. The cells were incubated for 30 minutes in PBS containing 5% FCS and 0.1 μg/mL Rh123 (Eastman Kodak, Rochester, NY) at 37 °C, or with 0.25 μg/mL fluoresceinated WGA (WGA-FITC;

Vector, Burlingame, CA) at room temperature, as previously described [15,25]. After sorting, the WGA-labeled cells were incubated for 30 minutes at 37 °C in 0.2 mol/L of the competitive sugar *N*-acetyl-*D*-glucosamine (Sigma) to remove surface-bound WGA. Cell sorting was performed on a FACS II (B-D Systems, Becton Dickinson, Sunnyvale, CA) at a rate of 2,500 cells per second using a single argon laser tuned at 488 nm (350 mW).

CAFC assay. Long-term BM cultures were established in 96-well plates for limiting dilution analysis of CAFC as previously described [20,22]. After irradiation the stroma was overlaid with 8 to 12 dilutions of the sorted or unseparated BMC, twofold apart, using 15 wells per concentration. The culture medium consisted of α-modified Dulbecco's modified Eagle's medium (Flow, McClean, VA) at 280 mOsmol/kg, containing 10^{-4} mol/L β-mercaptoethanol, 10^{-5} mol/L hydrocortisone 21-hemisuccinate (Sigma), 2 mmol/L L-glutamine, 10^{-7} mol/L sodium selenite, 100 IU/mL penicillin and 100 μg/mL streptomycin, supplemented with 20% horse serum. The wells were inspected every 2 to 3 days, between 3 and 35 days after inoculation, using a phase-contrast inverted microscope. Wells were scored positively if at least one cobblestone area was observed. The frequency of CAFC was calculated using the maximum likelihood solution [26,27].

CFU-S assay. The content of CFU-S-12 in unseparated and sorted BM was determined by intravenous infusion into 8-Gy-irradiated BALB/c mice [28], using eight mice per group. At day 12 the mice were killed and the macroscopic colonies were counted after fixation of the spleens in Telleyesniczky's solution.

Peripheral blood chimerism. Groups of four to eight sublethally irradiated female α-thalassemic mice were intravenously injected with sorted or unseparated BMC from normal male BALB/c. Red blood cell (RBC) chimerism was determined by analyzing the forward light-scatter distribution of the normal and microcytic thalassemic erythrocytes using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as previously described [19,29]. Values below 5% donor-type RBCs did not statistically differ from zero due to variation in the calibration curve required for FACScan analysis of RBC chimerism [29]. Nucleated blood cell chimerism was determined by fluorescent *in situ* hybridization (FISH) on blood smears of the sex-mismatched chimeric mice. The murine Y chromosome-specific probe M34 [30,31], kindly provided by Dr. L. Singh (Center for Cellular and Molecular Biology, Hyderabad, India), was labeled with biotin-16-deoxyuridine triphosphate (Boehringer-Mannheim, Mannheim, Germany) by nick-translation. Slides were fixed for 10 minutes at room temperature in 100% methanol and stored dry at -20°C until further use. FISH was performed using a modification of the protocol of Pinkel *et al.* [32] as previously described [33]. After hybridization, the cells were incubated with avidin-FITC (Vector, Burlingame, CA). The fluorescence signal was amplified with a biotinylated goat-anti-avidin antibody (Vector) followed by a second incubation with avidin-FITC. The slides were then dehydrated in ethanol, dried, mounted with antifade medium [33] and stored at -20 °C. The percentage of Y chromosome$^+$ cells was determined by counting 100 to 200 cells. False positive signals ranged between 1% and 2%.

Chimerism in hematopoietic organs and lineages. At 9 and 12 months after transplantation, individual recipient mice were studied for the organ distribution and extent of

lineage expression of the donor stem cells. Monocytes and granulocytes were sorted from BM within a medium to high forward and perpendicular light-scatter window, using antibody ER-MP20. B-lymphocytes were sorted from the spleen using antibody RA3-6B2 (anti-B220) [34] and T-lymphocytes were directly harvested from the thymus. For FISH, approximately 2×10^5 BMC, spleen cells, thymic lymphocytes or sorted cells were transferred onto slides and spread using a drop of serum. In addition, cytospin preparations of the sorted cells were stained with May-Grünwald/Giemsa for differential counting. BM CFU-S-12 chimerism was determined after transfer of 2 to 5×10^4 BMC from eight individual chimeric mice into 8-Gy-irradiated female BALB/c recipients (groups of 10 animals). Individual spleen colonies were excised at day 12, suspended in PBS, fixed in methanol/25% (vol/vol) glacial acetic acid and transferred onto slides. The percentage of donor-type colonies was determined by FISH.

RESULTS

Transient and persisting cobblestone area formation in the CAFC assay. Light-density BM from untreated and 5-fluorouracil-treated mice was separated on the FACS using Rh123 or WGA, and tested for its short and long-term repopulating ability (STRA and LTRA) *in vitro* using the CAFC assay. Normal light-density BM was depleted of contaminating monocytes and granulocytes by MACS or FACS using antibody ER-MP20. From this LD/ER-MP20$^-$ population, the 10% most Rh123^{bright}, 10% most Rh123^{dull} cells, and 4% most WGA^{bright} cells were sorted from within a light-scatter window as previously described [15,25,35] (Figure 3.1A). Light-density day-6 post-5-fluorouracil BM (LD/FU$_{6d}$BM) was not further depleted of monocytes and granulocytes because the percentages of these cells were already low. The WGA^{bright} and

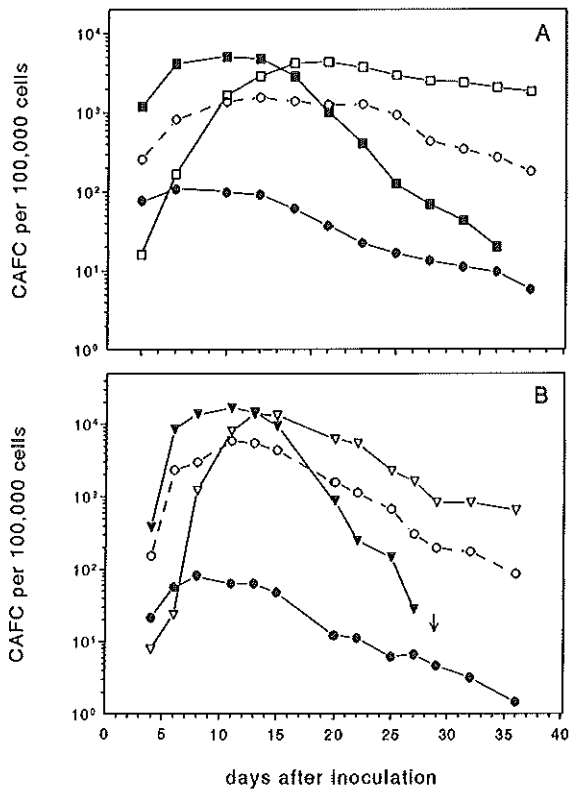

Figure 3.1 CAFC-frequencies of (A): unseparated BMC (●), light-density ER-MP20$^-$ cells (○), and the 10% most Rh123^{bright} (■) and Rh123^{dull} cells (□) sorted from the LD/ER-MP20$^-$ population; and (B): unseparated BMC (●), LD/FU$_{6d}$BM (○), and its 6% most WGA^{bright} (▼) and 6% WGA^{dim} (▽) subpopulations. Frequencies were calculated by limiting dilution analysis using Poisson statistics. Data represent 2 of 4 individual experiments.

WGA^{dim} cells, sorted from $LD/FU_{6d}BM$, each comprised 6% of the nucleated cells [10] (Figure 3.1B). The results show that both the $Rh123^{bright}$ and WGA^{bright} subpopulations were 50- to 200-fold enriched for cells that gave rise to an early but transient cobblestone area formation at day 10 after inoculation (CAFC-10; the *in vitro* equivalent to CFU-S-12 [20,21]). In contrast, the $Rh123^{dull}$ and WGA^{dim} subpopulations showed persisting cobblestone area formation and were 250- to 300-fold enriched for late CAFC (CAFC-28/35) as compared to unseparated BM. This indicates that the *in vitro* short-term and long-term repopulating ability can largely be separated using Rh123 retention or WGA affinity.

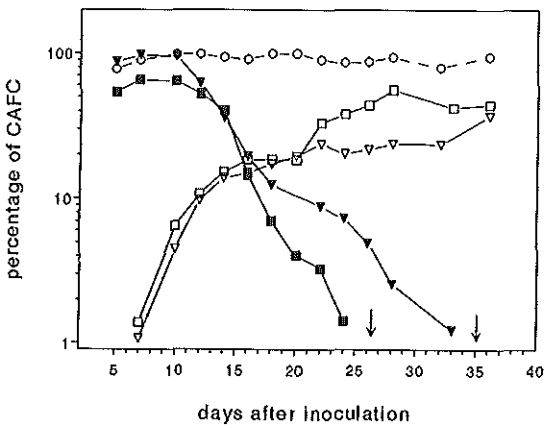

Figure 3.2 Percentages of: (o) the total number of CAFC in the ER-MP20$^-$ population as compared with light-density BM; the number of CAFC in the sorted $Rh123^{bright}$ (■), $Rh123^{dull}$ (□) and WGA^{bright} (▼) subpopulations as compared with LD/ER-MP20$^-$ cells; and the number of CAFC in the sorted WGA^{dim} (▽) subpopulation as compared with $LD/FU_{6d}BM$.

To investigate whether or not we had selected for subpopulations of short-term and long-term repopulating stem cells from light-density BM, the respective recoveries of the sorted populations were calculated from the CAFC data. The ER-MP20$^-$ population contained 95 to 100% of all early and late CAFC that were originally present in light-density BM (Figure 3.2). The $Rh123^{bright}$ and WGA^{bright} subpopulations represented 65% and 95%, respectively, of the early appearing CAFC from LD/ER-MP20$^-$ BM. The $Rh123^{dull}$ and WGA^{dim} cells, sorted from LD/ER-MP20$^-$ and $LD/FU_{6d}BM$, respectively, comprised 40 to 50% of the late appearing primitive CAFC and only 5% to 10% of the original CAFC-10. These results together indicate that the sorted populations were representative for almost all STRA (including the CFU-S-12) and about half of the LTRA that were contained in the light-density populations. Apparently, about 50% of the LTRA was contained in the Rh123 and WGA-FITC intermediately staining cell populations that were not sorted in our procedure.

Transient and persisting hematopoiesis *in vivo*. The BM subpopulations that were tested in the CAFC assay were in parallel analyzed for their STRA and LTRA *invivo*, using an α-thalassemic chimeric mouse model. Sorted and unseparated BMC, derived from normal male mice, were transplanted into groups of 3-, 4- or 5-Gy-irradiated female α-thalassemic mice (4 to 8 animals per group). In the subsequent 12 months the percentage of peripheral blood donor-type erythrocytes was determined by FACScan analysis. At each time point, the lines fitted through the mean % donor-type

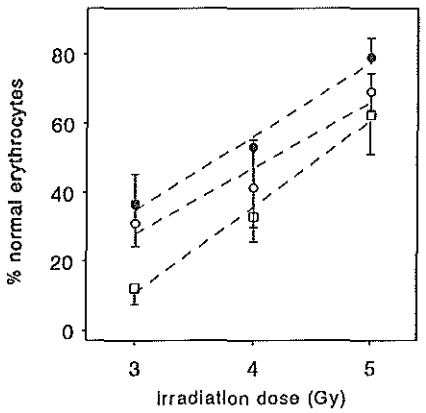

Figure 3.3 Percentage of donor RBCs at 6 weeks after transplantation of (●) $3x10^5$ unseparated BMC, (○) $15x10^3$ LD/ER-MP20$^-$ cells, or (□) 670 Rh123^{dull} cells, in groups of 3-, 4- or 5-Gy-irradiated female α-thalassemic mice (4 to 8 animals per group). The lines are fitted using linear-regression analysis. Bars denote 1 SD.

erythrocytes at 3, 4 and 5 Gy ran parallel for all stem cell populations. This is illustrated by Figure 3.3 at 6 weeks after transplantation. The mean repopulation data of all animals that received a dose of 5 Gy in each of the four individual experiments, are shown in Figure 3.4 (data of the 3 and 4 Gy groups are not shown). The enrichment for the *in vivo* erythroid repopulating ability of the sorted cells was calculated for each of the three irradiation groups. This was done by dividing the mean percentage of donor-type erythrocytes of the animals that received sorted cells by that of recipients of unseparated BM, after correction for the number of cells injected. Figures from the three individual irradiation groups were then combined to calculate the mean enrichments that are shown in Figure 3.5. Transplantation of 6,770 Rh123^{bright} or 4,700 WGA^{bright} cells into α-thalassemic recipients led to a transient donor-type repopulation lasting only 4 to 5 months. In contrast, infusion of 670 or 1,700 Rh123^{dull}, or 1,700 WGA^{dim} cells resulted in sustained chimerism of approximately 40% to 70% donor-type RBCs (Figure 3.4 and 3.5). These populations were 50 to 240 times enriched for *in vivo* long-term repopulating ability as compared with unseparated BM. In support of the *in vitro* data, these results show that also the *in vivo* STRA and LTRA have largely been separated. In addition, our observations indicate that the erythrocytic progeny of transiently repopulating cells can be measured up to 5 months after transplantation.

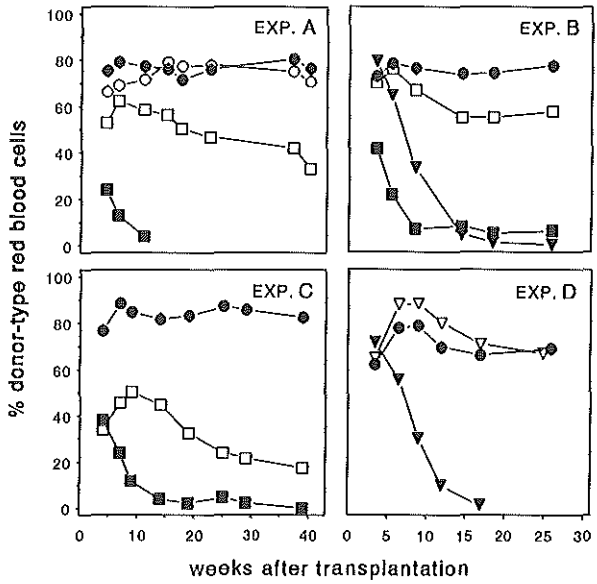

Figure 3.4 Percentage of donor RBCs in 5-Gy-irradiated α-thalassemic mice (4 to 8 animals per group) after transplantation of unseparated BMC or BMC sorted on the basis of Rh123 or WGA. (A) through (D) indicate four individual experiments. (●) $3x10^5$ unseparated BMC infused per recipient (in A through D); (○) $15x10^3$ LD/ER-MP20$^-$ cells (in A); (■) Rh123^{bright} cells (1,930, 5,950, 6,770 nc in (A) through (C), respectively); (□) Rh123^{dull} cells (670, 1,700, 2,000 nc in (A) through (C), respectively); (▼) WGA^{bright} cells (4,700 and 3,700 nc in (B) and (D), respectively); and (▽) 1,700 WGA^{dim} cells (in D). Cells in experiment (A) through (C) were sorted from normal BM, cells in (D) were sorted from 5-fluorouracil-treated animals.

Analysis of the number of CAFC-10, CAFC-28 and CFU-S-12 injected per mouse. The contribution of CFU-S-12 and primitive hematopoietic stem cells in the different grafts to long-term repopulation was analyzed by comparing the number of CAFC-10, CFU-S-12 and CAFC-28 with chimerism at 6 months after transplantation (Table 3.1). Injection of 1,930 to 6,770 Rh123^{bright}, or 3,700 to 4,700 WGA^{bright} cells per mouse, containing low numbers of CAFC-28 but as many as 281 CFU-S-12 and high numbers of CAFC-10, resulted in only short-term repopulation (Figure 3.4). Injection of 670 to 2,000 Rh123^{dull} cells, on the other hand, which contained low numbers of CAFC-10 (in one case even 0.7 CAFC-10, equalling about 0.1 CFU-S-12 injected per mouse [21]) led to stable long-term repopulation ranging from 24% to 58% at 6 months after transplantation (Table 3.1). In addition, injection of 189 WGA^{dim} cells from LD/FU$_{6d}$BM, which contained 0.9 CFU-S-12, led to more than 10% donor-type repopulation in 50% of the mice (Table 3.2), and more than 40% repopulation in 17% of the mice injected (not shown). Therefore, the data clearly show that long-term repopulation is independent of the number of CFU-S-12 injected and can be attributed to more primitive stem cells being Rh123^{dull} and WGA^{dim}. In the present report the ratio between CAFC-10 and CFU-S-12 varied from 2.0 to 9.7. This could be caused by

intrinsic differences between individual stromal layers, or differences in the spleen-seeding efficiency of the sorted cells. Previously, a more extensive regression analysis has shown that 4.7 to 6.7 CAFC-10 were detected for each CFU-S-12 [21].Using this particular transplantation model, the LTRA unit was defined as the number of male cells that had to be grafted to reach at least a sustained level of 10% donor-type RBCs in 5-Gy-irradiated female α-thalassemic mice. Because of the limitations of both the RBC and WBC analyses (see Materials and Methods), a signal lower than 5% was not considered to be indicative of donor-type engraftment. Using Poisson statistics and

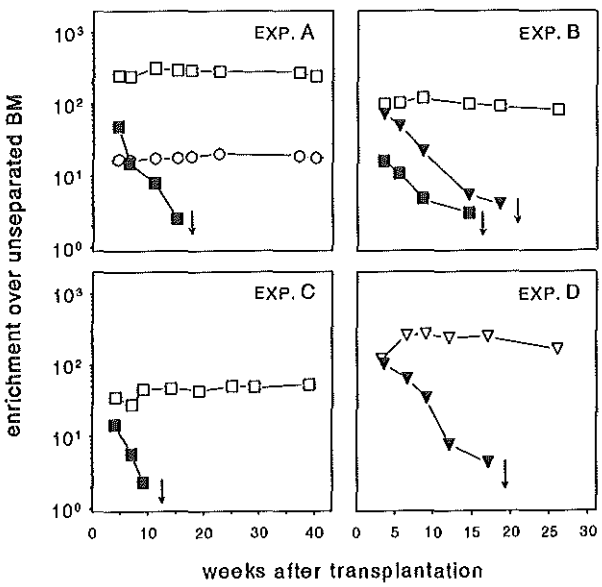

Figure 3.5 Mean enrichments for erythroid repopulating ability of sorted cells over unseparated BMC (see legend Figure 3.4 for the different populations) in groups of 3-, 4- or 5-Gy-irradiated α-thalassemic mice. Enrichments were separately calculated for each irradiation group, corrected for the number of cells injected, and then combined (see Results section). (A) through (D) indicate individual experiments. The control BMC level is at 1.

limiting dilution analysis, the frequency of the LTRA unit in the 160-fold enriched LD/FU_{6d} WGA^{dim} cells (Figure 3.5D) was calculated to be 1 in 412 (Table 3.2).

FISH. To test the concordance of the FISH technique, 120 peripheral blood slides were prepared from the same sex-mismatched chimeric mouse and stored at -20°C. Groups of slides were subsequently processed in three different FISH sessions. The y probe was derived from the six different nick-translation batches that we had used in our experiments in the past 2 years. Series of 100 as well as 200 cells were counted, some by two independent observers. Variance analysis and t-statistics (data not shown)

showed no significant differences between: (1) the different y-probe batches, (2) the different FISH sessions, (3) the counting of 100 or 200 cells, or (4) between observers. The standard deviation of the 120 samples, at a level of 63.3% male cells, was 10.2%.

Table 3.1. In vitro and in vivo repopulating ability of sorted cells.

		infused per recipient			% donor-type at 6 months	
population	number of cells	number of CAFC-10	number of CFU-S-12	number of CAFC-28	RBC	WBC
unseparated BMC	300,000	297	nd	18	76	31
	300,000	504	nd	24	87	32
	300,000	495	53	12	77	43
	300,000	213	73	12	69	41
LD/ER-MP20$^-$	15,000	106	nd	27	78	20
Rh123^{bright}	6,770	879	nd	<0.9	5	0.5
	5,950	532	63	<16.1	6	nd
	1,930	96	nd	<0.5	5	1.0
WGA^{bright}	4,700	1,592	171	<7.1	0	nd
	3,700*	555	281	<9.9	<2	nd
Rh123^{dull}	2,000	0.7	nd	4.3	24	11
	1,700	18.7	3.1	76.8	58	31
	670	11.2	nd	12.2	47	12
WGA^{dim}	1,700*	78.4	8.3	140.4	67	43
	567*	26.1	2.8	46.8	29	21
	189*	8.7	0.9	15.6	14	7
	63*	2.9	0.3	5.2	7	nd
	21*	1.0	0.1	1.7	2	nd

Light-density ER-MP20$^-$ cells from normal BM or day-6 post-5-fluorouracil BM (LD/ER-MP20$^-$ or LD/FU_{6d}BM, respectively) were sorted on the basis of their rhodamine-123 (Rh123) retention or wheat germ agglutinin (WGA) affinity. Long-term repopulation was determined after transplantation into 5-Gy-irradiated female α-thalassemic mice. Donor-type RBCs and white blood cells (WBCs) represent mean percentages of groups of 4 to 8 animals. RBC chimerism was determined by FACScan, WBC chimerism by FISH. * indicates the populations sorted from 5-fluorouracil-treated animals.
Abbreviation: nd, not determined.

Table 3.2. Frequency analysis of LTRA units, 6 months after transplantation of WGA^{dim} cells sorted from LD/FU$_{6d}$BM

No. of cells injected	Fraction of mice with less than 10% donor-type erythrocytes
1,700	0/4*
567	2/7
189	3/6
63	7/8
21	8/8
LTRA frequency	1/412
95% confidence limits	1/207-1/817

*The frequency of the LTRA unit responsible for 10% donor-type erythroid repopulation was calculated from the proportion of mice with less than 10% donor-type RBCs using limiting dilution analysis according to Poisson statistics. Mice were irradiated with 5 Gy and transplanted with WGA^{dim} cells from a light-density fraction of day-6 post-5-fluorouracil BM (LD/FU$_{6d}$BM).

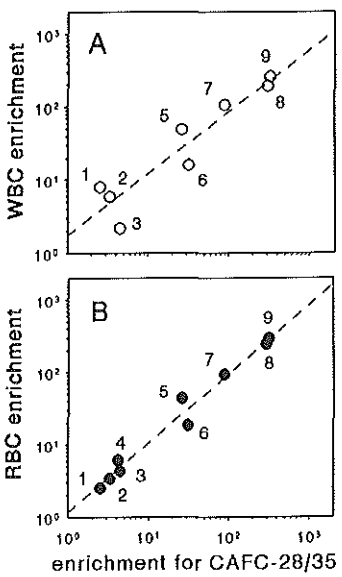

Figure 3.6 Correlation between the enrichment for CAFC-28/35 *in vitro* and (A): donor-type nucleated blood cells (WBC) (r=0.94; n=8; p<0.001), or (B): donor-type RBCs *in vivo* (r=0.99; n=9; p<0.001), at 4 months after transplantation. Symbols represent the mean enrichments of sorted cells over control BMC (12 to 24 mice per group). All three irradiation groups are included, as explained in the Results section. Numbers indicate different grafts: 1 to 2, Rh123^{bright}; 3 to 4, WGA^{bright}; 5,7 and 9, Rh123^{dull}; 6, LD/ER-MP20^{-} and 8, WGA^{dim}. RBC chimerism was determined by FACScan, nucleated blood cell chimerism by FISH using a murine Y chromosome-specific probe.

Comparison of the enrichment for LTRA *in vitro* and *in vivo*. To compare the enrichments of the sorted populations over unseparated BM, determined *in vitro* and *in vivo*, data from different experiments were combined. The percentage of donor-type nucleated cells was determined using FISH on blood smears at 2, 4, and 6 months, and

also in one experiment at 9 months after transplantation. Linear regression analyses showed a high and significant correlation between the enrichment for CAFC-28/35 *in vitro*, and donor-type RBCs and nucleated blood cells *in vivo* (Figure 3.6), supporting our earlier findings [10]. Data at 4 months after transplantation (Figure 3.6A and B) also showed that the enrichment for RBC and nucleated blood cell chimerism correlated strongly. The correlations at 2, 6 and 9 months gave similar results (not shown).

Table 3.3. Percentage donor RBCs and nucleated cells in 8 individual chimeric mice, 9 months after transplantation.

TBI (Gy)	grafted* cells	RBCs	WBCs	BM	spleen	thymus	CFU-S-12	
5	NBMC	72	46	31	46	77	24 ± 7.5	(8/33)
5	$Rh123^{dull}$	50	24	24	25	23	13 ± 11.7	(1/8)
5	$Rh123^{dull}$	25	4	2	10	4	<7	(0/14)
5	$Rh123^{dull}$	72	39	36	32	20	36 ± 10.3	(8/22)
4	NBMC	52	47	43	43	90	87 ± 5.0	(40/46)
4	$Rh123^{dull}$	43	27	31	22	10	35 ± 8.6	(11/31)
4	$Rh123^{dull}$	29	12	4	12	7	6 ± 10.8	(2/12)
4	$Rh123^{dull}$	9	10	5	14	26	<6	(0/16)

*$3x10^5$ unseparated BMC (NBMC) and 670 $Rh123^{dull}$ cells sorted from light-density ER-MP20$^-$ BM were transplanted per mouse after 4 or 5 Gy TBI as indicated. CFU-S-12 chimerism is presented as the percentage donor-type CFU-S-12 colonies (± SE) after secondary transplantation. Between brackets the number of male colonies per total number of spleen colonies analyzed. The SE of the proportion was approximated on the assumption of normally distributed data. RBC chimerism was determined by FACScan analysis. Other data were generated using FISH.

Therefore, the results indicate that RBC chimerism can be used to determine the LTRA of a graft *in vivo* as of 2 months after transplantation. The use of α-thalassemic mice reduced the time required for analyzing 100 samples from almost 4 days, which was required for FISH, to less than 1 hour for FACScan analysis of the peripheral RBCs. The method also provides a direct way to determine chimerism without the need for previous immunochemical or biochemical staining [29]. This makes the model extremely suitable for studying long-term repopulation using large groups of mice.

Multilineage donor-type reconstitution. In addition to the repopulation in the RBC compartment, chimerism was also determined in the major hematopoietic organs and differentiation lineages of individual mice. This was done at 9 and 12 months after transplantation of 3 x 10^5 unseparated BMC, 670 or 1,700 LD/ER-MP20$^-$ Rh123^{dull} cells (Table 3.3 and 3.4). Microscopic inspection of the May-Grünwald/Giemsa-stained cytospin preparations of the sorted monocytes and granulocytes proved them to be more than 95% pure (not shown). Peripheral blood cell chimerism, induced by unseparated BMC or Rh123^{dull} cells, was present in all lineages, whereas its level was comparable with that in most hematopoietic organs. The selective advantage of the erythroid repopulation has been described previously [19]. CFU-S-12 chimerism and nucleated blood cell chimerism were of the same magnitude (Table 3.3). The present results indicate that the LTRA cells, acquired by buoyant density centrifugation and sorting on the basis of their low Rh123 retention, are qualitatively comparable to the LTRA cells in unseparated BM and give rise to a stable long-term multilineage donor-type reconstitution.

Table 3.4. Percentage donor-type cells in the different hemopoietic lineages of 8 individual chimeric mice, 12 months after transplantation

grafted* cells	RBCs	WBCs	mono-cytes	granulo-cytes	B cells	T cells
NBMC	75	53	51	34	38	51
NBMC	86	56	62	45	30	58
NBMC	91	63	54	62	15	46
NBMC	56	36	44	30	10	60
Rh123^{dull}	93	42	22	52	18	nd
Rh123^{dull}	62	33	40	10	9	32
Rh123^{dull}	78	56	23	58	23	32
Rh123^{dull}	48	20	10	25	18	nd

*3x10^5 unseparated BMC (NBMC) or 1,700 Rh123^{dull} cells sorted from light-density ER-MP20$^-$ BM cells were transplanted in 5-Gy-irradiated mice. Monocytes and granulocytes were sorted from BM using ER-MP20, B cells using RA3-6B2 (anti-B220). T cells were harvested from the thymus without sorting. RBC chimerism was determined by FACScan analysis. Other data were generated by FISH. Abbreviation: nd, not determined.

DISCUSSION

The present results clearly demonstrate a lack of LTRA activity *in vivo* and *in vitro* in the populations 50- to 200-fold enriched for CFU-S-12 using Rh123 or WGA. The sorted $Rh123^{bright}$ and WGA^{bright} cells represented 65% and 95%, respectively, of all CFU-S-12 (CAFC-10) that were originally present in pre-enriched light-density BM. It has been previously reported that CFU-S are unable to maintain hematopoiesis in long-term BM culture [36-38]. In the CAFC assay, $Rh123^{bright}$ and WGA^{bright} sorted cells gave rise to an initial but short-lived hematopoietic burst [10,20,21,38]. Injection of as many as 281 WGA^{bright} CFU-S-12 in sublethally irradiated mice, equal to a calculated 2,800 day-12 spleen colony-forming cells (corrected for a 10% seeding efficiency), did not result in any significant repopulation beyond 4 to 5 months after transplantation. In contrast, the populations that were 160- to 240-fold enriched for LTRA (Figure 3.5) were severely depleted of CFU-S-12 activity. Injection of 670 to 2,000 $Rh123^{dull}$ or 200 to 500 WGA^{dim} cells with extremely low numbers of CFU-S-12 induced sustained chimerism (Table 3.1). In one experiment CFU-S-12 numbers approximated 0.1 injected per mouse. Chimerism involved all hematopoietic organs and lineages tested, including the BM CFU-S-12 as determined in secondary recipients, indicating that our sorting procedure enriched for LTRA subsets with similar multilineage expression ability as have LTRA cells in unseparated BM. Therefore, the results imply that hematopoietic stem cells contain a distinct stem cell subset with LTRA that can be separated physically from the vast majority of CFU-S-12. Although the data make it likely that LTRA stem cells have pre-CFU-S properties, we do not exclude that some LTRA cells may form a spleen colony.

Partial or total separation of LTRA from CFU-S has been reported earlier using plastic-adherence or counterflow elutriation [9,39]. However, final enrichment levels for the respective hematopoietic stem cell populations, were lower than presently reported. Frequency analysis of the plastic-adherent BMC, containing 30% of the long-term repopulating stem cells as detected by competitive repopulation in lethally irradiated animals, showed about equal numbers of CFU-S-12 and LTRA cells [39]. The graft that induced long-term engraftment still contained 15 CFU-S-12, whereas we observed long-term repopulation with only 0.1 to 3.1 CFU-S-12 injected per animal.

Previously, the population of Thy-1.1^{lo} Lin^- Sca-1^+ cells has been thought to comprise stem cells purified to almost unit efficiency [1,7,8,40]. These cells have been attributed with the capacity to protect lethally irradiated animals as well as provide short-term and long-term repopulation. However, it was recognized that most of the Thy-1.1^{lo} Lin^- Sca-1^+ cells possessed STRA, with only a minority being true LTRA cells [6]. The number of CFU-S-12/13 in the Lin^- Sca-1^+ population, calculated from their frequency of 1 in 21 cells, comprised almost 50% of the population [41,42]. Further analysis of the phenotype of the Lin^- Sca-1^+ cells, using several lectins and antibodies, showed a heterogeneity for Thy-1 expression, WGA affinity and Rh123

retention [40,41,43]. Although Lin^- $Sca\text{-}1^+$ cells contained functionally different $Rh123^{lo}$ and $Rh123^{med/hi}$ subpopulations; responding differently to hematopoietic growth factors [43]; the use of neither Rh123 nor Thy-1 allowed for a separation of CFU-S activity from LTRA [8,40,43]. However, analysis of the fluorescence profile of Lin^- $Sca\text{-}1^+$ cells labeled with WGA, showed a clearly distinguishable large peak of WGA^{bright} cells [41], comprising about 50% of the population at a fluorescence level characteristic for CFU-S-12 in our laboratory. The remaining 50% WGA^{dim} cells appeared highly similar to our definition of LTRA cells, a population depleted of CFU-S-12 [10]. Therefore, this observation suggests that the Thy-1.1^{lo} Lin^- $Sca\text{-}1^+$ population could be divided into two subpopulations containing either WGA^{bright} CFU-S or the more primitive WGA^{dim} non-CFU-S LTRA cells, using the criteria applied in the present study.

The enrichment for LTRA *in vivo* correlated strongly with the enrichment for the CAFC-28/35 *in vitro*, as did the enrichments for CFU-S-12 and CAFC-10 (data not shown). This supports earlier findings that the CAFC assay can be used as a tool for the simultaneous and quantitative assessment of populations with STRA as well as LTRA [10,12,21]. Using either competitive repopulation or the CAFC assay, the frequency of the LTRA cell in marrow has been estimated to range from 0.5 to 2.5 per 10^5 normal BMC [7,10,44,45]. In the present study, using sublethally irradiated α-thalassemic recipients in a sex-mismatched transplantation setting, the frequency of the *in vivo* LTRA cell could not be determined directly, but was estimated by defining the number of cells capable of inducing a sustained level of 10% donor-type RBCs as an LTRA unit. For the 160-fold enriched LD/FU_{6d} WGA^{dim} population (Figure 3.5D), the LTRA unit was calculated to contain 412 cells. Therefore, the frequency of the LTRA unit in unseparated BM approximates 1 to 2 per 10^5 cells, which is in agreement with previous estimates of *in vivo* LTRA cells in different mouse models [7,44,45].

At 12 months after transplantation of 1,700 Rh^{dull} cells, containing 2 to 3 LTRA units, clear differences can be seen in their contribution to the monocytic and granulocytic lineages in individual mice (Table 3.4). Such a segregation of monocytic and granulocytic lineage expression has been observed earlier using retrovirally marked stem cell clones [46]. It was suggested that in addition to the stochastic mechanisms operating at the level of stem cell commitment, lineage specific demands might influence the clonal expansion of particular lineages by microenvironmental and humoral factors [46-48]. Also, the percentage of donor-type cells in the thymus, which varied considerable when compared with the other hematopoietic tissues (Table 3.3), might be influenced by these mechanisms. The presence of intra-thymic long-lived T cell clones may play an additional role. Therefore, the contribution to the T-cell lineage might temporarily differ from the expression in other lineages.

In summary, we achieved a stringent separation of CFU-S activity from stem cells that are capable of inducing a stable long-term multilineage reconstitution of sublethally irradiated mice. Both as determined *in vitro* and *in vivo*, LTRA cells were 160- to 240-

fold enriched as compared with unseparated BM. A highly enriched population of CFU-S-12, on the other hand, did only contribute to repopulation in the first months after transplantation. This transient role of CFU-S in repopulation has also been suggested by other investigators [12,39,49,50]. The spleen colony-forming cells could be held responsible for the strong short-term clonal fluctuations observed in retroviral transfection experiments in the first 4 to 6 months after transplantation [46,51-53]. Therefore, to study the early clonal engraftment of the LTRA cells, separation from CFU-S populations is desirable. Such a study on the *in vivo* behavior of the LTRA cells could eventually provide clues to design more effective protocols for gene therapy.

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CHAPTER 4

IDENTIFICATION OF HEMATOPOIETIC STEM CELL SUBSETS ON THE BASIS OF THEIR PRIMITIVENESS USING ANTIBODY ER-MP12

Johannes C.M. van der Loo[1], Walentina A.T. Slieker[2],
Dorinde Kieboom[1] and Rob E. Ploemacher[1]

1. Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
2. Department of Immunology, Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

Monoclonal antibody ER-MP12 defines a novel antigen on murine hematopoietic stem cells. The antigen is differentially expressed by different subsets in the hematopoietic stem cell compartment and enables a physical separation of primitive long-term repopulating stem cells from more mature multilineage progenitors. When used in two-color immunofluorescence with ER-MP20 (anti-Ly-6C), six subpopulations of bone marrow (BM) cells could be identified. These subsets were isolated using magnetic and fluorescence-activated cell sorting, phenotypically analyzed, and tested *in vitro* for cobblestone area-forming cells (CAFC) and colony-forming units in culture (CFU-C; M/G/E/Meg/Mast). In addition, they were tested *in vivo* for day-12 spleen colony-forming units (CFU-S-12), and for cells with long-term repopulating ability using a recently developed α-thalassemic chimeric mouse model. Cells with long-term repopulating ability (LTRA) and day-12 spleen colony-forming ability appeared to be exclusively present in the two subpopulations that expressed the ER-MP12 cell surface antigen at either an intermediate or high level, but lacked the expression of Ly-6C. The ER-MP12^{med}20^{-} subpopulation (comprising 30% of the BM cells, including all lymphocytes) contained 90-95% of the LTRA cells and immature day-28 CAFC (CAFC-28), 75% of the CFU-S-12, and low numbers of CFU-C. In contrast, the ER-MP12^{hi}20^{-} population (comprising 1-2% of the BM cells, containing no mature cells) included 80% of the early and less primitive CAFC (CAFC-5), 25% of the CFU-S-12, and only 10% of the LTRA cells and immature CAFC-28. The ER-MP12^{hi} cells, irrespective of the ER-MP20 antigen expression, included 80-90% of the CFU-C (day-4 through day-14), of which 70% were ER-MP20^{-} and 10-20% ER-MP20$^{med/hi}$. In addition, erythroblasts, granulocytes, lymphocytes and monocytes could almost be fully separated on the basis of ER-MP12 and ER-MP20 antigen expression. Functionally, the presence of 5 μg/mL ER-MP12 in a long-term BM culture did not affect hematopoiesis, as was measured in the CAFC assay. Our data demonstrate that the ER-MP12 antigen is intermediately expressed in the long-term repopulating hematopoietic stem cell. Its level of expression increases upon maturation towards CFU-C, to disappear from mature hematopoietic cells, except from B and T lymphocytes.

INTRODUCTION

The bone marrow (BM) hematopoietic stem cell compartment contains a hierarchically organized continuum of stem cell subsets, ranging from pluripotent hematopoietic stem cells to *in vitro* clonable multilineage progenitors [1,2]. The most immature hematopoietic stem cells are functionally defined by their extensive self-renewal capacity and ability to provide multilineage long-term repopulation (LTRA) in sublethally or lethally irradiated animals, which was studied using retrovirally marked stem cell clones [3-7]. It has been shown that LTRA cells can physically be separated from the large majority of day-12 spleen colony-forming units (CFU-S-12) on the basis of cell size and density, using centrifugal counterflow elutriation (CCE) [8,9]. The lack of stem cell specific markers, however, makes it difficult to highly enrich LTRA cells and simultaneously separate them from the short-term repopulating cells. Separation would benefit the search for stem cell specific genes [10,11], and aid to the development of protocols for more efficient gene transfer in hematopoietic stem cells.

To achieve high enrichments, techniques such as CCE, density centrifugation and magnetic sorting for lineage marker-negative cells, had to be combined with fluorescence-activated cell sorting [12,13]. The different protocols included separation on the basis of rhodamine-123 (Rh123) retention [14-19], Hoechst 33342 fluorescence [19,20], and wheat germ agglutinin (WGA) affinity [21-24]. This resulted in a 850 to 2000-fold enrichment for multilineage long-term repopulating stem cells with relatively low numbers of co-purified CFU-S-12 [19,22,25,26]. However, the most primitive and more mature hematopoietic stem cell subsets could not be discriminated on the basis of cell surface antigens. Apart from stem cell purification, antigens that are selectively expressed by the most primitive hematopoietic stem cells could reveal information on the complex interactions of these cells with their specific microenvironment. We therefore set out to find cell surface markers that are differentially expressed on the different subsets of the hematopoietic stem cell compartment.

Recently, we produced two novel rat monoclonal antibodies (mAbs), ER-MP12 and ER-MP20 [27,28], revealing six distinct subpopulations of murine BM cells when used in two-color immunofluorescence [29]. ER-MP12 recognizes a yet unknown 140 kDa single-chain glycoprotein on murine hematopoietic cells. ER-MP20 was shown to be directed against differentiation antigen (Ag) Ly-6C [30]. After sorting the different subsets on the basis of ER-MP12 and ER-MP20 Ag expression, the highest frequency of thymus-seeding and repopulating cells was found in the subpopulation (1-2% of BM cells) that lacked Ly-6C but expressed the ER-MP12 Ag at a high level (ER-MP12^{hi}20^{-}) [29,31]. After intravenous transfer, however, both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations generated B lymphocytes and myeloid cells in addition to T lymphocytes, indicating the presence of pluripotent hematopoietic cells. Although frequencies and recoveries of these pluripotent cells were not assessed, it showed that ER-MP12 could possibly be used as a tool in dissecting the hematopoietic stem cell compartment.

In the present report we therefore investigated the expression of the ER-MP12 Ag on the various hematopoietic stem cell and progenitor cell subsets in the BM. Using magnetic and fluorescence-activated cell sorting, BM cells were separated into six subpopulations on the basis of their differential expression of the ER-MP12 and ER-MP20 antigens. The subpopulations were phenotypically analyzed and subsequently tested for cobblestone area forming cells (CAFC-7 through CAFC-35) [32,33] and long-term *in vivo* repopulating cells, using an α-thalassemic chimeric mouse model as was recently described [23]. In addition, the subsets were tested for CFU-S-12 and for *in vitro* clonable progenitors, including macrophage, granulocyte, erythroid, megakaryocyte and mast cell colony-forming units.

Our data demonstrate that the ER-MP12 Ag is differentially expressed by the various subsets in the BM stem cell compartment, with LTRA cells expressing the ER-MP12 Ag at an intermediate level. With development towards CFU-C, the Ag expression gradually increases, to disappear from most lineages in the course of their final maturation, except from T and B lymphocytes. The present results identify the ER-MP12 Ag as a novel positive marker on hematopoietic stem cells with a level of expression that is inversely related to the primitiveness of the cells in the stem and progenitor cell compartment.

MATERIALS AND METHODS

Animals. Male inbred BALB/cAnCrlRij mice, and female heterozygous α-thalassemic BALB/c ($Hba^{th/+}$) mice [34], were bred at the former Institute of Applied Radiobiology and Immunology, Rijswijk, The Netherlands. C57BL/6-Ly5.1-pep^{3b} (Thy-1.2, Ly-5.1) and (CBA♂ x C57Bl♀)F1 mice were bred at the Central Animal Department of the Erasmus University. All mice, 12 to 25 weeks of age, were bred maintained under specific pathogen free conditions and received acidified water (Ph 2.8) and food pellets ad libitum. The C57BL/6-Ly5.1-pep^{3b} breeding pairs were kindly provided by Dr. I.L. Weissman, Stanford University, Stanford, CA.

Monoclonal antibodies and conjugates. Rat monoclonal antibodies (mAbs) ER-MP12 and ER-MP20 (both IgG2a isotypes) were purified from culture supernatants by ammonium sulphate precipitation. ER-MP20 was conjugated to fluorescein isothiocyanate (FITC; isomer I, Sigma, St.Louis, MO)(ER-$MP20^{FITC}$), while ER-MP12 was biotinylated (ER-$MP12^{bio}$) using N-hydroxy-succinimidyl-biotin (Sigma) according to standard procedures [35]. Streptavidin-conjugated R-Phycoerythrin (SAV-PE) and Streptavidin-conjugated TRICOLOR (SAV-TRI) were obtained from Caltag Laboratories (South San Francisco, CA). Hybridoma supernatants were used for mAbs RA3-6B2 (anti-B220) [36], RB6-8C5 (anti-GR-1) [37] and M1/70 (anti-Mac-1) [38]. Erythroid lineage specific mAb TER-119 [39] was kindly provided by Dr. T. Kina, Kyoto University, Kyoto, Japan. mAb PH2-99, an anti-E.Coli-β-galactosidase (rat IgG2a) was made at the department of Immunology, Erasmus University, Rotterdam, The Netherlands. As a second stage antibody R-Phycoerythrin-conjugated mouse-adsorbed goat anti-rat IgG (H+L) (GαRa-PE; Caltag) was used. Antibodies and conjugates were titrated for optimal staining of mouse BM cells.

Preparation of cell suspensions. Preparation of the BM cells, and (in specific experiments) buoyant density centrifugation, using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient, was performed as previously described [33]. Cells with a density of 1.069-1.075 g/mL (2% to 6% of total BM) were collected from the interphase, washed in PBS containing 5% fetal calf serum (FCS), and maintained on ice throughout the staining and purification procedure. For long-term repopulation experiments, low-density BM cells were depleted of ER-MP20-positive cells by magnetic separation using the MACS® (Miltenyi Biotec, Bergisch-Gladbach, Germany), prior to fluorescence-activated cell sorting. Cells were incubated for 30 min with ER-$MP20^{bio}$ in PBS containing 0.01% (wt/vol) NaN_3, washed, and subsequently incubated for 15 min with streptavidin-conjugated MACS microbeads (Miltenyi Biotec) in PBS containing 0.01% NaN_3 and 5 mmol/L EDTA (Titriplex III; Merck, Darmstadt, Germany). After incubation, the cells were washed in PBS supplemented with 0.01% NaN_3, 5 mmol/L EDTA and 1% (wt/vol) bovine serum albumin (BSA, Fraction V; Sigma) and separated using the MACS column B2 (Miltenyi Biotec) at a flow-rate of 0.2 to 0.3 mL/min. MACS beads were sterilized by filtration through a 0.22 μm filter. The non-magnetic, ER-MP20-negative population (ER-$MP20^-$) was collected and maintained on ice in PBS containing 5% FCS.

Immunofluorescence staining and cell sorting. For two-color immunofluorescence staining, unseparated or low-density BM cells (5 x 10^6 cells per 50 μL) were incubated with

ER-MP12^{bio}, washed in PBS containing 0.01% NaN_3 and 0.5% BSA, and subsequently incubated with ER-MP20^{FITC} and SAV-PE. Washing buffer was used for all dilutions. For three-color analysis the cells were first incubated with hybridoma supernatant (mAbs see above). This was followed by an incubation with Gαra-PE containing 2% normal mouse serum to avoid non-specific binding. The cells were subsequently washed with buffer containing 2% normal rat serum to block any free binding sites, and incubated with ER-MP12^{bio} followed by ER-MP20^{FITC} and SAV-TRI. For *in vivo* experiments low-density BM cells were first depleted of ER-MP20-positive cells by MACS and then stained with ER-MP12^{bio} and SAV-PE for further sorting. All incubations were performed for 30 min at 0 °C. Cells were analyzed using a FACScan flowcytometer (Becton Dickinson, Mountain View, CA) or sorted on either a FACS 440 or a FACS Vantage (Becton Dickinson) at a rate of 2,500 cells per second using a single argon ion laser tuned at 488 nm (100 mW). Viable cells were counted using a Bürker hemocytometer.

Colony assay. The number of *in vitro* clonable progenitors (colony-forming units in culture, CFU-C) was determined by culturing either 1 to 2 x 10^4 unseparated BM cells or varying numbers of sorted cells in 1 mL cultures. The culture medium consisted of the α-modification of Dulbecco's modified Eagle's medium (DMEM; Gibco) at an osmolarity of 280 mOsmol/kg. The medium was supplemented with 1.2% (wt/vol) methylcellulose (Methocel MC, Fluka Chemie, Buchs, Switzerland), 20% horse serum (Gibco), 1% BSA, 80 U/mL penicillin, 80 μg/mL streptomycin, 3.2 mmol/L L-glutamine (Merck), 8 x 10^{-8} mol/L sodium selenite (Merck) and 8 x 10^{-5} mol/L β-mercaptoethanol (at final concentrations). The cultures were either stimulated by 10% (vol/vol) pokeweed mitogen-stimulated mouse spleen-conditioned medium (PWM-MSCM) and 2 U/mL recombinant human erythropoietin (Epo; Merckle, Ulm, Germany), or contained 2 U/mL Epo, 50 U/mL murine IL-3, 15 U/mL murine steel factor (SF), 50 U/mL human IL-11, and 2 ng/mL murine IL-12. The recombinant murine cytokines IL-3, SF, IL-11, and IL-12 were kindly provided by Dr. S. Neben of the Genetics Institute, Cambridge, MA. The cultures were kept at 37 °C, 5% CO_2 and 100% humidity. Colonies containing 50 cells or more were counted after 4 to 14 days of culture.

CAFC-assay. Long-term BM cultures were established in 96-well plates for limiting dilution analysis of cobblestone-area-forming cells (CAFC) as previously described [32,40]. To determine the effect of ER-MP12 on hematopoiesis *in vitro*, unseparated BM cells were tested in the CAFC assay in the presence or absence of 5 μg/mL ER-MP12 (rat IgG2a) or control mAb PH2-99 (rat-anti-E.Coli-β-galactosidase IgG2a). To this purpose BM cells were first labeled with one of the mAbs and then inoculated. Half of the medium, including the mAbs, were half changed every 3 to 4 days. CAFC frequencies were determined over a 4 week period.

Spleen colony-forming unit (CFU-S) assay. The number of CFU-S-12 in unseparated and sorted BM cells was determined by intravenous injection into 8-Gy-irradiated BALB/c recipients (8 to 12 mice per group) [41]. At day 12 the spleens were excised and fixed in Telleyesniczky's solution. The macroscopic colonies were counted.

Long-term repopulating ability (LTRA). Unseparated BM and sorted cells, derived from male BALB/c donor mice, were intravenously injected into 5-Gy-irradiated female

α-thalassemic BALB/c ($Hba^{th/+}$) mice (6 to 8 mice per group). Red blood cell chimerism was determined from 6 weeks up to 1 year after transplantation. Using the forward light-scatter as a measure of erythrocyte size, the distribution of normal-sized (donor-type) and microcytic thalassemic (recipient-type) erythrocytes was analyzed using a small drop of peripheral blood as previously described [42,43].

RESULTS

Phenotypic characterization of ER-MP12 and ER-MP20 labeled BM subsets. To study the distribution of the hematopoietic lineages, murine BM cells were sorted into six subpopulations, based on the expression of the ER-MP12 and ER-MP20 antigens in a two-color immunofluorescence analysis (Figure 4.1) [29]. Four of the

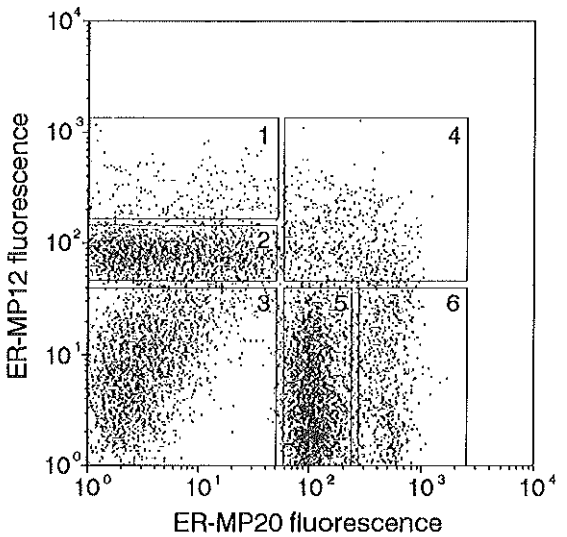

Figure 4.1 Two-color immunofluorescence analysis of mouse BM cells stained with monoclonal antibodies ER-MP12 and ER-MP20. Window 1 (ER-MP12^{hi}20^{-}) contained 2.1 ± 0.1% of the cells, window 2 (ER-MP12^{med}20^{-}) 30.0 ± 1.9%, window 3 (ER-MP12^{-}20^{-}) 28.1 ± 2.0%, window 4 (ER-MP12^{+}20^{+}) 9.2 ± 1.2%, window 5 (ER-MP12^{-}20^{med}) 25.6 ± 1.8%, and window 6 (ER-MP12^{-}20^{hi}) 5.0 ± 0.2% (average ± 1 SEM; based on 10 experiments).

subpopulations expressed one of the two antigens, at either an intermediate or a high level. The ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets contained 2% and 30% of the nucleated cells, respectively, whereas the ER-MP12^{-}20^{med} and ER-MP12^{-}20^{hi} subsets included 26% and 5%, respectively. The subset that expressed both antigens, irrespective of the level of expression, was designated ER-MP12^{+}20^{+} and contained 9% of the BM cells. The remaining population that lacked both antigens and contained 28% of the cells was denoted as ER-MP12^{-}20^{-}. The distribution of the hematopoietic subsets was calculated by taking into account the sizes of the subpopulations in unseparated BM (Table 4.1). Early myeloid cells all expressed the ER-MP20 Ag, whereas the early erythroid cells were found predominantly in the ER-MP12^{-}20^{-}, and for a smaller part in the ER-MP12^{+}20^{+} population. Although the ER-MP12^{hi}20^{-} subpopulation consisted for 93% of blast cells, of which 50% undifferentiated (data not shown), undifferentiated blasts could quantitatively be recovered from all subpopulations. Interestingly, all late erythroid cells (98%), mature granulocytes (97%), lymphocytes (96%), and the majority

of the monocytes (76%), could be recovered from the ER-MP12$^-$20$^-$, ER-MP12$^-$20^{med}, ER-MP12^{med}20$^-$, and ER-MP12$^-$20^{hi} subpopulations, respectively.

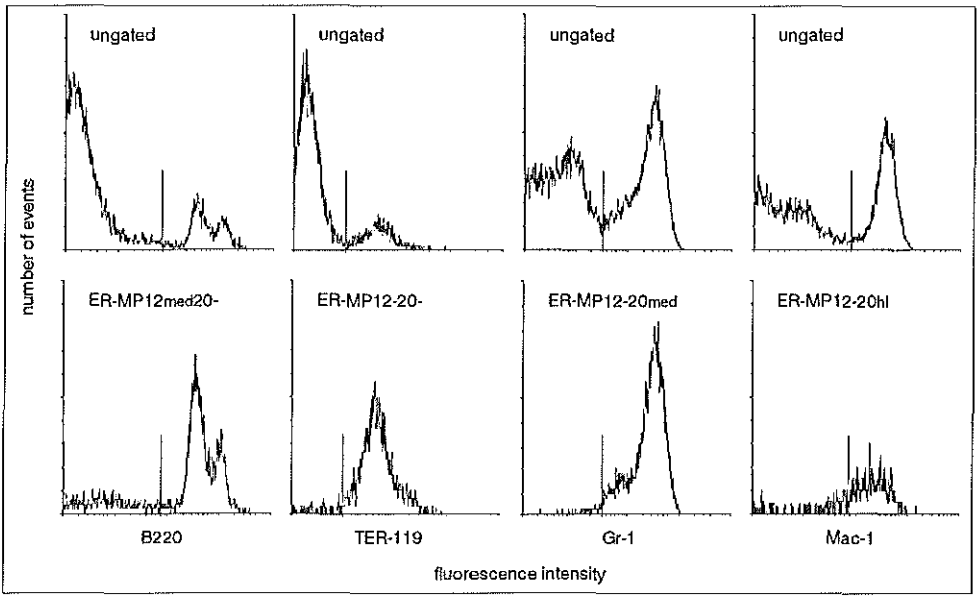

Figure 4.2 Expression of B220, TER-119, Mac-1 and Gr-1 on total BM cells (ungated), and BM cells gated for different ER-MP12/ER-MP20 subpopulations. For three color-immunofluorescence analysis, cells were first incubated with hybridoma supernatants subsequently followed by Gαra-PE, ER-MP20^{FITC}, ER-MP12^{bio} and SAV-TRI. Subpopulations were gated according to the windows in Figure 4.1. Background fluorescence, as determined with an isotype-matched control mAb, is indicated for each marker.

To verify the presence of the morphologically identified lymphocytes, granulocytes and monocytes, the subpopulations were tested in a three-color immunofluorescence analysis for the expression of B220, Mac-1, or Gr-1. Erythroblasts were labeled with TER-119 (Figure 4.2). The ER-MP12^{med}20$^-$ subset almost completely consisted of B220$^+$ lymphocytes. Also, cells expressing high levels of CD4 and CD8 were exclusively found in this subset (data not shown) [31]. All ER-MP12$^-$20$^-$ cells appeared to express the TER-119 Ag, while the ER-MP12$^-$20^{med} cells all expressed Gr-1. The majority of the cells in the ER-MP12$^-$20^{hi} subpopulation, containing a high frequency of morphologically recognizable monocytes (Table 4.1), expressed Mac-1. These data therefore confirm the morphological analyses and show that the mature hematopoietic lineages in murine BM represent distinct cell classes on the basis of their expression of the ER-MP12 and ER-MP20 antigens.

In the following, we successively studied the distribution of the ER-MP12 and ER-MP20 antigens on the different CAFC subsets, *in vivo* long-term repopulating stem cells, CFU-S-12 and *in vitro* clonable progenitors, respectively.

Table 4. 1. Distribution of hematopoietic lineages in 6 subpopulations defined on the basis of ER-MP12 and ER-MP20 Ag expression

population*	frequency in NBMC (%)	granuloid‡		erythroid		lymphoid	monocytes	blasts
		early	late	early	late			
ER-MP12^{hi}20^{-}	2.1	2.0	0	6.4	0.3	1.7	0.4	10.0
ER-MP12^{med}20^{-}	30.0	0	0	1.4	0	96.2	7.8	20.5
ER-MP12^{-}20^{hi}	5.0	21.9	0.1	0.6	0.1	0	76.3	3.2
ER-MP12^{-}20^{med}	25.6	30.6	96.8	0	0	0	5.3	8.6
ER-MP12^{+}20^{+}	9.2	45.5	2.7	28.1	1.8	1.8	10.2	33.8
ER-MP12^{-}20^{-}	28.1	0	0.4	63.5	97.8	0.3	0	23.9

*BM was sorted into 6 different subpopulations on the basis of their ER-MP12 and ER-MP20 Ag expression.
‡Sorted cells were stained with May-Grünwald/Giemsa and 400 cells per group were differentiated by microscope. Figures show the percentage recovery of the hematopoietic lineages with columns amounting to 100%. Myeloblasts and myelocytes were classified as early, bands and segmented granulocytes as late granuloid cells. Early erythroid cells included pro- and basophilic erythroblasts, late erythroid cells represent polychromatophils and normoblasts. Undifferentiated blast-like cells are denoted as blasts. Megakaryocytes are left out as their frequency was very low.

CAFC subsets differentially express the ER-MP12 Ag. Using the CAFC assay, we determined the frequency of hematopoietic precursors in the six subpopulations (Fig 1). We previously showed that CAFC frequencies determined at 10 days after inoculation (CAFC-10) correlate highly with the number of CFU-S-12, while frequencies determined after 4 weeks (CAFC-28/35) correlate highly with the long-term *in vivo* repopulating ability of a graft [22-24,33,44]. Compared with unseparated BM, the ER-MP12^{hi}20^{-}, ER-MP12^{med}20^{-}, and ER-MP12^{+}20^{+} subsets were enriched for CAFC (Figure 4.3A). The ER-MP12^{hi}20^{-} subset contained the highest frequency of early as well as late CAFC (Figure 4.3A). Although this subpopulation quantitatively included 80% of the early CAFC, it contained only 10% of the more immature CAFC-28 (Figure 4.3B). In contrast, the ER-MP12^{med}20^{-} subpopulation contained only 5% of the more

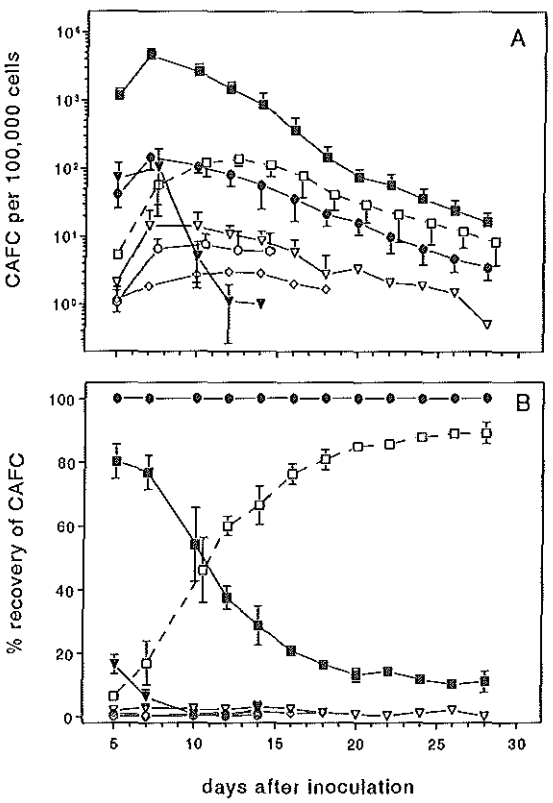

Figure 4.3 **(A):** Cobblestone-area-forming cell (CAFC) frequencies (mean ± 1 SD) of cells sorted on the basis of ER-MP12 and ER-MP20 antibodies: (●) unseparated BM cells, (■) ER-MP12^{hi}20^{-}, (□) ER-MP12^{med}20^{-}, (◇) ER-MP12^{-}20^{-}, (▼) ER-MP12^{+}20^{+}, (▽) ER-MP12^{-}20^{med}, (○) ER-MP12^{-}20^{hi} (average of 3 experiments). **(B):** Relative distribution (mean ± 95% confidence limits) of the CAFC subsets in the sorted populations (symbols as in Figure 4.3A). Total recovery in the three experiments at different time points varied for from 80% to 140%. Recoveries in individual experiments were normalized at 100% and then averaged.

mature progenitors but included nearly 90% of the CAFC-28. The ER-MP12$^+$20$^+$ subpopulation contained only transient CAFCs that disappeared within the first 10 days. Therefore, CAFC-10 (CFU-S-12) as well as the more immature CAFC-28 (LTRA cells) were found only in the subpopulations that expressed the ER-MP12 Ag but lacked the expression of ER-MP20. More specifically, most immature stem cells (CAFC-28) expressed ER-MP12 at an intermediate level, whereas upon maturation from CAFC-28 up to CAFC-5 an increasing percentage was found in the subpopulation expressing ER-MP12 at a high level.

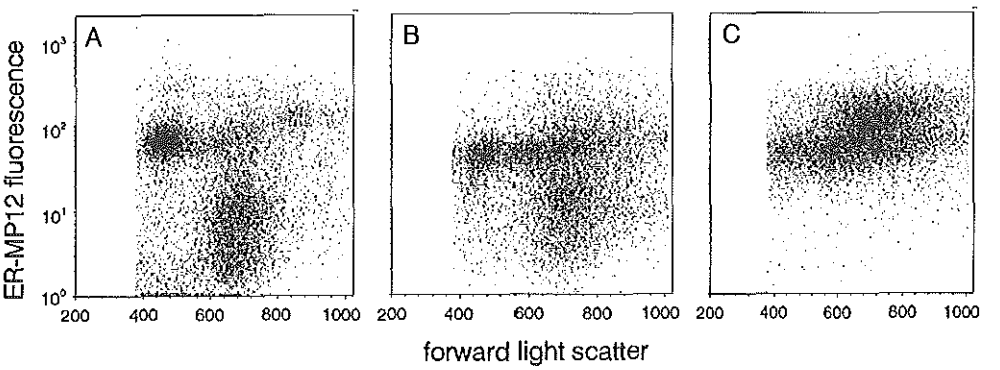

Figure 4.4 Dotplots of the forward light-scatter against the ER-MP12 fluorescence of **(A)**: unseparated BM cells, **(B)**: low-density (1.069 to 1.075 g/mL) BM cells, **(C)**: low-density BM cells depleted of ER-MP20-positive cells by MACS (i.e. ER-MP20$^-$). The ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subsets comprised 26% and 69% of the low-density cells, respectively.

CAFC subsets in low-density and in unseparated BM are equally distributed with respect to ER-MP12 Ag expression. To achieve high numbers of purified cells for combined *in vitro* and *in vivo* studies, BM had to be pre-enriched by density centrifugation. Figure 4.4 shows the forward angle light-scatter against the ER-MP12 expression in (A) unseparated BM, (B) low-density BM, and (C) MACS-depleted ER-MP20$^-$ low-density BM. The procedure effectively enriched for blast-like cells with a high and intermediate ER-MP12 expression. Furthermore, the ER-MP12$^-$20$^-$ subpopulation was almost depleted by the density cut (Figure 4.4C) as it mainly consisted of erythroblast and normoblasts (Table 4.1 and Figure 4.2). To relate the distribution of the LTRA cells in low-density BM (see next section) to the other data, sorted cells from low-density BM were also tested in the CAFC assay, in parallel with the *in vivo* experiments. Low-density BM was approximately 10.5-fold enriched for all CAFC compared with unseparated BM (data not shown). Like in unseparated BM, the low-density ER-MP12^{hi}20$^-$ subset contained the more mature precursors that gave rise to an early CA-formation, whereas 90% to 95% of the CAFC-28 could be recovered in the ER-MP12^{med}20$^-$ subpopulation (data not shown). Therefore, the distribution of the most immature CAFC subsets with respect to the expression of the ER-MP12 Ag had not changed by the pre-enrichment procedure.

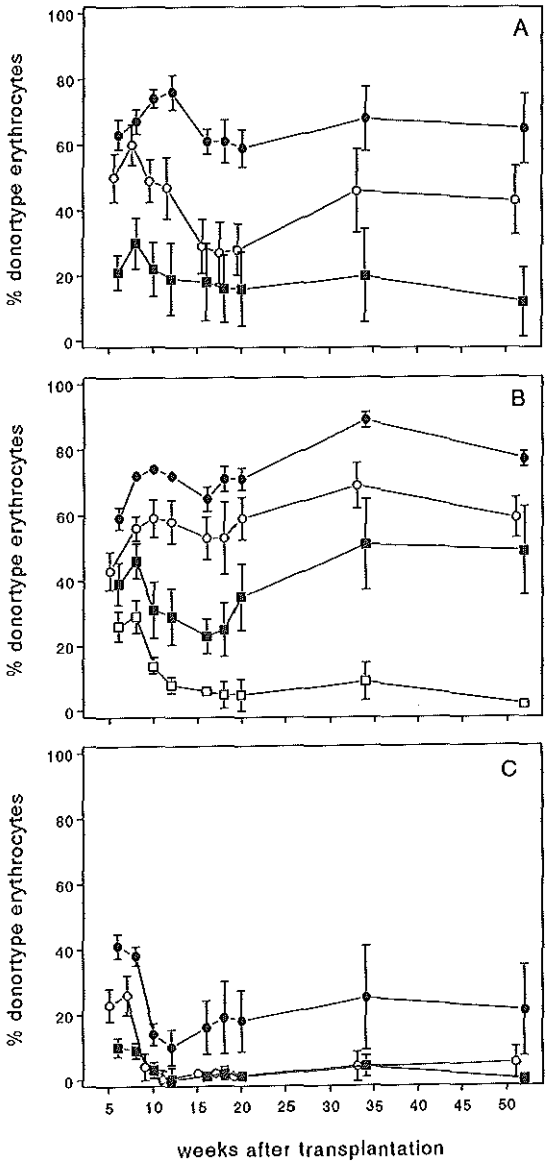

Figure 4.5 *In vivo* repopulating ability of unseparated BM cells, low-density ER-MP12^{hi}20^{-} cells, and low-density ER-MP12^{med}20^{-} cells (for dotplot see Figure 4.4C), after i.v. transplantation into 5-Gy-irradiated α-thalassemic mice. Percentage of donor-type red blood cells (mean ± 1 SEM; 6 mice per group) after injection of: **(A)** unseparated BM cells: (●) 3x10^5, (○) 1x10^5, (■) 3x10^4 cells per mouse; **(B)** low-density ER-MP12^{med}20^{-}: (●) 116,000, (○) 39,000, (■) 13,000, (□) 4,300 cells; and **(C)** low-density ER-MP^{hi}20^{-}: (●) 39,000, (○) 13,000, (■) 4,300 cells.

Majority of *in vivo* long-term repopulating stem cells express ER-MP12 intermediately. To investigate whether the above defined subpopulations contained long-term or transiently *in vivo* repopulating stem cells, the subsets were intravenously injected into sublethally irradiated α-thalassemic mice. Donor-type repopulation was defined by the percentage of normal-sized erythrocytes in the peripheral blood of the microcytic recipients as previously described [23,42]. Different numbers of unseparated BM cells (Figure 4.5A) and low-density ER-MP12^{med}20^{-} and ER-MP12^{hi}20^{-} BM cells (Figures 4.5B and C, respectively), were transplanted per mouse. Chimerism was followed up to a year after transplantation. All fractions induced an initial period (0-4 months) of transient repopulation. On average, the ER-MP12^{med}20^{-} subset induced a higher level of stable chimerism (4-12 months) when compared to the level of transient repopulation than the ER-MP12^{hi}20^{-} subset, indicating it contained more LTRA cells relative to the number of STRA cells. The frequencies of the LTRA unit responsible for 20% donor-type repopulation at one year after transplantation were estimated by

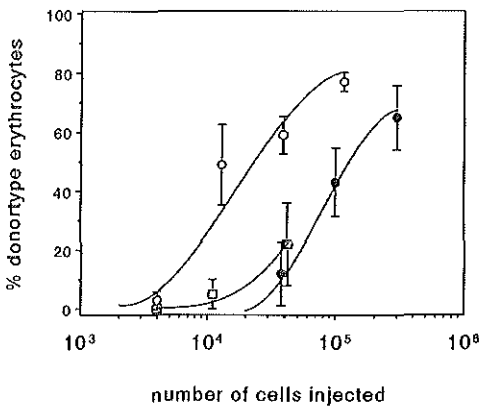

Figure 4.6 Donor-type red blood cells 52 weeks after transplantation of: (●) unseparated BM cells, (○) low-density ER-MP12^{med}20^{-}, or (□) low-density ER-MP12^{hi}20^{-} BM cells; in 5-Gy-irradiated α-thalassemic mice (6 mice per group).

extrapolation (Figure 4.6). Total BM contained 1 LTRA unit per 55,000 cells, the low-density ER-MP12^{hi}20^{-} subset 1 per 40,000, and the ER-MP12^{med}20^{-} subset 1 per 8,000 cells. The ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets were therefore 1.4-fold and 6.9-fold enriched over total BM, respectively, and thus contained 7% and 93%, respectively, of the LTRA cells in low-density BM. This result corresponds with the CAFC data in that both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets contained *in vivo* LTRA cells, and that 90% to 95% of the stem cells (CAFC-28/35 and LTRA cells) were found within the subset expressing intermediate levels of the ER-MP12 Ag.

CFU-S-12 are heterogeneous with respect to ER-MP12 Ag expression. The distribution of CAFC-10 suggested that CFU-S-12 would be present in both the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations (Figure 4.3B). To calculate the enrichment and recovery of CFU-S-12, the two subpopulations were injected into 8-Gy-irradiated animals and tested for their day-12 spleen colony-forming ability (Table 4.2). The other subpopulations contained no significant number of CAFC-10 (Figure 4.3A) and were therefore not tested for CFU-S-12. Compared with unseparated BM, the ER-MP12^{hi}20^{-} subpopulation was 11.4-fold enriched for CFU-S-12. On the basis of their recovery, it was calculated that the ER-MP12^{hi}20^{-} subset contained about 25% of the CFU-S-12, whereas 75% was found in the ER-MP12^{med}20^{-} subpopulation. Apparently, labeling and sorting of the cells did not affect their spleen colony-forming ability. Therefore, the data show that all BM CFU-S-12 can be recovered from the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets, while the majority expressed the ER-MP12 Ag at an intermediate level.

Table 4. 3. Recovery of day-7 and day-14 colony-forming units in culture (CFU-C) in ER-MP20^{-} BM cells separated on the basis of ER-MP12 Ag expression

population	frequency in NBMC*	day-7 CFU-C per 10^4 cells†	recovery (%) day-7 CFU-C	day-14 CFU-C per 10^4 cells	recovery (%) day-14 CFU-C
NBMC	100.0	15.2 ± 2.7	100.0	23.6 ± 4.2	100.0
ER-MP12^{hi}20^{-}	2.3	460.2 ± 85.9	70.7 ± 18.2	676.0 ± 55.5	66.6 ± 13.0
ER-MP12^{med}20^{-}	22.5	13.5 ± 2.2	20.0 ± 4.8	14.4 ± 6.0	13.7 ± 6.2
ER-MP12^{-}20^{-}	19.5	< 0.2	< 0.3	0.8 ± 0.4	0.7 ± 0.3

*Average frequencies in these particular experiments.
†Normal BM cells (NBMC) were separated on the basis of ER-MP12 and ER-MP20 Ag expression. Cultures contained 20% horse serum, 1% BSA and were stimulated by 10% PWM-MSCM and 2 U/mL Epo. Colony numbers from 2 experiments, 4 dishes per group, are given as the mean ± 1 SD.

Myeloid progenitors all highly express the ER-MP12 Ag. To determine the number of mature progenitors in the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets, the subpopulations were tested for day-7 and day-14 CFU-C in standard methylcellulose cultures (Table 4.3). The ER-MP12^{hi}20^{-} subset was 30-fold enriched when compared to unseparated BM, and contained 67% to 71% of the CFU-C. The ER-MP12^{med}20^{-}, on the other hand, was not enriched and contained 14% to 20% of the CFU-C. The ER-MP12^{-}20^{-} subset contained less than 1% of the CFU-C. Significant differences between day 7 and day 14, with respect to recovery and enrichment of CFU-C, were not observed.

Table 4. 2. Distribution of CFU-S-12 on the basis of ER-MP12 and ER-MP20 Ag expression

population contribution[§]	injected per mouse	CFU-S-12 per spleen*	colonies per 10^5 cells (± SD)	mean (± SEM)	enrichment factor	recovery of CFU-S-12[‡]	relative
NBMC	$5x10^4$	6.8 ± 2.8	13.5 ± 5.6				
		9.2 ± 3.2	18.4 ± 6.4				
	$4x10^4$	6.3 ± 2.5	15.8 ± 6.4	15.8 ± 0.8	1	100%	
		6.1 ± 2.2	15.2 ± 5.6				
	$2.5x10^4$	4.0 ± 1.2	16.0 ± 4.6				
ER-MP12^{hi}20$^-$	1500	3.1 ± 1.4	206.7 ± 91.3				
		3.1 ± 1.3	206.7 ± 86.0	179.6 ± 20.9	11.4 ± 1.4	27.3%	24.9%
		1.8 ± 1.9	118.5 ± 128.1				
	1250	2.3 ± 1.6	186.4 ± 128.3				
ER-MP12^{med}20$^-$	$2.5x10^4$	6.2 ± 2.4	24.8 ± 9.6				
	$2.0x10^4$	9.1 ± 2.2	45.5 ± 11.2	48.6 ± 8.9	3.1 ± 0.6	82.5%	75.1%
		11.9 ± 2.2	59.4 ± 11.0				
		12.9 ± 2.2	64.5 ± 11.2				

*Sorted or unseparated BM cells (NBMC) were injected into groups of 7 to 15 (8 Gy) irradiated recipients. Spleens were taken out and fixed in Telleyesniczky after 12 days. Data represent the mean of 4 to 5 separate experiments (± 1 SD) and were corrected for the number of endogenous colonies observed in the control irradiated groups (0.1 colony/spleen).

‡The recovery and relative distribution of CFU-S-12 in the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ populations were calculated using their average frequencies of 2.4% and 26.8%, respectively.

§The relative contribution of CFU-S-12 in the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations was calculated using their respective recoveries.

Part of the more mature progenitors, day-7 CFU-C (data not shown) and early but transient CAFC (Figure 4.3B), have been found to express intermediate or high levels of the ER-MP20 Ag. To determine the distribution of the ER-MP12 Ag on all myeloid progenitors, BM was separated on the basis of ER-MP12 Ag expression alone. The cultures were stimulated by Epo, IL-3, SF, IL-11 and IL-12; a combination of cytokines that has shown to specifically support multilineage colony formation [45,46]. On average, 80% to 90% of the day-7 and day-14 CFU-C expressed ER-MP12 at a high level (Table 4.4). To determine the distribution of specific progenitors, colonies were individually picked after 14 days of culture, were stained with May-Grünwald/Giemsa and differentiated by microscope. Individual CFU-C colonies nearly all contained cells of the monocyte-macrophage lineage (Table 4.4). Although differences between the

Table 4. 4. *In vitro* colony formation and lineage expression after sorting on the basis of ER-MP12

	NBMC[§]	ER-MP12^{hi}	ER-MP12^{med}	ER-MP12^{-}
Number and distribution of day-7 and day-14 CFU-C*				
day-7 CFU-C	16.8 ± 1.6	125.8 ± 17.2	2.4 ± 0.5	0.2 ± 0.1
day-14 CFU-C	17.8 ± 4.8	202.5 ± 24.6	7.3 ± 2.0	0.2 ± 0.1
% day-7 CFU-C		87.0 ± 15.9	11.0 ± 2.8	2.0 ± 1.4
% day-14 CFU-C		79.4 ± 13.0	19.2 ± 5.7	1.4 ± 0.7
Percentage of CFU-C-14 colonies with specific lineage expression[‖]				
granuloid	12.0	6.3	22.9	4.5
mono/maph	100.0	99.2	94.0	95.5
megakaryocytic	16.9	10.2	26.5	9.1
erythroid	14.5	10.2	25.3	9.1
mast cells	4.8	12.6	12.0	0.0
percentage of colonies with uni- or multilineage character[§]				
1 lineage	73.5	78.7	53.0	81.8
2 lineages	9.6	11.8	26.5	9.1
3 lineages	10.8	6.3	12.1	4.6
4 lineages	6.0	2.4	7.2	4.6
5 lineages	0.0	0.8	1.2	0.0

*Number of day-7 and day-14 CFU-C per 10^4 cells inoculated (mean ± 1 SD). Cultures were stimulated by Epo, IL-3, SF, IL-11 and IL-12. Colonies were counted in 6 replicate dishes with a maximum of 40 colonies/dish. The relative contribution (mean ± 1 SD) of the different CFU-C subsets was calculated on the total number of CFU-C recovered.

[‡]CFU-C colonies were individually picked at day 14. Colonies were transferred to slides, stained with May-Grünwald/Giemsa and differentiated by microscope.

[§]Number of colonies picked: NBMC, 83; ER-MP12^{hi}, 127; ER-MP12^{med}, 83; and ER-MP12^{-}, 22.

[‖]The number of lineages per CFU-C colony was determined irrespectively of the particular lineage type.

recoveries of individual lineages were small, the ER-MP12^{med} subpopulation gave rise to an overall higher percentage of colonies containing granulocytes, megakaryocytes and erythroblasts. After counting the number of lineages per colony, irrespective of the type of lineage, 47% of the colonies grown from the ER-MP12^{med} subpopulation contained more than one lineage (Table 4.4). This is in difference with the other subpopulations that contained only 18% to 21% multilineage CFU-C, which indicates that the ER-MP12^{med} subpopulation contains the more immature progenitors. In conclusion, our data show that 80% to 90% of all BM CFU-C, including both multilineage and lineage-restricted progenitors, express the ER-MP12 Ag at a high level.

Stroma-associated hematopoiesis *in vitro* could not be blocked by ER-MP12. To investigate whether the ER-MP12 Ag plays a role in hematopoiesis we studied the effect of mAb ER-MP12 on cobblestone area formation. Unseparated BM cells were labeled with ER-MP12 or with rat isotype control mAb PH2-99 (anti-E.Coli-β-galactosidase) and tested in the CAFC assay for 4 weeks, in the presence of 5 μg/mL mAb (ER-MP12 or PH2-99). Antibodies were added every 3-4 days by replacing half of the medium. Compared to control cultures, their was no significant effect on the CAFC frequencies (data not shown). A change in the size of individual cobblestone areas, that would reveal an effect on the proliferative capacity of CAFCs, was also not observed. Therefore, replating studies were not performed. In conclusion, these data do not support a functional role of the ER-MP12 Ag in hematopoiesis *in vitro*.

DISCUSSION

ER-MP12 and ER-MP20, two novel mAbs that have been raised against an immature BM macrophage precursor line [28], allowed the identification of six distinct subpopulations of murine BM cells when used in two-color immunofluorescence [29,31]. We previously reported that the ER-MP12^{hi}20^{-} BM subpopulation, as opposed to the ER-MP12^{med}20^{-} subset, contained a high frequency of precursor cells with thymus repopulating ability, upon intra-thymic injection [29]. Upon intravenous transfer, however, cells of both subsets gave rise to T, B and myeloid repopulation [31]. These observations prompted us to define the expression of the ER-MP12 and ER-MP20 antigens among other hematopoietic cells and on hematopoietic stem cell subpopulations. The present study shows that the ER-MP12 Ag was intermediately expressed by LTRA cells, of which 90% to 95% were recovered from the ER-MP12^{med}20^{-} subpopulation. With differentiation, the ER-MP12 Ag expression increased, reached a maximum in the *in vitro* clonable progenitors (CFU-C), and disappeared from most lineages during final maturation, with the exception of T and B lymphocytes (Figure 4.7). Hence, ER-MP12 recognizes a novel Ag on hematopoietic stem cells, which expression is related to the primitiveness of the stem cells within the hematopoietic stem cell hierarchy. The ER-MP20 Ag Ly-6C was expressed on only a small percentage of the more mature *in vitro* clonable progenitors [47], but was absent on primitive CAFC, CFU-S-12 and LTRA cells.

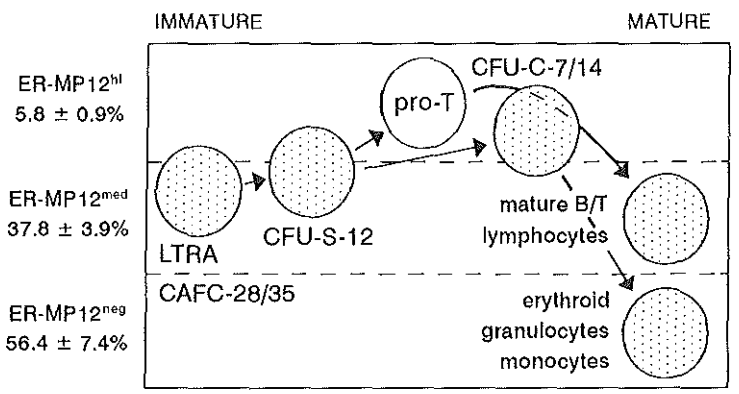

Figure 4.7 Schematic representation of the ER-MP12 Ag expression during hematopoietic differentiation. Circle areas depict the distribution (%) of several subsets with respect to the different levels of Ag expression as determined in the present study. For clarity, the overlap between different subsets is not taken into account. Frequencies are averaged from 4 experiments (± 1 SEM). Pro-T defines a subset that has the ability to repopulate the thymus 21 days after intravenous injection into sublethally irradiated animals [data from Slieker et al. 1993 [31]]. The distribution of the LTRA was determined on low-density cells.

ER-MP12 and ER-MP20 enable a separation of the four major hematopoietic lineages in the BM, as was demonstrated by FACScan analysis and differential

counting. Granulocytes and monocytes did not express the ER-MP12 Ag, but expressed the ER-MP20 Ag Ly-6C at intermediate and high levels, respectively [30,47]. We previously demonstrated that LTRA cells have a low affinity for the lectin WGA, while the large majority of CFU-S-12 have a high affinity for WGA, allowing high enrichment factors for both CFU-S-12 and LTRA cells when used for cell sorting [22-24]. The ER-MP12^{med}20^{-} subpopulation, that contains the LTRA cells and the majority of CFU-S-12, consists for about 90% of lymphocytes that do not bind WGA [22]. Monocytes, granulocytes and erythroblasts, on the other hand, show variable affinity for WGA, but do not bind ER-MP12, as was shown in the present study. Therefore, if combined with sorting on the basis of WGA affinity, ER-MP12 may enhance and simplify the purification procedure for LTRA cells, which is currently being investigated. This combination would enable a separation of LTRA cells from the large majority of CFU-S-12 and at the same time exclude all mature BM cells, without the use of an additional panel of lineage-specific mAbs [19,26,48-51], or intracellular dyes like rhodamine-123 and Hoechst 33342 [18-20].

To investigate whether the ER-MP12 Ag is functionally important for hematopoiesis, BM cells were cultured in the presence of ER-MP12 or an isotype control mAb, in the CAFC assay. The protocol and concentration of the mAbs were comparable to that used for the inhibition of hematopoiesis in LTBMC by mAbs against c-*kit* and Pgp-1/CD44 [52,53]. However, no effect of ER-MP12 on the CAFC frequencies was observed. These data indicate that ER-MP12 does either not interfere with hematopoiesis in LTBMC, or does not block a functional epitope on the ER-MP12 Ag. Although not conclusive, these data do not support a functional role of the ER-MP12 Ag in the regulation of stroma-dependent hematopoiesis *in vitro*.

We previously showed that both the ER-MP12 and ER-MP20 antigens were expressed by mice of different MHC haplotypes (H-2^{b}, H-2^{d}, H-2^{k}, H-2^{s}, H-2$^{b/q}$) with only slight variations in the distribution of the nucleated cells among the six BM subpopulations [31]. Which contrasts with the expression of the Ly-6A/E (Sca-1) and Thy-1 antigens that are haplotype restricted [54]. In the present study no differences were found in the distribution of CAFC subsets, CFU-S-12 and CFU-C between C57Bl/6-Ly-5.1 (H-2^{b}) and BALB/c (H-2^{d}) mice, with respect to ER-MP12 and ER-MP20 Ag expression. Therefore, mAbs ER-MP12 and ER-MP20 may be applicable for sorting using a wide range of haplotype mice.

Recently, a common lymphoid and dendritic cell precursor population has been identified in the adult mouse thymus by the phenotype Sca-2^{+} Thy-1.1^{lo} Sca-1^{+} CD4^{lo} (the "low-CD4" precursor), that did not have spleen colony-forming capacity upon i.v. infusion [55-57]. Upon intrathymic transfer, however, this precursor took less time to generate CD4^{+}CD8^{+} thymocytes than do Sca-2^{-} Thy-1.1^{lo} Sca-1^{+} Lin^{-} BM cells [58], which are highly enriched for CFU-S-12 [59]. This observation suggests that the average CFU-S is more primitive than the thymic and BM [60] pre-T cell, which is

supported by the recent observation that individual spleen colonies were able to give rise to T and B lymphocytes upon i.v. and intrathymic transfer [61]. Such a differentiation sequence would be consistent with the observed increase in ER-MP12 Ag expression as was found in the present study (Figure 4.7).

Phenotypically, LTRA cells have been identified by a very low expression of the lineage markers B220, TER-119, CD4, and Gr-1 [25,26,62-64], a low expression of Thy-1.1, and a low to negative expression of Thy-1.2 [25,48,49,54,65]. In addition, they were positively identified by the expression of a high level of *c-kit* [26,50-52,66,67], and a high level of the major histocompatibility class 1 Ag H-2K [25,68]. However, all these markers were indiscriminately expressed by the different subsets of the hematopoietic stem cell compartment. Two antigens that could partly distinguish primitive from the more mature hematopoietic stem cell subsets were identified by mAbs Sca-1 (Ly-6A/E) [48] and Fall-3 [69], which were expressed by 7-10% and 15-30% of the BM cells, respectively (Table 4.5). The Sca-1$^+$ cells contained all LTRA cells and about half of the CFU-S-12, while the Sca-1$^-$ cells contained most of the committed myelo-erythroid progenitors [48,49,59,70,71].

Table 4. 5. Comparison of the distribution of hematopoietic stem cell and progenitor cell subsets with respect to the expression of the ER-MP12 Ag, Ly-6A/E (Sca-1) and Fall-3.

population	%BMC	%BMC Lin$^-$	%pre-T*	%pre-B‡	%CFU-C§	%CFU-S-12	%LTRA‖
Sca-1$^+$	5-6	0.2	50-80¶	n.d.	10¶ (65)	~50¶	~100¶
Sca-1$^-$	94-95	n.d.	0¶	n.d.	90¶ (35)	low¶	0¶
Fall-3$^+$	15-30	10-25	n.d.	7	48	65	most if not all
Fall-3$^-$	70-85	n.d.	n.d.	93	52	35	almost none
ER-MP12^{hi}	2-4	1-2	majority	n.d.	80-90	25	5-10
ER-MP12^{med}	30-35	2-3	minority	n.d.	10-20	75	90-95
ER-MP12$^-$	61-68	1-2	none	n.d.	1-2	0	0

Figures on the expression and distribution of Sca-1 are from multiple references [48,49,54,59,70,71]. The distribution of Fall-3 is described in Müller-Sieburg (1991) [69], while the ER-MP12 data come from Slieker *et al.* (1993) [29], and were described in the present study.

* Determined by intrathymic transfer into sublethally irradiated animals.

‡ Determined at 2 weeks in Whitlock-Witte cultures.

§ Sca-1: distribution of day-7 and day-14 CFU-C stimulated with IL-3. Between brackets the distribution after stimulation with IL-3, SF, IL-1 and IL-6. Fall-3: distribution of day-10 CFU-C stimulated by IL-3; ER-MP12: day-4 through day-14 CFU-C stimulated by IL-3, SF, IL-11 and IL-12.

‖ Frequency analysis by CAFC (day-28/35) *in vitro*, and LTRA (>5 months) *in vivo*.

¶ Data refer to the (Thy-1^{lo} Lin$^-$) Sca-1$^+$ and (Thy-1^{lo} Lin$^-$) Sca-1$^-$ subsets of BM.

Abbreviation: nd, not determined or unknown.

Similarly, Fall-3 was expressed by the majority of LTRA cells and by 65% of the CFU-S-12, while the Fall-3$^-$ subset contained 52% of the *in vitro* clonable (IL-3 responsive) progenitors [69]. In addition, the Fall-3$^-$ subset included 93% of the B cell precursors, as was tested in 2 week Whitlock-Witte cultures. However, Fall-3 did not allow the identification of discretely stained subpopulations which might have been the reason that some radioprotective and long-term repopulating ability have been reported in the Fall$^-$ subset [69]. The present data show that the distribution of the ER-MP12 Ag partly overlaps with that of Fall-3 and is comparable with the distribution of Sca-1 (Table 4.5). The expression of the ER-MP12 Ag and Sca-1 differ in that the Lin$^-$ Sca-1$^+$ subset contained only 0.2% of all BM cells including 50% of the CFU-S-12, while the Lin$^-$ ER-MP12^{med} subset comprised 2-3% of the BM cells, including 75% of the CFU-S-12. FACScan analysis showed that ER-MP12 was heterogeneously expressed on Sca-1$^+$ BM cells and that 30% of both the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells expressed Sca-1 [31], suggesting that ER-MP12 could be used to identify subsets within the Thy-1^{lo} Lin$^-$ Sca-1$^+$ subpopulation, which remains to be tested.

In conclusion, the present study identifies the ER-MP12 Ag as a novel positive marker on adult mouse BM hematopoietic stem cells. Like the previously defined stem cell antigens Fall-3 and Sca-1, mAb ER-MP12 can be used for a further dissection of the hematopoietic stem cell compartment and identification of cell surface molecules on functionally different hematopoietic stem cell subsets.

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CHAPTER 5

ER-MP12 ANTIGEN, A NEW CELL SURFACE MARKER ON MOUSE BONE MARROW CELLS WITH THYMUS-REPOPULATING ABILITY:

II. THYMUS-HOMING ABILITY AND PHENOTYPIC CHARACTERIZATION OF ER-MP12-POSITIVE BONE MARROW CELLS

Walentina A.T. Slieker[1], Johannes C.M. van der Loo[2], Marella F.T.R. de Rijk-de Bruijn[1], Dale I. Godfrey[3], Pieter J.M. Leenen[1], and Willem van Ewijk[1]

Departments of Immunology[1], and Hematology[2], Erasmus University, Rotterdam, The Netherlands, and DNAX Research Institute[3], Palo Alto, CA.

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ABSTRACT

Previously, we have shown that six distinct subsets of bone marrow (BM) cells can by identified using the mAbs ER-MP12 and ER-MP20 in two-color immunofluorescence analysis. Upon *intrathymic* transfer into sublethally irradiated mice, thymus-repopulating ability was restricted to ER-MP20$^-$ BM cells expressing either high or intermediate levels of the ER-MP12 antigen (1-2% and ~30% of BM nucleated cells respectively). The highest frequency of thymus-repopulating cells was found in the minor subset of ER-MP12^{hi}20$^-$ BM cells. In the present study, we demonstrate that upon *intravenous* transfer, thymus-homing and -repopulating BM cells are exclusively confined to the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM subpopulations, the highest frequency being detected among ER-MP12^{hi}20$^-$ BM cells. Analysis of the peripheral blood leukocytes of reconstituted mice showed that not only progenitors of T cells, but also progenitor cells of the B cell lineage as well as the myeloid lineage were present within both subsets. Three-color flow cytometric analysis revealed that ER-MP12^{hi}20$^-$ BM cells in particular were phenotypically heterogeneous with respect to the expression of the cell surface markers Thy-1, Sca-1, CD44, B220, and *c-kit*. In conclusion, our data demonstrate that ER-MP12 positively identifies BM cells with the ability to home to and repopulate the thymus. The phenotypic heterogeneity displayed by the ER-MP12^{hi}20$^-$ BM subset, containing the highest frequency of thymus-homing and -repopulating cells, provides a basis for further separation of prothymocyte activity from other hematopoietic activities in the bone marrow of the mouse.

INTRODUCTION

The thymus is the major site for T cell development. In this organ, complex and poorly understood selection processes, likely directed by an intricate network of heterogeneous stromal cell populations, shape the TCR specificity repertoire of the maturing thymocytes [1-6]. The mature, selected thymocytes descend from a minute population of intrathymic precursor cells which undergo extensive proliferation and differentiation in the subcapsular zone of the thymic cortex [1,7,8]. In adult life this intrathymic pool of T cell precursors is maintained by bone marrow (BM) -derived progenitor cells (prothymocytes) [9-11] which seed the thymus continuously at a low rate [12]. So far, the characterization of prothymocytes is being hampered by a low frequency of these cells in the BM, the absence of distinguishing morphological features, and, above all, the paucity of mAbs detecting cell surface antigens specific for T cell progenitors. Hence, the phenotype of thymus-repopulating cells is still poorly characterized. It is not clear as yet whether these progenitor cells are either multipotent stem cells, lymphoid lineage-restricted progenitor cells or progenitor cells exclusively committed to the T cell lineage.

In a previous paper [13] we showed that six distinct subpopulations of BM cells could be identified using the recently developed mAbs ER-MP12 and ER-MP20 [14] in two-color immunofluorescence analysis (Figure 4.1, *Chapter 4*). As assessed by *intrathymic* transfer thymus-repopulating ability appeared to be restricted to ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells. The highest frequency of thymus-repopulating cells was found in the subpopulation of ER-MP12^{hi}20^{-} BM cells.

In the present study we transferred the six BM subpopulations isolated on the basis of different levels of ER-MP12 and ER-MP20 antigen expression, *intravenously* into sublethally irradiated Ly-5/Thy-1 congenic mice in order to determine whether the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subsets have the capacity to home to the thymus *in vivo*. Our data indicate that BM cells which express the ER-MP12 antigen but not the ER-MP20 antigen indeed meet these physiological criteria of T cell precursors. The highest frequency of thymus-homing and -repopulating cells was detected in the ER-MP12^{hi}20^{-} BM subpopulation. Furthermore, phenotypic analysis of peripheral blood leukocytes of reconstituted mice revealed that not only thymus-repopulating cells, but also progenitor cells of the B cell lineage and the myeloid lineage were present among ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells. Finally, three-color flow cytometric analysis revealed that ER-MP12^{hi}20^{-} BM cells particularly were heterogeneous with respect to the expression of the cell surface antigens Thy-1, Sca-1, CD44, B220 and *c-kit*, indicating that this subpopulation can be further divided into smaller subsets. This phenotypic heterogeneity provides a basis for further attempts to separate prothymocytes from progenitor cells of other hematopoietic lineages.

MATERIALS AND METHODS

Mice. C57BL/6-Ly-5.1-Pep^{3b} (Thy-1.2, Ly-5.1) mice (kindly provided by Dr I. L. Weissman, Stanford University, Stanford, CA, USA), C57BL/Ka Bl-1 (Thy-1.1, Ly-5.2), BCBA(F1) (CBA/Rij x C57BL/Ka), BALB/c, B10.D2/n, BALB.k, and SJL/J mice were cesarean derived, foster reared, raised and maintained under clean conventional conditions with free access to food and water (acidified to pH 2.8) in the mouse facilities of the Department of Immunology.

Monoclonal antibodies and fluorescent reagents. The mAbs used in the present study are listed in Table 5.1. (**references** [14-23]) ACK2 (anti-*c-kit*) and A20-1.7 (anti-Ly5.1) were kindly provided by Dr S. Nishikawa, Kumamoto University Medical School, Kumamoto, Japan and Dr S. Kimura, Sloan-Kettering Cancer Center, New York, NY, respectively. The mAbs were used either (partially) purified, conjugated to biotin or FITC (Sigma, St. Louis, MO) by standard procedures [24] or as culture supernatant. In some instances anti-Thy-1.2 mAb conjugated to 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS, Boehringer Mannheim, Germany) according to the manufacturer's procedure was used. Goat anti-rat Ig absorbed with mouse Ig and conjugated to phycoerythrin (GαRa-PE) (Caltag Laboratories, San Francisco, CA), rabbit anti-rat(Fab)$_2$ fragments coupled to FITC (RαRa-FITC) (Cappel, Organon Teknika, Turnhout, Belgium), streptavidin-phycoerythrin (SAV-PE) (Caltag Laboratories), and streptavidin-TRICOLOR (SAV-TRI) (Caltag Laboratories) were used as second stage fluorescent reagents.

Preparation of cell suspensions. Suspensions of BM cells and thymuses were prepared as described [13] using Dutton's balanced salt solution (GIBCO, Breda, The Netherlands) supplemented with 5% FCS (Dutton-FCS). Peripheral blood was obtained by heart puncture and collected in tubes containing heparin-coated glass beads. Erythrocytes were depleted by hypotonic lysis using a 17 mmol/L Tris buffer (pH 7.2) supplemented with 0.144 mol/L ammonium chloride (4 volumes buffer per 1 volume blood). After 10 min at 4 °C, the cells were washed 3 times with PBS.

Immunofluorescence staining. Two-color immunofluorescence staining using conjugated mAb was performed as described before [13]. PBS supplemented with 0.5% BSA and 20 mmol/L NaN_3 (PBS-BSA-NaN_3) was used for washing as well as diluting the reagents to optimal concentrations. When fluorescein-conjugated second stage anti-rat Ig were used in two or three-color stainings the cells were first incubated with hybridoma culture supernatant, washed, and subsequently incubated with RαRa-FITC (two-color stainings) or GαRa-PE (three-color stainings) supplemented with 2% normal mouse serum to avoid non-specific binding. After 2 washes the cells were incubated and washed in the presence of 2% normal rat serum to block any free anti-rat Ig binding sites of the conjugate. Next the cells were incubated with biotin-conjugated mAb, washed, and finally incubated with SAV-PE (two-color stainings) or SAV-TRI and FITC-conjugated mAb (three-color stainings). For cell sorting experiments BM cells were stained as previously described [13].

Flow cytometric analysis and cell sorting. Cell surface fluorescence was analyzed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). Erythrocytes and dead

cells were excluded from analysis by electronic gating on the basis of light scatter characteristics. Cell sorting was performed using a FACS 440 (Becton-Dickinson) as described previously [13].

Table 5.1 Monoclonal antibodies

mAb	Antigen	Reference
ER-MP12	ER-MP12 Ag	14
ER-MP20	ER-MP20 Ag	14
59-AD2.2	Thy-1	15
H129.19	CD4	16
53-6.72	CD8	15
KT3	CD3	17
RA3-6B2	B220	18
RB6-8C5	Gr-1	19
M1/70	Mac-1	20
IM7.8.1	CD44	21
ACK2	*c-kit*	22
E13 161-7	Sca-1	23
30H12	Thy-1.2	15
A20-1.7	Ly-5.1	Dr S. Kimura

Cell transfer and analysis of repopulation. C57Bl/Ka Bl-1 recipient mice (aged 10-15 weeks) were exposed to 7.5 Gy (unless otherwise stated) of γ irradiation using two opposing ^{137}Cs sources (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) at a dose rate of 1.01 Gy/min. Two hours after irradiation different numbers of unseparated or sorted congenic BM cells were injected i.v. into mice via their tail veins. Three weeks after i.v. transfer the recipient mice were killed. Thymocytes were stained with directly conjugated anti-Thy-1.2 mAb to detect donor-derived thymocytes. In one experiment recipient mice were irradiated 8.0 Gy and reconstituted; after 4 weeks they were killed and their peripheral blood was analyzed for the presence of donor-derived T and B lymphocytes and myeloid cells. Peripheral blood leukocytes were dually labeled with either anti-B220 (B cells), anti-CD3 (T cells), or anti-Mac-1 (myeloid cells) (followed by RαRa-FITC as a second stage reagent) and donor-specific biotinylated anti-Ly-5.1 followed by SAV-PE. Cell surface fluorescence was analyzed on a FACScan flow cytometer (Becton-Dickinson). Cell suspensions with $\geq$ 1% donor-derived cells in thymus or peripheral blood were scored as positive for donor-type repopulation.

RESULTS

Thymus-homing and -repopulating ability of BM subpopulations sorted on the basis of a different expression of the ER-MP12 and ER-MP20 antigens. As has previously been shown [13], BM cells can be separated into six subpopulations on the basis of difference in ER-MP12 and ER-MP20 antigen expression (Figure 4.1, *Chapter 4*). Using the *intrathymic* transfer assay thymus-repopulating ability appeared to be exclusively confined to ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells, with the highest frequency of thymus-repopulating cells being found in the former subset (constituting 1-2% of total nucleated BM cells). In the present study we assessed the thymus-homing ability of the six BM subpopulations after *intravenous* transfer in order to investigate whether functional prothymocyte activity is confined to the ER-MP12^{hi}20^{-} and ER-12^{med}20^{-} subsets or maybe one of these. The six BM subpopulations, each characterized by a different expression of the ER-MP12 and ER-MP20 antigens, were isolated by fluorescence-activated cell sorting and transferred i.v. into sublethally irradiated Ly-5/Thy-1 congenic mice. Twenty-one days after cell transfer the thymi of the recipient mice were analyzed for the presence of donor-derived (i.e. Thy-1.2^{+}) thymocytes (Table 5.2). As a control the thymus-repopulating ability of unseparated BM cells was determined. In nine out of 39 mice (23%) reconstituted with total BM cells donor-derived thymocytes were detected after i.v. injection of 25,000 cells. In recipient mice injected i.v. with 25,000 separated BM cells thymus-homing and -repopulating cells appeared to be restricted to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations. In experiments 3 and 4, thymus-repopulating cells were only detected in the BM subpopulation with a high level of ER-MP12 antigen expression, while in the second experiment, only a low percentage (3.5% ± 1.6%) of donor-derived thymocytes was detected in eight out of 15 mice reconstituted with ER-MP12^{med}20^{-} BM cells. Re-analysis of the sorted BM fractions revealed the same degree of purity (>96%) for the ER-MP12^{med}20^{-} subset in each experiment (data not shown), indicating that the differences between the individual experiments can not be explained by the presence of contaminating ER-MP12^{hi}20^{-} cells.

Results were more clear-cut when only 5000 cells were injected i.v. Under these conditions thymus-repopulating ability was exclusively confined to ER-MP12^{hi}20^{-} BM cells (Table 5.2). These data demonstrate that: (i) BM cells expressing the ER-MP12 antigen but not the ER-MP20 antigen are able to home to and repopulate the thymus of sublethally irradiated mice after i.v. transfer; (ii) the ER-MP12^{hi}20^{-} BM subset contains the highest frequency of thymus-homing and -repopulating BM cells.

Multi-lineage reconstitution potential of ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells. To assess the developmental potential of the subsets of ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells more extensively we i.v. transferred sorted ER-MP12^{hi}20^{-} or ER-MP12^{med}20^{-} BM cells, or unseparated BM cells into 8 Gy irradiated Ly-5/Thy-1 congenic recipient mice. Four weeks later peripheral blood nucleated cells were

Table 5.2 Thymus-homing and -repopulating ability of sorted BM cell subpopulations

Cell source	% of BM cells[a]	Number of cells transferred	Experiment number	Positive mice/ total mice[b]	% donor-derived thymocytes[c]
total BM		25×10^3		9/39	29.6 ± 7.8
		5×10^3		0/10	
ER-MP12^{med}20$^-$	30.7 ± 4.9	25×10^3	1	4/10	45.3 ± 6.1
			2	8/15	3.5 ± 1.6
			3	0/9	
			4	0/14	
		5×10^3	3	0/10	
			4	0/10	
ER-MP12^{hi}20$^-$	2.1 ± 0.4	25×10^3	1	5/7	24.0 ± 10.2
			2	5/5	44.5 ± 15.7
			3	5/6	36.2 ± 11.6
		5×10^3	3	4/6	25.4 ± 11.5
			4	10/15	33.2 ± 8.3
ER-MP12$^+$20$^+$	9.2 ± 3.1	25×10^3		1/9	2.4
ER-MP12$^-$20^{med}	26.8 ± 6.6	25×10^3		0/18	
ER-MP12$^-$20^{hi}	4.8 ± 1.7	25×10^3		0/14	
ER-MP12$^-$20$^-$	28.6 ± 5.6	25×10^3		0/14	

C57BL/Ka BI-1 (Ly-5.2, Thy-1.1) recipient mice were irradiated 7.5 Gy and injected i.v. with donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Thymus reconstitution was analyzed 21 days after cell transfer.

[a] The relative distribution of the BM subpopulation among nucleated BM cells (mean percentage ± SEM of 14 experiments).

[b] Thymus suspensions with ≥ 1% donor-derived thymocytes were scored as positive.

[c] The mean percentage of donor-derived (Thy-1.2$^+$) cells ± SEM was calculated from 4-10 mice.

Table 5.3 Thymus and peripheral blood chimerism after i.v. reconstitution with sorted ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM subpopulations

			peripheral blood			
Cell source	Cell number (n)[a]	% donor-derived thymocytes (Thy-1.2^{+})[b]	% donor-derived cells (Ly-5.1^{+})[c]	% T cells (CD3^{+}) of donor origin (Ly-5.1^{+})[d]	% B cells (B220^{+}) of donor origin (Ly-5.1^{+})[d]	% myeloid cells (Mac-1^{+}) of donor origin (Ly-5.1^{+})[d]
ER-MP12^{hi}20^{-}	10 x 10^{3} (7)	89.5 ± 1.9	66.5 ± 6.4	16.4 ± 3.3	84.9 ± 3.0	83.6 ± 8.3
	5 x 10^{3} (7)	39.9 ± 12.0	31.0 ± 8.0	6.2 ± 1.8	76.5 ± 3.3	44.0 ± 15.3
ER-MP12^{med}20^{-}	25 x 10^{3} (9)	72.3 ± 6.4	51.5 ± 6.8	5.9 ± 0.8	74.4 ± 3.2	74.1 ± 6.4
	10 x 10^{3} (6)	61.8 ± 10.3	34.7 ± 10.5	5.3 ± 1.7	52.7 ± 9.2	55.9 ± 13.7
	5 x 10^{3} (4)	24.9 ± 13.8	13.5 ± 4.8	1.4 ± 0.4	42.5 ± 2.6	23.0 ± 12.3
total BM	25 x 10^{3} (6)	49.9 ± 15.7	23.1 ± 6.8	4.1 ± 1.6	48.6 ± 9.4	33.5 ± 10.4
	10 x 10^{3} (2)	35.9	6.7	1.2	23.0	8.6

C57BL/Ka Bl-1 (Ly-5.2, Thy-1.1) recipient mice were irradiated 8.0 Gy and reconstituted intravenously with donor cells of C57BL/6-Ly-5.1-Pep^{3b} (Ly-5.1, Thy-1.2) mice. Thymus and peripheral blood chimerism was analyzed 28 days after cell transfer.

[a]n: Number of mice analyzed.

[b]The average percentage ± SEM of donor-derived thymocytes (Thy-1.2^{+}).

[c]The average percentage ± SEM of donor-derived (Ly-5.1^{+}) peripheral blood nucleated cells.

[d]The average percentage ± SEM of either peripheral T cells (CD3^{+}), B cells (B220^{+}), and myeloid cells (Mac-1^{+}) which were of donor origin (Ly-5.1^{+}).

analyzed for the presence of donor-derived (i.e. Ly-5.1$^+$) cells. In each sample the percentage of T lymphocytes (CD3$^+$ cells), B lymphocytes (B220$^+$ cells), and myeloid (Mac-1$^+$) cells of donor origin was determined. These results are shown in Table 5.3. Donor-derived cells were detected in all recipient mice. The average percentage of Ly-5.1$^+$ peripheral blood nucleated cells appeared to be maximal after i.v. transfer of ER-MP12^{hi}20$^-$ BM cells (e.g. 31.0% versus 13.5% after transfer of 5000 ER-MP12^{med}20$^-$ cells). Similarly, the highest percentage of donor-derived T lymphocytes in peripheral blood was detected after transfer of ER-MP12^{hi}20$^-$ BM cells (e.g. 6.2% versus 1.4% after transfer of 5000 ER-MP12^{med}20$^-$ cells).

At this time point (i.e. 28 days after i.v. transfer) the percentage of donor-derived peripheral T cells was still low. Yet high percentages of donor-derived (Thy-1.2$^+$) thymocytes were detected after transfer of ER-MP12^{hi}20$^-$ cells as well as ER-MP12^{med}20$^-$ cells (Table 5.3). The considerable percentage of donor-derived thymocytes in mice reconstituted with 5000 ER-MP12^{med}20$^-$ cells at 28 days is in sharp contrast with the absence of donor-derived thymocytes at 21 days after transfer of these cells (Table 5.2). This apparent discrepancy may be explained by the presence of more primitive hematopoietic stem cells within the ER-MP12^{med}20$^-$ subset (*Chapter 4* [25]). These cells most likely need additional maturational events in the bone marrow and may therefore need more time to mature into thymocytes (i.e. 28 days instead of 21 days). Donor-derived cells belonging to the B cell lineage and myeloid lineage were also found after i.v. transfer of either subset (Table 5.3). The highest percentage of both donor-derived B lymphocytes and myeloid cells was found after transfer of ER-MP12^{hi}20$^-$ BM cells. Taken together our results show that both ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM subsets contain not only T cell progenitors but also progenitor cells capable of differentiating into B lymphocytes and myeloid cells.

Cell surface phenotype of ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells. The cell surface phenotype of both BM subsets was examined using three-color immunofluorescence analysis in order to investigate to what extent ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells are phenotypically heterogeneous and resemble previously described BM subpopulations enriched for hematopoietic stem cells and progenitor activity [26-28]. The staining profiles of ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM subpopulations with a panel of mAb against different cell surface antigens is shown in Figures 5.1A and B, respectively. For comparison the profiles of ungated nucleated BM cells are shown (Figure 5.1C). We analyzed the expression of the mature lineage markers Gr-1, Mac-1, CD4, CD8 and B220 by ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM cells in order to compare the current progenitor fractions with those isolated by others using depletion of BM cells for the mentioned mature markers [26,27,29,30]. Compared to unseparated BM cells (Figure 5.1C) both ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ BM subpopulations were depleted of cells that strongly expressed Gr-1 and Mac-1 (Figures 5.1A and B, respectively). Lymphoid cells expressing high levels of CD4 and CD8, most likely mature T lymphocytes, were only detected in the ER-MP12^{med}20$^-$ subset

(Figure 5.1B). In both subpopulations cells were detected which stained dimly for CD4. In this context it is noteworthy that low levels of CD4 were recently detected on pluripotent stem cells [31,32] and on the earliest subset of thymocytes detectable [7,8]. About 90% of the ER-MP12^{med}20^{-} BM cells expressed B220 (Figure 5.1B), suggesting

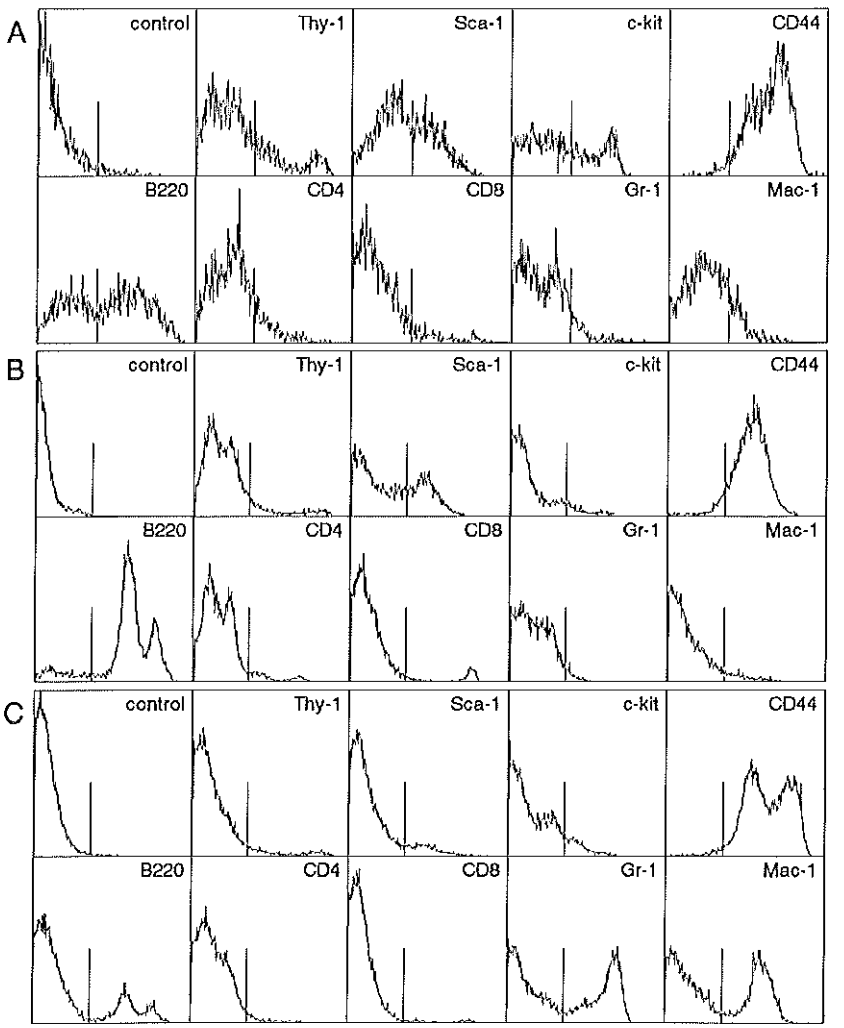

Figure 5.1 Cell surface phenotype of ER-MP12^{hi}20^{-} **(A)**, ER-MP12^{+}20^{-} **(B)** and unseparated BM cells **(C)**. BM cells were stained with mAb specific for either of the indicated Ag (second stage reagent: G*a*Ra-PE), FITC-conjugated ER-MP20, and biotinylated ER-MP12 (followed by SAV-TRI). Control: isotype-matched control mAb (IgG2a or IgG2b). Life gates were set on either ER-MP12^{hi}20^{-} (A) or ER-MP12^{+}20^{-} cells (B) and the expression of the indicated markers was analyzed. Histograms in (A), (B) and (C) were generated from 4000, 10,000, and 14,0000 events, respectively. The results of one representative experiment are shown.

that the majority of the cells in the ER-MP12^{med}20^{-} BM subset belong to the B cell lineage. In contrast, ER-MP12^{hi}20^{-} cells differed strikingly from the ER-MP12^{med}20^{-} subset in that about 50% lacked the expression of B220 (Figure 5.1A). Analysis of the forward light scatter against the fluorescence distribution revealed that the B220^{-} cells in the ER-MP12^{hi}20^{-} subset were mainly blast-like cells (Fig 2A). Interestingly the ER-MP12^{hi}20^{-} BM cells expressing the highest level of B220 antigen appeared to be large blast-like cells while ER-MP12^{hi}20^{-} cells with an intermediate level of B220 expression showed a scatter profile characteristic for lymphoid cells (Figure 5.2A) Both subsets contained cells that expressed low levels of Thy-1 as well as cells lacking detectable levels of this cell surface marker (Figures 5.1A and B). Low levels of the Thy-1 antigen have been detected on BM cells with thymus-repopulating ability and other hematopoietic progenitors and stem cells [26,33-35]. A small percentage of ER-MP12^{hi}20^{-} BM cells (2.5-7%) expressed high levels of the Thy-1 antigen (Fig 1A). Analysis of the forward light scatter against the fluorescence distribution revealed that the majority of the Thy-1^{hi} ER-MP12^{hi}20^{-} cells were large blast-like cells (Figure 5.2B), suggesting that these cells are probably not T lymphocytes. In contrast, the majority of the Thy-1^{hi} cells in the ER-MP12^{med}20^{-} BM subset (Figure 5.1B) appeared to be small lymphoid cells (data not shown), most likely representing mature T cells.

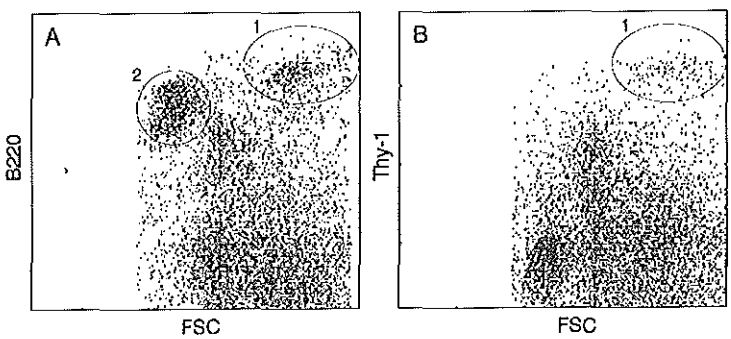

Figure 5.2 Forward light scatter versus log fluorescence distribution of ER-MP12^{hi}20^{-} BM cells stained with B220 **(A)** and Thy-1 **(B)**. BM cells were stained as described in the materials and methods section (three-color staining). Life gates were set on ER-MP12^{hi}20^{-} cells and the expression of the third marker was analyzed. [1]: blast-like cells expressing high levels of either B220 (A) or Thy-1 (B). [2]: lymphoid cells expressing B220. Dotplots were generated from 8000 events in one representative experiment.

It has been shown previously that thymus-repopulating ability is exclusively confined to BM cells expressing Sca-1 (Ly-6A/E [27]). Our data demonstrate that about30% of both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells expressed Sca-1 (Figures 5.1A and B, respectively) compared to about 10% of ungated BM cells (Figure 5.1C). The only other cells in BM that expressed this cell surface marker were found in the ER-MP12^{+}20^{+} subset (data not shown). Thus, in accordance with the thymus-

repopulating activity the expression of Sca-1 appeared to be restricted to BM cells expressing the ER-MP12 antigen.

ER-MP12^{hi}20^{-} cells differed markedly from ER-MP12^{med}20^{-} cells in the expression of *c-kit*, the receptor for stem cell factor [36,37], which has been found on hematopoietic stem cells and progenitor cells [28,38,39]. Two percent of the cells in ungated BM and in the ER-MP12^{med}20^{-} subpopulation expressed high levels of *c-kit* (Figures 5.1C and B, respectively). In contrast, 35-40% of the ER-MP12^{hi}20^{-} cells, mainly blast-like cells (data not shown), expressed this cell surface marker at high levels (Fig 1A), suggesting a high frequency of progenitor cells in this latter subpopulation.

CD44 is differentially expressed by most hematopoietic cells [40]. Multipotent stem cells and progenitor cells, including thymus-repopulating cells, express intermediate levels of this surface marker [21,27,40] while the earliest intrathymic T cell precursors express high levels [7]. Our results show that all ER-MP12^{med}20^{-}BM cells expressed intermediate levels of this antigen (Figure 5.1B). Analysis of the ER-MP12^{hi}20^{-} subpopulation revealed cells expressing intermediate levels as well as high levels (more than 50%) of CD44 (Figure 5.1A).

Table 5.4 Relative distribution of BM subsets characterized by distinct patterns of ER-MP12 and ER-MP20 antigen expression in various mouse strains

Mouse strain	MHC H-2	12^{-}20^{-}	12^{med}20^{-}	12^{hi}20^{-}	12^{-}20^{med}	12^{-}20^{hi}	12^{+}20^{+}
C57BL/6-Ly-5.1	b	26.6	26.8	1.7	29.5	5.2	10.2
C57BL/Ka BL-1	b	22.6	20.5	1.6	42.2	7.1	5.8
BCBA(F1)	b/q	29.7	16.2	1.3	40.7	5.7	6.4
BALB/c	d	22.4	30.9	1.5	36.2	4.5	4.5
BALB.k	k	28.5	21.5	1.3	40.6	4.0	4.4
SJL/J	s	26.9	32.7	1.5	27.5	5.5	6.2

BM cells were simultaneously stained with ER-MP12 and ER-MP20. The percentages of nucleated BM cells among the 6 different subpopulations were determined by FACScan analysis.

In summary, our data demonstrate that: (i) selection against ER-MP20 and for ER-MP12 antigen expression results in the depletion of BM cells expressing high levels of the myeloid lineage differentiation markers Gr-1 and Mac-1; (ii) both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} cells are heterogeneous with respect to the expression of several cell surface markers; (iii) ER-MP12^{hi}20^{-} cells are phenotypically more heterogeneous than ER-MP12^{med}20^{-} BM cells.

Expression of ER-MP12 and ER-MP20 antigens by different mouse strains. The potential usefulness of the ER-MP12 and ER-MP20 antigens for the enrichment of BM cells with thymus-repopulating ability depends, partly, upon the expression of these cell surface markers by a wide variety of mouse strains. Therefore we examined the expression of the ER-MP12 and ER-MP20 antigens by BM cells from mice of different haplotypes (Table 5.4). Our results show that both antigens are at least expressed by mice of H-2^b, H-$2^{b/q}$, H-2^d, H-2^k, and H-2^s haplotypes. The distribution of cells among the BM subsets characterized by different expression of the ER-MP12 and ER-MP20 antigens varied only slightly between the different mouse strains. Most importantly, approximately the same percentage (1.3-1.7% of total nucleated BM cells) of ER-MP$12^{hi}20^-$ BM cells were detected in all tested strains.

DISCUSSION

In our previous paper we showed that by using the mAb ER-MP12 and ER-MP20 in two-color immunofluorescence analysis six distinct subpopulations of BM cells could be identified [13]. Upon intrathymic transfer into sublethally irradiated recipient mice we found that thymus-repopulating ability was exclusively confined to BM cells that expressed either high or intermediate levels of the surface antigen ER-MP12 but lacked the expression of the surface marker ER-MP20. The highest frequency of thymus-repopulating cells was found in the minor subset of ER-MP12^{hi}20^{-} BM cells (1-2% of total nucleated BM cells). However, by means of intrathymic transfer the ability of the injected cells to home to the thymus, which is a prerequisite for prothymocytes, could not be assessed.

In the present study we transferred the six BM subpopulations i.v. into sublethally irradiated mice in order to investigate whether thymus-homing and -repopulating cells are contained within the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM subpopulations. Our results demonstrate that T cell progenitors (assayed 21 days after i.v. transfer) are indeed restricted to the ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subset of BM cells. The highest frequency of thymus-homing and -repopulating cells was detected in the ER-MP12^{hi}20^{-} subpopulation. Analysis of the peripheral blood leukocytes of mice reconstituted 28 days earlier with either ER-MP12^{hi}20^{-} or ER-MP12^{med}20^{-} cells revealed the presence of donor-derived T lymphocytes, B lymphocytes and myeloid cells, indicating that not only thymus-repopulating cells, but also progenitor cells of the B cell lineage as well as the myeloid lineage were present in both subpopulations. At this later time point of analysis (i.e. 28 days) high percentages of donor-derived thymocytes were found after i.v. transfer of either 5000 ER-MP12^{hi}20^{-} or 5000 ER-MP12^{med}20^{-} cells, whereas at 21 days donor-derived thymocytes were only detected in mice reconstituted with the ER-MP12^{hi}20^{-} subset. This apparent discrepancy is most likely explained by the presence of multipotent stem cells within the ER-MP12^{med}20^{-} subset (*Chapter 4* [25]) which need to undergo additional maturational events in the BM before acquiring the ability to home to the thymus. Therefore, these cells need more time to mature into thymocytes, resulting in the detection of donor-derived thymocytes at a later time point (i.e. 28 days after i.v. transfer). In contrast the thymus-repopulating cells in the ER-MP12^{hi}20^{-} subpopulation probably already have acquired the ability to home to the thymus, and therefore will give rise to progeny at an earlier time point (i.e. 21 day after i.v. transfer). Our results demonstrate that, although the ER-MP12^{hi}20^{-} subset of BM cells contains a high frequency of T cell progenitors, the separation of restricted prothymocytes from other hematopoietic activities will depend upon the use of additional cell surface markers.

Cell surface phenotyping revealed that both ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} BM cells were heterogeneous with respect to the expression of several cell surface markers. Both subpopulations lacked cells expressing high levels of Gr-1 and Mac-1, indicating

that ER-MP20 is extremely suitable for depleting at least mature myeloid cells in the enrichment procedure of hematopoietic progenitor cells. As the Mac-1 antigen has been detected on a subset of thymus-repopulating cells [26,27,41], it remains to be tested whether Mac-1^{lo} cells, constituting a small percentage of both ER-$MP12^{hi}20^{-}$ and ER-$MP12^{med}20^{-}$ cells, are capable of differentiating into the T cell lineage. Cells expressing high levels of CD4 were depleted of the ER-$MP12^{hi}20^{-}$ subset, indicating that mature T lymphocytes (at least $CD4^{+}CD8^{-}$ cells) are excluded by selecting for high levels of ER-MP12 antigen expression. In contrast, mature T cells lymphocytes were detected among ER-$MP12^{med}20^{-}$ cells. Cells expressing low levels of CD4 were found in both subsets. Recent reports have shown that both hematopoietic stem cells and the earliest detectable thymocytes express CD4 at a low level [7,8,31,32]. Therefore the use of anti-CD4 mAbs in BM purging protocols should be avoided, because this may lead to the depletion, rather than the enrichment of progenitors and stem cells.

Almost all of the ER-$MP12^{med}20^{-}$ BM cells expressed B220. These cells therefore most probably belong to the B cell lineage. In contrast, half of the ER-$MP12^{hi}20^{-}$ BM cells were $B220^{-}$. Surprisingly the ER-$MP12^{hi}20^{-}$ cells expressing high levels of B220 appeared to be blast-like cells. Further research is required to determine whether this $B220^{hi}$ subpopulation of ER-$MP12^{hi}20^{-}$ BM cells is restricted to B cell lineage development. On the other hand the presence of thymus-repopulating cells in this $B220^{hi}$ subset may not be excluded, because at least some T cell progenitor activity has been detected in a subset of the Thy-1^{lo} Sca-1^{+} BM cells expressing B220 and Mac-1 [41].

The majority of ER-$MP12^{hi}20^{-}$ and ER-$MP12^{med}20^{-}$ cells are Thy-1^{-}, while both subsets contain cells expressing low levels of Thy-1. Progenitor cells, including thymus-repopulating cells, and hematopoietic stem cells have been found to express low levels of Thy-1 [26,27,30]. However, it has recently been shown that only in Thy-1.1 genotype mouse strains was the stem cell activity restricted to Thy-1^{lo} BM cells, while in mouse strains expressing the Thy-1.2 allele (i.e. the common form of the Thy-1 locus, found in mice used in this study) this activity was detected in both Thy-1^{-} and Thy-1^{lo} subsets [42]. Although the thymus-repopulating ability of the Thy-1.2^{-} and Thy-1.2^{lo} cells was not assessed in that study [42], a low level of Thy-1 expression is maybe not a valid criterion for the isolation of prothymocytes from the BM of Thy-1.2 genotype mice. Interestingly, some ER-$MP12^{hi}20^{-}$ BM cells expressed high levels of Thy-1.2. These cells are most likely immature, because they have a blast-like appearance in the scatter profile. In addition, as about 50% of the NK cells express Thy-1 [43], the presence of these cells within the Thy-1^{hi} subset of ER-$MP12^{hi}20^{-}$ cells may not be excluded.

Cells expressing the Sca-1 antigen were present in both subsets. It has been shown previously that Sca-1 is expressed by thymus-repopulating cells and other hematopoietic progenitors and stem cells [27,30,41]. This cell surface marker however is not an ideal marker for the isolation of thymus-repopulating cells, because Sca-1 is constitutively

expressed only by BM cells of Ly-6^b haplotype mice [44]. In contrast, our data show that the ER-MP12 and ER-MP20 antigens are expressed on a broad range of mouse strains with only slight differences in the size of the six BM subpopulations.

Cells expressing high levels of the proto-oncogene *c-kit* were markedly enriched among ER-MP$12^{hi}20^-$ BM cells. This cell surface marker has been detected on hematopoietic stem cells and progenitor cells [28,38-40], also including the earliest intrathymic precursor cells [45]. Therefore separation of the ER-MP$12^{hi}20^-$ BM cells on the basis of *c-kit* expression may lead to a further purification of prothymocytes.

ER-MP$12^{hi}20^-$ cells expressed either high or intermediate levels of CD44 while all ER-MP$12^{med}20^-$ BM cells expressed this cell surface marker at an intermediate level. An intermediate level of CD44 has been detected on pluripotent stem cells and Thy-1^{lo} Lin^- Sca-1^+ BM cells [27,40], while the earliest intrathymic T cell precursors express high levels of CD44 [7,8]. In this context separation of ER-MP$12^{hi}20^-$ BM cells on the basis of high levels of CD44 expression may also lead to a further enrichment of thymus-repopulating cells.

Finally, cell surface markers such as Fall-3, which distinguishes pluripotent stem cells from B cell progenitors [46], or the heterogeneity of different stem cell and progenitor subsets for rhodamine 123 retention and wheat germ agglutinin affinity [47-50] (*Chapters 2 and 3 and 6*) may prove to be useful for the separation of the pro-T cell activity from other hematopoietic activities in the ER-MP$12^{hi}20^-$ subset.

In summary our data demonstrate that progenitor cells with the capacity to home to and repopulate the thymus are confined to the ER-MP$12^{hi}20^-$ and ER-MP$12^{med}20^-$ subsets of BM cells. The highest frequency of thymus-homing and -repopulating BM cells was found among ER-MP$12^{hi}20^-$ cells which heterogeneously expressed the cell surface markers Thy-1, Sca-1, CD44, B220, and *c-kit*. This phenotypic heterogeneity provides a basis for the further purification of thymus-homing and -repopulating cells from the ER-MP$12^{hi}20^-$ subpopulation.

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CHAPTER 6

USE OF ER-MP12 AS A POSITIVE MARKER FOR THE ISOLATION OF MURINE IN VITRO LONG-TERM REPOPULATING STEM CELLS

Johannes C.M. van der Loo[1], Walentina A. T. Slieker[2]
and Rob E. Ploemacher[1]

Institutes of Hematology[1] and Immunology[2], Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

Monoclonal antibody ER-MP12, which was recently described, defines an antigen on murine hematopoietic stem cells that is differentially expressed by the various subsets in the hematopoietic stem cell compartment. To test whether ER-MP12 could be an asset for further subfractionation of these subsets, we physically sorted our previously defined low-density ER-MP20$^-$ (i.e. Ly-6C$^-$) Rhodamine (Rh123)^{dull} and wheat germ agglutinin (WGA)^{dim} stem cell populations on the basis of ER-MP12 antigen expression. In addition, we determined the distribution of the ER-MP12 antigen on day-6 post-5-fluorouracil bone marrow. Long-term and transiently repopulating stem cell subsets were both identified *in vitro* using the cobblestone area forming cell (CAFC) assay. The data show that sorting on the basis of ER-MP12 improved the separation of primitive and more mature stem cell subsets in the Rh123^{dull}, but not in the WGA^{dim} subpopulation. However, the combination of sorting cells on the basis of an intermediate ER-MP12 expression and a low WGA affinity (ER-MP12^{med} WGA^{dim}) did allow an 840-fold enrichment for day-28 CAFC (*in vitro* LTRA) when compared to unseparated bone marrow. The distribution of the ER-MP12 antigen on post-5-fluorouracil bone marrow stem cells was found to be similar to that in normal bone marrow stem cells, suggesting that the level of antigen expression is not cell cycle stage dependent. In conclusion, the combination of ER-MP12 and WGA offers the advantage of a positive selection strategy for hematopoietic stem cells, allowing different stem cell subsets to be distinguished on the basis of their primitivity. Since no mature bone marrow cells are found within the WGA^{dim} ER-MP12^{med} subpopulation, the combination of ER-MP12 and WGA enables hematopoietic stem cells to be highly enriched and makes the use of a cocktail of lineage-specific antibodies redundant.

INTRODUCTION

For the purification of hematopoietic stem cells a diversity of methods have been used over the years, as was reviewed by Spangrude and by Visser and Van Bekkum [1,2]. Among the various parameters used, only few are differentially expressed in the murine stem and progenitor cell compartment, e.g. Thy-1, rhodamine-123 (Rh123) and wheat germ agglutinin (WGA). Thy-1 is highly expressed on *in vitro* clonable progenitors, like high proliferative potential colony-forming cells (HPP-CFC), erythroid burst-forming units (BFU-E) and CFU-C, whereas expression is lost upon maturation [3-7]. Cells with long-term *in vivo* repopulating ability (LTRA) express low levels of Thy-1.1, or low to negative levels of Thy-1.2, depending on the allelic variant [8-13]. Rh123, on the other hand, is a supravital fluorescent dye that preferentially binds to the mitochondrial membrane of living cells [14,15]. Primitive hematopoietic cells in both human and mouse are characterized by a low Rh123 retention [16-24], which could be related directly to a high expression of the multidrug resistance P-glycoprotein [25,26]. The third parameter, WGA, has been routinely used for the positive selection of hematopoietic stem cells and progenitors [27-30]. We have previously shown that cells with marrow repopulating ability (MRA) and LTRA could be separated from the large majority of day-12 spleen colony-forming units (CFU-S-12) on the basis of differences in their WGA affinity [31-33]. A powerful pre-enrichment step, when combined with sorting on the basis of WGA affinity, is the *in vivo* treatment of mice with 5-fluorouracil (5FU) [32]. As 5FU is selectively cytotoxic for cycling cells it depletes most of the progenitors and maturing cells but spares the more primitive hematopoietic stem cells [34-37]. 5FU treatment has also been used by others as a standard procedure to enrich LTRA cells in the BM prior to a further separation by fluorescence-activated cell sorting [10,13,38,39].

Recently, we identified a novel antigen (Ag) on murine hematopoietic stem and progenitor cells which was recognized by monoclonal antibody (mAb) ER-MP12 [40]. It has been shown that its level of expression is inversely related to the primitiveness of the various subsets within the hematopoietic stem cell compartment. The majority of the cells with LTRA express the ER-MP12 Ag at an intermediate level (ER-MP12^{med}). However, with development towards colony-forming units in culture (CFU-C), the Ag expression increases, to disappear from most cells with final maturation, except from B and T lymphocytes. The subset that highly expresses the ER-MP12 Ag (ER-MP12^{hi}) contains the majority of thymus-seeding and repopulating cells [41,42], most CFU-C, and part of the CFU-S-12 population [40]. Thus, although the ER-MP12 Ag is not stem cell specific, we anticipated that it may be a useful marker for the separation of primitive and more mature hematopoietic stem cell subsets.

In the present report we set out to evaluate the use of ER-MP12 as a tool for the purification of hematopoietic stem cells in combination with other methods that are able to discriminate subsets on the basis of their primitiveness. Subsequently, we studied the distribution of the ER-MP12 Ag with respect to Thy-1 expression, Rh123 retention, and WGA affinity. In addition, we investigated the value of ER-MP12 selection in our

previously defined stem cell purification protocols, using low density BM sorted on the basis of Rh123 and WGA, and compared the ER-MP12 expression on marrow hematopoietic stem cells sorted from normal versus 5FU-treated mice. The different hematopoietic stem cell subsets were identified on the basis of differences in the initiation and duration of clonal expansion on an irradiated stromal layer using the cobblestone area forming cell (CAFC) assay. As we have previously shown using different transplantation models, CAFC day-10 frequencies can be used to enumerate CFU-S-12 numbers, while the CAFC day-28 to day-35 content of a cell suspension directly relates to its ability to induce stable chimerism *in vivo* [32,33,43-47].

MATERIALS AND METHODS

Animals. Male inbred BALB/c, C57BL/6-Ly5.1-pep^{3b}, and (CBA x C57BL)F1 mice (BCBA), 12 to 25 weeks of age, were bred and maintained under specific pathogen free conditions at the Central Animal Department of the Erasmus University, and received acidified water (pH 2.8) and food pellets ad libitum. Breeding pairs of the C57BL/6-Ly5.1-pep^{3b} mice were kindly provided by Dr. I.L. Weissman, Stanford University, Stanford, CA.

Monoclonal antibodies and conjugates. Rat monoclonal antibodies (mAbs) ER-MP12 and ER-MP20, which were originally raised against a macrophage precursor cell line [48], were purified from hybridoma supernatant and conjugated to either biotin, using N-hydroxy-succinimidyl-biotin (Sigma, St.Louis, MO), or fluorescein isothiocyanate (FITC; isomer I, Sigma), according to standard procedures [49]. Streptavidin-conjugated R-Phycoerythrin (SAV-PE; Caltag Laboratories, South San Francisco, CA) and Streptavidin-conjugated TRICOLOR (SAV-TRI; Caltag) were used as second stage reagents. Biotinylated anti-Thy-1.2 mAb 30H12 [50] was used for immunofluorescence analysis. Antibodies and conjugates were titrated for optimal staining of mouse BM cells.

Preparation of the BM cells. BM cells were harvested from tibiae and femurs as previously described [32]. In one experiment, male BALB/c mice were intravenously injected with 150 mg 5-fluorouracil (5FU; Sigma) in phosphate-buffered saline (PBS) per kg body weight, 6 days before BM harvest. In specific experiments, BM cells from untreated and 5FU-treated animals were further separated using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient, as previously described [44]. Cells with a density of 1.069-1.075 g/mL (normal BM), or 1.069-1.078 g/mL (5FU BM), were collected from the interphases, washed in PBS containing 5% fetal calf serum (FCS) and maintained on ice throughout the staining and sorting procedure.

Labeling and cell sorting. For immunofluorescence analysis and cell sorting, unseparated normal BM cells, or low-density post-5FU BM (LD/FU$_{6d}$BM) cells were subsequently labeled with biotinylated ER-MP12 (ER-MP12^{bio}), fluoresceinated ER-MP20 (ER-MP20^{FITC}) and SAV-PE. Incubations were performed at a concentration of 5 x 10^6 cells per 50 μL. For dilution and washing we used PBS containing 0.01% (wt/vol) NaN_3 and 0.5% (wt/vol) bovine serum albumin (BSA; fraction V; Sigma). In some experiments, ER-MP20$^+$ cells were depleted from low-density BM cells by magnetic separation using the MACS® (Miltenyi Biotec, Bergisch-Gladbach, Germany), prior to further sorting on the fluorescence-activated cell sorter (FACS). For this procedure cells were incubated for 30 min with ER-MP20^{bio} in PBS containing 0.01% NaN_3, washed, and incubated for 15 min with streptavidin-conjugated MACS microbeads (Miltenyi Biotec) in PBS, containing 0.01% NaN_3 and 5 mmol/L EDTA (Titriplex III; Merck, Darmstadt, Germany). After incubation, the cells were washed in PBS supplemented with 0.01% NaN_3, 5 mmol/L EDTA and 1% BSA, and separated using the MACS column B2 (Miltenyi Biotec) at a flow-rate of 0.3 mL/min. MACS microbeads were sterilized by filtration through a 0.22 μm filter. For further separation by FACS, ER-MP20$^-$ cells were incubated with either 0.25 μg/mL fluoresceinated wheat germ agglutinin (WGA-FITC; Vector, Burlingame, CA), or 0.1 μg/mL rhodamine-123 (Rh123; Eastman Kodak,

Rochester, NY), followed by ER-MP12^{bio} and SAV-TRI labeling. WGA-FITC and Rh123 were incubated for 30 min at room temperature or 37 °C, respectively. Rh123-labeled cells were washed and allowed to stabilize their intracellular dye concentration for 30 min at 37 °C. After sorting, WGA-labeled cells were incubated for 30 minutes at 37 °C in 0.2 mol/L of the competitive sugar *N*-acetyl-*D*-glucosamine (Sigma) to remove surface-bound WGA. Cells were analyzed using a FACScan (B-D Systems, Becton Dickinson, Sunnyvale, CA). Sorting was performed on a FACS II and FACS 440 (B-D Systems) at a rate of 2500 cells per second using a single argon laser tuned at 488 nm (350 mW).

CAFC-assay. Long-term BM cultures were established with BM from BCBA mice for limiting dilution analysis of CAFC as previously described [43,51]. For limiting dilution analysis, the irradiated stroma was overlaid with 8 to 12 dilutions of a cell suspension, two-fold apart, using 15 wells per concentration. Wells were inspected 1 to 2 times per week, from 1 to 5 weeks after inoculation, using a phase-contrast inverted microscope. Wells were scored positively when at least one hematopoietic clone (cobblestone area, CA, containing five or more dark-phase cells) was observed. The frequency of CAFC was calculated from the proportion of negative wells using Poisson statistics [52].

RESULTS

The ER-MP12^{med}20^{-} subpopulation is heterogeneous for CAFC subsets. Using mAbs ER-MP12 and ER-MP20 in two-color immunofluorescence, different subpopulations of BM cells can be identified (Figure 6.1). The ER-MP12 Ag is expressed on all hematopoietic stem cells and progenitors, while the ER-MP20 Ag (Ly-6C) is primarily present on mature granulocytes and monocytes and their committed progenitors (window 6 and 7, respectively) [40]. As previously shown, 90%

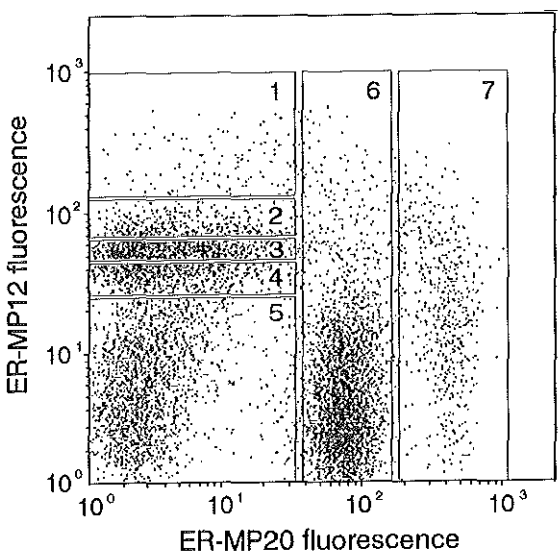

Figure 6.1 Two-color immunofluorescence analysis of mouse BM cells stained with monoclonal antibodies ER-MP12 and ER-MP20. Windows 1 through 5 contained 3%, 19%, 19%, 19% and 40%, respectively, of the ER-MP20^{-} BM cells. Window 6 (ER-MP20^{med} subset) contained 33.6%, and window 7 (ER-MP20^{hi} subset) 6.2% of all nucleated cells.

to 95% of the LTRA cells in BM could be recovered from the subpopulation that intermediately expressed the ER-MP12 Ag but lacked Ly-6C (ER-MP12^{med}20^{-}, as indicated in Figure 6.2A, lower window). We have reported earlier that labeling of the cells with ER-MP12 or ER-MP20 did not affect their *in vivo* and *in vitro* colony-forming ability [40]. The majority of the *in vitro* clonable progenitors (CFU-C) expressed the ER-MP12 Ag at a high level and were recovered from the ER-MP12^{hi}20^{-} subpopulation (Figure 6.2A, upper window). However, considerable overlap has been found between the recoveries of CFU-S-12 and LTRA cells [40].

To study the distribution of the ER-MP12 subsets in detail, ER-MP20$^-$ BM cells were sorted into 5 fractions of different ER-MP12 Ag expression (Figure 6.1). Cells that expressed the ER-MP12 Ag at an intermediate or high level were divided into 4

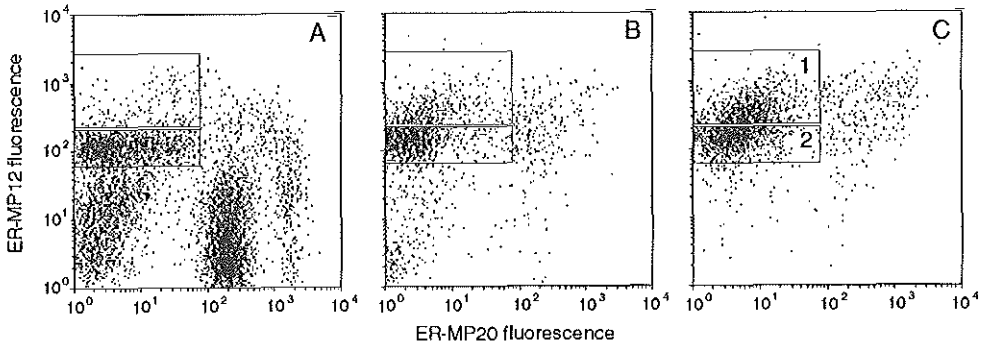

Figure 6.2 Dotplots of the ER-MP12 against the ER-MP20 fluorescence of **(A):** unseparated BM cells, **(B):** day-6 post-5FU BM ($FU_{6d}BM$), **(C):** low-density $FU_{6d}BM$ ($LD/FU_{6d}BM$). The ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations are indicated in (A) through (C). Sort windows [1] and [2] in (C) contained 18% and 64% of the $LD/FU_{6d}BM$ cells, respectively.

fractions: a fraction corresponding with the top half of the previously defined ER-MP12^{hi}20$^-$ subpopulation [40-42], containing 3% of the ER-MP20$^-$ cells (Figure 6.1; window 1), and 3 equal fractions containing 19% of the cells (Figure 6.1; window 2, 3 and 4, respectively) subdividing the previously defined ER-MP12^{med}20$^-$ subpopulation. The fraction with the lowest expression (Figure 6.1; window 5) contained 40% of the ER-MP20$^-$ cells. The sorted cells were subsequently tested in the CAFC assay (Figure 6.3A). CAFC frequencies were used to calculate the relative recoveries of the 5 fractions compared to unseparated BM (Figure 6.3B).

The fraction with the highest ER-MP12 expression (fraction 1) appeared to contain extremely short-term *in vitro* repopulating CAFC that disappeared within the first 14 days after inoculation. Fraction 2, representing the cells with a relative high ER-MP12 expression, contained the highest percentage of CAFC-10 (equivalent to the *in vivo* CFU-S-12) [44], but a relatively low percentage of the CAFC-28 compared to fraction 3 and 4. Fraction 4 was relatively enriched for the more primitive stem cell subsets as it contained 40% of the CAFC-28 but only 10% of the CAFC-10. In summary, the recovery of early appearing cobblestone areas (CAFC-7 through CAFC-15) gradually increased with increasing ER-MP12 expression, while the more primitive CAFC-28 were distributed in similar percentages over the three intermediately staining ER-MP12 subpopulations (fraction 2, 3, and 4). On average, CFU-S-12 (CAFC-10) express the ER-MP12 Ag at a higher level than the more primitive stem cell subsets (CAFC-28). However, compared with the previously defined ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ sort windows (Figure 6.2A, upper and lower window, respectively), the data show that a

better separation of the primitive and more mature stem cell subsets could not be achieved without a significant reduction in recovery.

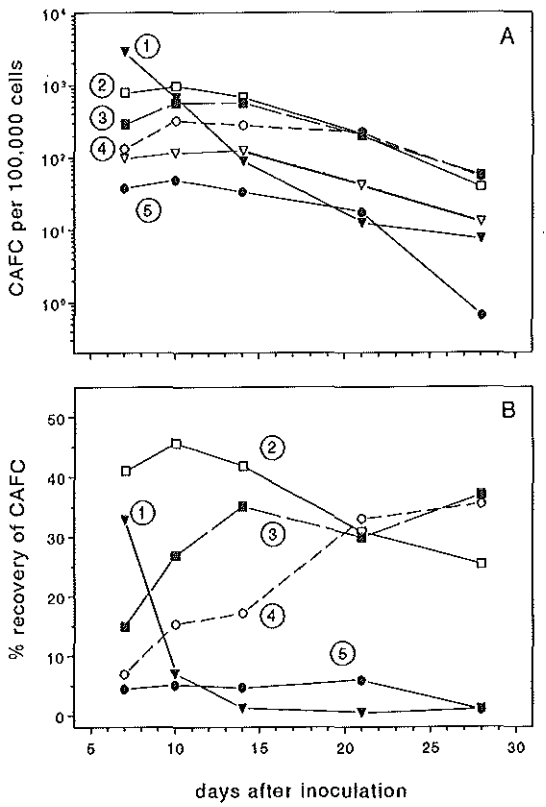

Figure 6.3 **(A):** CAFC frequencies of ER-MP20$^-$ BM sorted on the basis of ER-MP12 Ag expression (sort windows 1 through 5, Figure 6.1): (1) 93-100 percentile (●), (2) 78-93 percentile (○), (3) 59-78 percentile (■), (4) 40-59 percentile (□), and (5) 0-40 percentile (▼). Control (unseparated) BM cells (▽). For clarity, the 5 subpopulations are numbered and indicated in the graph and error bars are left out. The standard error approximated 25% to 30% for most data points. **(B):** Relative distribution of CAFC subsets compared to unseparated BM [symbols see (A)]. Recoveries are calculated on the basis of CAFC frequencies and the percentage of cells in the sort windows.

Phenotypic analysis of the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20 subpopulations. Unseparated BM and sorted ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations were analyzed with respect to their Rh123 retention, WGA affinity and Thy-1.2 expression in order to investigate whether these probes would enable a further separation of the primitive and more mature hematopoietic subsets (Figure 6.4). Sort windows for the ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulations are indicated in Figure 6.2A. To visualize small differences in WGA binding and Thy-1.2 expression, a linear instead of a logarithmic scale was used. The indicated classification of Rh123 retention and

WGA-binding subsets in Figure 6.4 corresponds to our previously reported criteria [32,33]. Compared to unseparated BM, the majority of the ER-MP12^{hi}20^{-} BM cells had a high retention of Rh123, expressed on average a high level of Thy-1.2, and contained a clearly distinguishable population of WGA^{bright} cells, which are the typical features of CFU-S-12, CAFC-10 and transiently *in vivo* repopulating stem cells [3,33]. The ER-MP12^{med}20^{-} subpopulation, on the other hand, was more heterogeneous with respect to Rh123 retention. This subpopulation contained a subset of blast cells that otherwise had the phenotypic characteristics of primitive stem cells, i.e. a low retention of Rh123

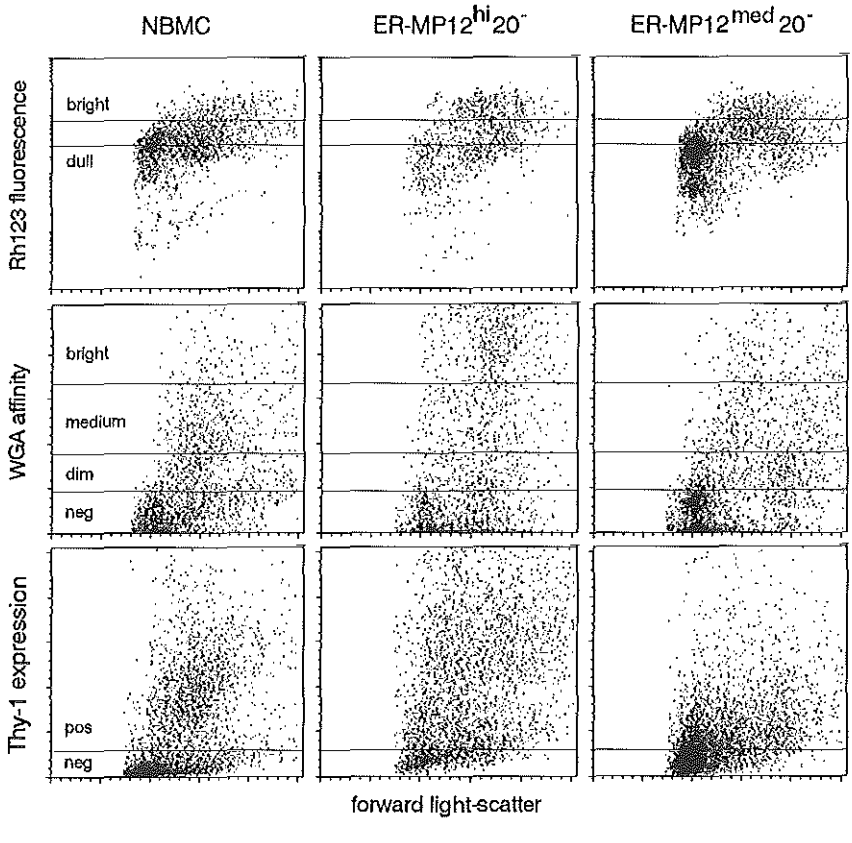

Figure 6.4 FACScan analysis of unseparated BM cells and of the sorted ER-MP12^{hi}20^{-} and ER-MP12^{med}20^{-} subpopulations stained with either Rh123, WGA-FITC or Thy-1.2 (sort windows are indicated in Figure 6.2A). The classification of the different fluorescence levels is indicated. Thy-1 expression and WGA affinity are given on a linear scale.

(Rh123^{dull}), a low affinity for WGA (WGA^{dim}), and a low expression of Thy-1.2 [3,32,33]. Also, subpopulations of small mononuclear-sized cells with different Rh123 retention could be observed. Thy-1 appeared less suitable for further sorting as it detected only little heterogeneity within the ER-MP12^{med}20^{-} subpopulation. Taken

together, FACScan analysis showed that the ER-MP12^{med}20^{-} subpopulation could be further separated on the basis of Rh123 retention and WGA affinity which may contribute to a further separation of LTRA cells and CFU-S-12.

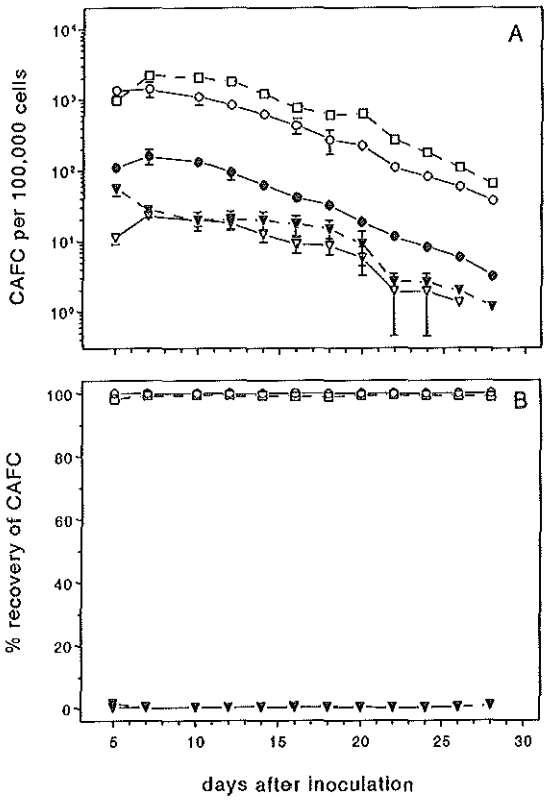

Figure 6.5 **(A):** CAFC frequencies of (1.069-1.075 g/mL) low-density BM separated on the basis of mAb ER-MP20: (●) unseparated BM cells, (○) low-density BM cells, (□) low-density ER-MP20^{-}, (▼) low-density ER-MP20^{med}, (▽) low-density ER-MP20^{hi}. Bars denote standard errors. **(B):** Distribution of CAFC subsets relative to low-density BM [symbols see (A)].

ER-MP12 Ag expression on Rh123^{dull} or WGA^{dim} CAFC subsets from normal BM. To study whether ER-MP12 could be used to improve the purity of our previously defined Rh123^{dull} and WGA^{dim} stem cell subsets [32,33], we separated low-density Rh123^{dull} and WGA^{dim} BM cells on the basis of their ER-MP12 Ag expression. Prior to cell sorting, low-density BM was depleted of ER-MP20-positive granulocytes and monocytes by MACS, as previously described [32,33]. Density centrifugation equally enriched for all CAFC subsets, as was shown in the CAFC assay (Figure 6.5A), and 99% of all CAFC could be recovered from the ER-MP20^{-} subset of low-density BM (Figure 6.5B). The sort windows for Rh123^{dull} and WGA^{dim} cells contained 25%

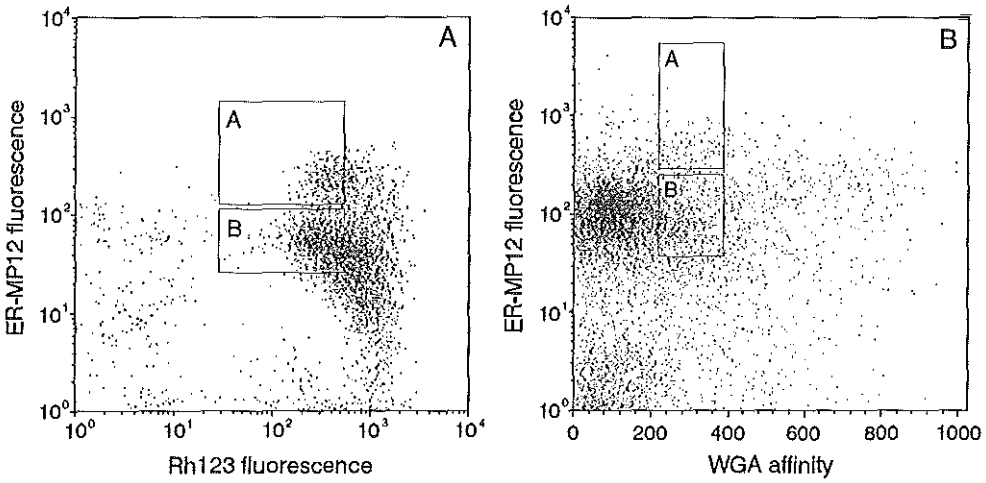

Figure 6.6 Dotplots of the ER-MP12 Ag expression against **(A)**: Rh123 fluorescence of low-density ER-MP20$^-$ BM, and **(B)**: WGA-FITC affinity of low-density ER-MP20$^-$ BM. The sort windows for the ER-MP12^{hi} and ER-MP12^{med} subsets are indicated by [A] and [B], respectively. The Rh123^{dull} and WGA^{dim} subpopulations, irrespectively of the level of ER-MP12 Ag expression, contained 25% and 12%, respectively, of all low-density ER-MP20$^-$ cells.

and 11% of the low-density ER-MP20$^-$ BM cells, respectively. The Rh123^{dull} and WGA^{dim} subpopulations were sorted into their respective ER-MP12^{hi} and ER-MP12^{med} subsets as indicated in Figures 6.6A and B (sort windows A and B, respectively), and were subsequently tested in the CAFC assay (Figures 6.7A and 6.8A). Recoveries of the ER-MP12^{hi} and ER-MP12^{med} CAFC were calculated relatively to the total number of CAFC in the Rh123^{dull} and WGA^{dim} subpopulations (Figures 6.7B and 6.8B). All Rh123^{dull} and WGA^{dim} CAFC activity was contained in the ER-MP12^{hi} and ER-MP12^{med} fractions as no CAFC could be detected in the ER-MP12$^-$ fraction [40]. The data show that most of the mature progenitors in the Rh123^{dull} subset that gave rise to early appearing cobblestone areas (CAFC-5 through CAFC-15) could be separated from the more immature progenitors (CAFC-21 through CAFC-32) on the basis of ER-MP12 Ag expression (Figure 6.7B). Immature and more mature CAFC within the WGA^{dim} subpopulation, however, could not be further separated by ER-MP12 as practically all WGA^{dim} CAFC already expressed the ER-MP12 Ag at an intermediate level (Figure 6.8B). Therefore, mAb ER-MP12 detects heterogeneity at the precursor cell level in the Rh123^{dull} but not in the WGA^{dim} subset of low-density BM. However, when combined with sorting on the basis of WGA affinity, LTRA cells could be 840-fold enriched compared to unseparated BM (Figure 6.8A), which is comparable to our highest enrichment for LTRA cells as achieved by sorting WGA^{dim} cells from low-density 5FU-BM [32].

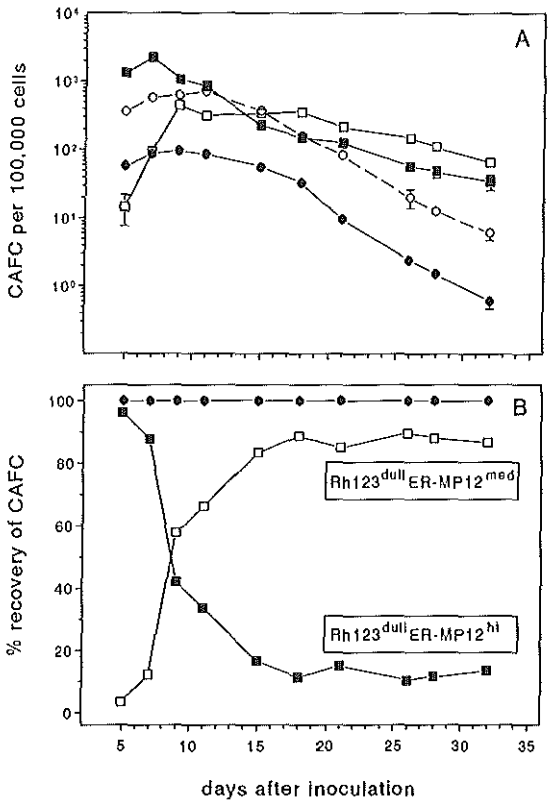

Figure 6.7 **(A):** CAFC frequencies of low-density ER-MP20$^-$ Rh123^{dull} BM cells sorted on the basis of ER-MP12 Ag expression: (●) unseparated BM cells; (○) low-density BM; (■) low-density Rh123^{dull} ER-MP12^{hi}; (□) low-density Rh123^{dull} ER-MP12^{med}. Bars denote standard errors. **(B):** Relative distribution of the ER-MP12^{hi} (■) and ER-MP12^{med} (□) subsets compared to Rh123^{dull} ER-MP20$^-$ low-density BM (●), which was set at 100%.

The distribution of the ER-MP12 Ag on CAFC cells in post-5FU BM. 5FU-treatment represents a powerful pre-enrichment step in our recently described LTRA purification protocol [32]. To investigate whether the ER-MP12 Ag expression on stem cells would be affected by 5FU-treatment, we labeled day-6 post-5FU low-density BM (LD/FU$_{6d}$BM) with ER-MP12 and ER-MP20, and sorted these cells into an ER-MP12^{hi}20$^-$ and ER-MP12^{med}20$^-$ subpopulation (Figure 6.2C, window 1 and 2). FACScan analysis shows the level of expression of both the ER-MP12 and ER-MP20 antigens in normal BM, FU$_{6d}$BM and LD/FU$_{6d}$BM (Figure 6.2; A through C). Most of the erythroblasts, granulocytes and monocytes, that were present in the ER-MP12$^-$20$^-$, ER-MP12$^-$20^{med} and ER-MP12$^-$20^{hi} subpopulations, respectively [40], had disappeared as a result of the 5FU-treatment and subsequent density step, whereas the highly

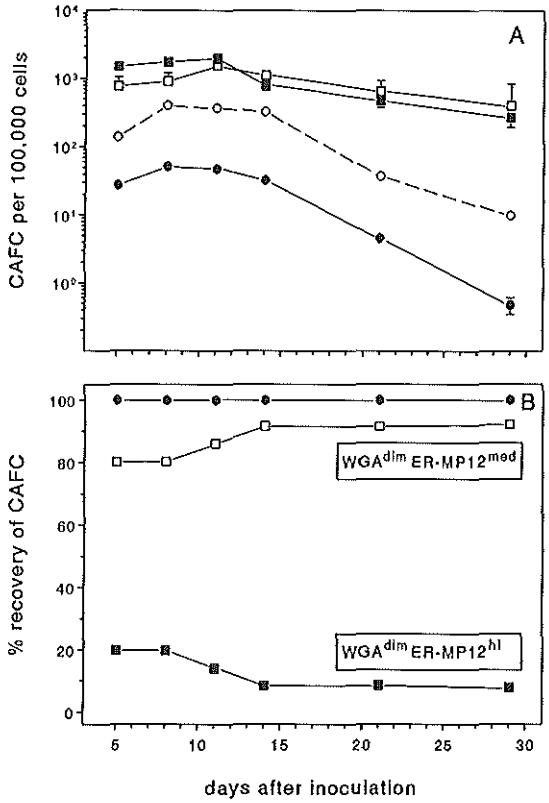

Figure 6.8 **(A):** CAFC frequencies of low-density ER-MP20$^-$ WGA^{dim} BM cells sorted on the basis of ER-MP12 Ag expression: (●) unseparated BM cells; (○) low-density BM; (■) low-density WGA^{dim} ER-MP12^{hi}; (□) low-density WGA^{dim} ER-MP12^{med}. CAFC-28 are 840-fold enriched compared to unseparated BM. Bars denote standard errors. **(B):** Relative distribution of the ER-MP12^{hi} (■) and ER-MP12^{med} (□) subsets compared to WGA^{dim} ER-MP20$^-$ low-density BM (●), which was set at 100%.

ER-MP12-Ag-expressing cells were significantly enriched. The CAFC frequency curves

(Figure 6.9A) revealed no qualitative differences between the two subpopulations in that enrichments for CAFC-10 and CAFC-47 were comparable. Eighty to ninety percent of the CAFC-14 through CAFC-47 were recovered from the ER-MP12^{med}20$^-$ subpopulation (Figure 6.9B), indicating that the majority of the most primitive hematopoietic stem cells in LD/FU$_{6d}$BM expressed the ER-MP12 Ag at an intermediate level, which is identical to the distribution of stem cells in normal BM [40]. Therefore, we conclude that 5FU-treatment had not changed the distribution of the ER-MP12 Ag on LTRA cells.

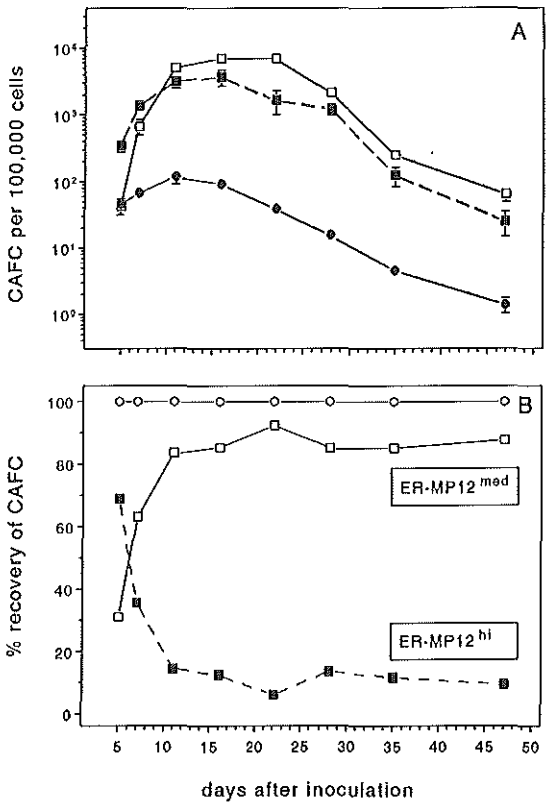

Figure 6.9 **(A):** CAFC frequencies of ER-MP20$^-$ LD/FU$_{6d}$BM sorted on the basis of ER-MP12 Ag expression: (●) unseparated BM cells; (■) ER-MP12^{hi} ER-MP20$^-$ LD/FU$_{6d}$BM; (□) ER-MP12^{med} ER-MP20$^-$ LD/FU$_{6d}$BM. Sort windows are indicated in Figure 6.2C. Bars denote standard errors. **(B):** Relative distribution of the ER-MP12^{hi} (■) and ER-MP12^{med} (□) subsets compared to ER-MP20$^-$ LD/FU$_{6d}$BM (○).

DISCUSSION

In the present study we evaluated the use of ER-MP12 for the purification of long-term repopulating BM hematopoietic stem cells and their physical separation from CFU-S-12 and more mature progenitors. The ER-MP12 Ag expression was subsequently studied in comparison to WGA affinity, Rh123 retention, and Thy-1 expression. We show that ER-MP12 detects heterogeneity within our previously described $Rh123^{dull}$ and WGA^{dim} stem cell subpopulations [32,33]. The combination of WGA and ER-MP12 enabled long-term *in vitro* repopulating cells (CAFC-28/35) to be 840-fold enriched over unseparated BM. This high enrichment is an improvement compared to sorting low-density normal BM on the basis of WGA alone [32], and is comparable to our highest reported enrichment achieved by sorting WGA^{dim} cells from $LD/FU_{6d}BM$ [32]. Furthermore, separation of primitive (CAFC-28/35) from more mature CAFC subsets (CAFC-7/14) in the $Rh123^{dull}$ subpopulation, which is in itself more heterogeneous than the WGA^{dim} subpopulation [33], could be enhanced by separation on the basis of ER-MP12. Finally, Thy-1 could not be used for further sorting as it was not heterogeneously expressed on $ER\text{-}MP12^{med}20^{-}$ cells. In conclusion, the present data show that ER-MP12, in combination with WGA, can be used as a positive selection criterion to highly enrich primitive hematopoietic stem cells.

Previously, it has been demonstrated that 5FU is able to trigger most LTRA cells into the cell cycle, however, without an apparent loss of total number in the marrow [53,54]. The present data show that the level of ER-MP12 Ag expression and the distribution of the *in vitro* LTRA cells (CAFC-28 through CAFC-46) had not changed by 5FU treatment 6 days previously, when compared to normal BM [40]. This suggests that the expression of the ER-MP12 Ag is not related to the cell cycle status of the hematopoietic stem cells, and shows that ER-MP12 can be applied for stem cell sorting under conditions of activation. This might be useful for gene therapy as cycling (post-5FU) LTRA cells have been shown to have a high transfection rate [38,39,55,56].

The combination of sorting on the basis of WGA affinity and ER-MP12 Ag expression has several advantages over the use other markers that can separate subsets on the basis of their primitivity. Differences in Rh123 retention have been used extensively for the purification of murine hematopoietic stem cells. In both mouse [19-23,33] and human [24,57-59] the most primitive hematopoietic cells have been shown to be among the cells with a low Rh123 retention. However, a major disadvantage of Rh123 is that its retention is also influenced by the cycling and metabolic status of the cells. This is reflected by the activity, size and number of mitochondria per cell, and can vary with the glucose concentration in the medium [13-15,60-62]. Consequently, all protocols and conditions that change the cycling behavior of the target cells, including exposure to infectious agents, or treatments that specifically activate the BM stem cell compartment, influence Rh123 retention. In addition, BM cells from different mouse strains also intrinsically differ in their Rh123

retention as the maintenance of stem cell populations in mice has been shown to be genetically determined [26]. Apparently, a low Rh123 retention is not characteristic for the stem cell, but merely reflects a physiological state. Therefore, standardization of Rh123 retention for the application of stem cell purification might be difficult, especially in man being an outbred species.

Mouse hematopoietic stem cells have also been phenotypically identified by the expression of the Ly-6A/E (Sca-1) antigen, low expression of Thy-1 and very low expression or absence of lineage-specific markers (Thy-1^{lo} Lin^- Ly-$6A/E^+$) [9,11]. However, the combination of Ly-6A/E (in Ly-6^b haplotype mice) and Thy-1.1 has only a limited application for the purification of murine hematopoietic stem cells as both antigens are haplotype restricted and, in most mouse strains, are not simultaneously expressed [12]. In contrast, the ER-MP12 Ag is similarly expressed on BM cells in mice of various different MHC haplotypes [42]. In addition, ninety percent of the long-term *in vitro* stem cells in BALB/c and C57Bl/6-Ly5.1 mice express the ER-MP12 Ag at an intermediate level, as was demonstrated in the CAFC assay [40]. This indicates that ER-MP12 is a widely applicable mAb for the separation of mouse hematopoietic cells. In addition, hematopoietic stem cells of different mouse strains (BALB/c, BCBA(C57BLxCBA), C57BL/6Jlco and C57BL/6-Ly5.1-pep^{3b}) have also been found to all have a low affinity for WGA, as demonstrated in our present and previous reports [32,33,63]. Furthermore, a WGA-positive population of both mouse and rhesus monkey low-density BM has been shown to contain *in vitro* colony-forming cells and natural suppressor activity [64,65], indicating that differences in WGA affinity may be applied for the dissection of the hematopoietic stem and progenitor cell compartment in not only different mouse strains, but also in other species. The combination of sorting stem cells on the basis of a low affinity for WGA and intermediate ER-MP12 Ag expression has the additional advantage that mature BM cells are simultaneously excluded in the procedure. Erythroblasts, mature granulocytes and monocytes all lack the ER-MP12 Ag [40], while the majority of the mature lymphocytes have no affinity for WGA [32]. The omission of a negative selection step for mature BM cells, that normally involves the use of a panel of lineage-specific mAbs, simplifies the purification procedure.

In summary, the data show that ER-MP12 and WGA are widely applicable probes that enable a positive selection of mouse hematopoietic stem cells and allow them to be highly enriched. Furthermore, both probes enable a separation of the hematopoietic stem cell subsets on the basis of their primitivity, and thus contribute to resolving the heterogeneity of the hematopoietic stem cell compartment.

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CHAPTER 7

MARROW AND SPLEEN SEEDING EFFICIENCIES OF ALL MURINE HEMATOPOIETIC STEM CELLS ARE DECREASED BY PREINCUBATION WITH HEMATOPOIETIC GROWTH FACTORS

Johannes C.M. van der Loo and Rob E. Ploemacher

Institute of Hematology, Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

The cobblestone-area forming cell (CAFC) assay permits a direct measurement of the seeding of primitive and more mature murine hematopoietic stem cell subsets by comparing the number of CAFC in the original transplant with the number of CAFC retrieved from bone marrow and spleen following transplantation. We found no differences in seeding efficiency between the more mature and primitive CAFC subsets, nor between seeding efficiencies of stem cells from low-density fractions of normal and day-6 post-5-fluorouracil bone marrow. The data show that 18% to 20% of all intravenously transplanted stem cell subsets seed to the bone marrow while 8% to 10% seed to the spleen. In addition, similar seeding efficiencies were found for CFU-S-12 as was determined by retransplantation. Previously, it has been reported that a 2-3 hour preincubation of bone marrow with IL-3 enhances the *in vivo* repopulating ability of a graft. To test whether hematopoietic growth factors affected this increased engraftment by enhancing the seeding of the transplanted marrow, we assessed the 16-18 hour seeding efficiency of short- and long-term *in vivo* repopulating stem cell subsets to bone marrow and spleen using the CAFC assay, after preincubation with or without hematopoietic growth factors. A 2-3 hour preincubation with IL-3, or a combination of IL-3, IL-12 and steel factor (SF), at 37°C, led to a substantial decrease in seeding compared to control (which was kept on ice) of all hematopoietic subsets measured, both in spleen and bone marrow. In concert with these data, the long-term *in vivo* repopulating ability of growth-factor incubated bone marrow was also decreased when compared to control. In conclusion, the present results indicate that the seeding efficiency of different hematopoietic stem cell subsets seems not to change with differentiation as all subsets had an equal seeding. In addition, we have been unable to observe a beneficial effect of growth factor preincubation on the repopulating ability of a graft.

INTRODUCTION

The homing of hematopoietic stem cells and progenitors and binding to the bone marrow (BM) stroma is mediated by a complex of interactions, involving stromal cells as well as components of the extracellular matrix (ECM) [1-4]. These components include the different types of collagen [5], several glycoproteins, such as fibronectin [6-11], hemonectin [12,13], thrombospondin [14,15], the intercellular and vascular cell adhesion molecules ICAM-1 [10,16] and VCAM-1 [10,17-19], and the family of proteoglycans with their different glycosaminoglycan side chains [20,21]. Human and murine hematopoietic stem cells and progenitors have been shown to interact with the stromal microenvironment using several pathways simultaneously. The $\beta 1$ integrins VLA-4 ($\alpha 4\beta 1$) and VLA-5 ($\alpha 5\beta 1$) mediate the binding to stromal fibronectin by interacting with its heparin and cell binding domains, respectively [7-10]. In addition, VLA-4 also interacts with VCAM-1 [10,19] while fibronectin can bind chondroitin-sulfate produced by the progenitor cell [22,23]. Other pathways involve the interaction of a $\beta 2$ integrin with ICAM-1 [10], and CD44/Pgp-1 with hyaluronic acid [24-26].

Ample evidence exists on the role of a membrane lectin in the specific homing of intravenously transplanted stem cells to the marrow [3,27,28]. This homing receptor, which has been purified from the cloned progenitor cell lines FDCP-1 and B6SUT [29,30], is a 110 kD glycoprotein heterodimer with specificity for galactosyl and mannosyl residues of a membrane-associated glycoconjugate on marrow stromal cells [31-36]. It has been shown that the expression of homing receptors on FDCP-1 and FDCP-mix cells can be upregulated by a brief incubation with IL-3 or GM-CSF [37,38]. In addition, it has been reported that an incubation of mouse BM cells or sheep or monkey fetal liver cells with hemopoietic growth factors (either IL-3, GM-CSF or phytohemagglutinin-stimulated lymphocyte-conditioned medium) prior to transplantation, significantly enhances the engraftment when compared with preincubation without these factors [37,39,40]. It has been suggested that this enhancement could be attributed to an increased seeding efficiency of the transplanted stem cells as a result of an upregulation of homing protein expression [37].

In the present study we investigated the BM and spleen seeding efficiency of day-12 spleen colony-forming units (CFU-S-12) and of short-term and long-term *in vivo* repopulating stem cell subsets as assessed by the cobblestone area forming cell (CAFC). In addition, we determined the effect of a brief 2-3 hour preincubation of BM cells with rIL-3, or with a combination of IL-3, IL-12 and steel factor (SF), on the CAFC seeding efficiency and long-term repopulating ability of the graft *in vivo*. The results show that all hematopoietic stem cell subsets tested, in normal and day-6 post-5FU low density BM, have similar seeding efficiencies. In addition, we show that a brief 2-3 hour preincubation of BM cells at 37°C with hematopoietic growth factors has a negative effect on the seeding of hematopoietic stem cells and progenitors to BM and spleen. Furthermore, we show that this preincubation did not enhance the long-term *in vivo* engraftment of the BM when compared to BM that was not incubated but kept on ice during the whole procedure.

MATERIALS AND METHODS

Animals. Male and female inbred B6CBA and (CBA x C57BL)F1 mice (BCBA), 14 to 35 weeks of age, were bred and maintained under specific pathogen free conditions at the Central Animal Department of the Erasmus University, and received acidified water (Ph 2.8) and food pellets ad libitum. In specific experiments male B6CBA mice were injected intravenously with 150 mg 5-fluorouracil (5FU; Sigma, St Louis, MO) in phosphate-buffered saline (PBS) per kg body weight 6 days before harvest of the BM.

Preparation of BM cells and density centrifugation. BM from untreated or 5FU-treated animals was harvested from tibiae and femurs and separated by buoyant density centrifugation using a discontinuous Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient as previously described [41]. Cells with a density of 1.069-1.078 g/mL (10-15% of total BM cells, 5-6% of post-5FU BM cells) were collected from the interphases, washed in cold Dutton's balanced salt solution (Dutton; Gibco, Breda, The Netherlands) containing 5% fetal calf serum (FCS), and maintained on ice.

Incubation with hematopoietic growth factors. To determine the effect of growth factor preincubation on spleen and BM seeding, normal low-density (LD) BM cells were incubated for 2 to 3 hours at 37°C at a density of 5 x 10^6 cells/mL, in Dutton containing 5% FCS, with or without specific growth factors. The control group (1) was maintained on ice. Other groups contained either (2) 100 U/mL murine IL-3, (3) 100 U/mL IL-3, 10 ng/mL murine IL-12 and 100 ng/mL murine steel factor (SF), (4) 10 ng/mL IL-12 and 100 ng/mL SF, or (5) no additional growth factors. After incubation the cells were washed, counted, and used immediately for transplantation. Part of the cells were kept on ice until transfer into the different *in vitro* assays (within 2 hours). All groups were tested for *in vitro* clonable myeloid progenitors (CFU-GM) and viability of the cells was tested using propidium iodide staining and FACScan analysis. Groups (1) to (3) were also tested for cobblestone area formation in the CAFC assay, for CFU-S-12 and for long-term *in vivo* repopulating ability (LTRA). In addition, the cells in groups (1) to (3) were tested for their spleen and BM seeding efficiency (next section). The recombinant factors used were kindly provided by Dr. S. Neben of the Genetics Institute, Cambridge, MA.

Determination of the seeding in femur and spleen. The seeding of normal LD-BM, after preincubation with or without growth factors, and post-5FU LD-BM, was determined at 16-18 hour after transplantation, as is schematically shown in Figure 7.1. Male B6CBA recipients (4-5 mice per group, 15 weeks of age) received 11.1 Gy total body irradiation (*gamma*) at a dose rate of 0.98 Gy/min, 7-9 hours before transplantation. The dose was chosen to maximally reduce residual hematopoietic activity allowing the detection of low numbers of seeded stem cells. Mice were transplanted with 4 x 10^6 post-5FU or 1.3 x 10^7 normal LD-BM cells, respectively, by injection of 0.5 mL of single cell suspensions into a lateral tail vein. Part of the cells were used to determine the input number of transplanted CFU-S-12 and CAFC subsets (CAFC-7 through CAFC-28) in the CFU-S and CAFC assay, respectively. Spleens and femurs were removed under sterile conditions 16-18 hours after transplantation, which is 24-26 hours after irradiation. Cells from spleens and femurs were collected

quantitatively and were pooled per organ per group. Single cell suspensions were prepared by gently sieving the cells through 100 μm and 30 μm sterile nylon gauze. Spleen and BM cells were subsequently tested for CFU-S-12 and CAFC. The BM and spleen seeding efficiencies were then calculated on the basis of the number of input CAFC and CFU-S-12, and number of output CFU-S-12 and CAFC per spleen or femur. The transplanted post-5FU LD-BM cells, as well as the spleen and BM cells from recipients of post-5FU LD-BM, were tested only in the CAFC assay.

Spleen colony-forming unit (CFU-S) assay. The number of CFU-S-12 in the primary transplant and in BM and spleen was determined by intravenous injection of the cells into 8.6 Gy irradiated male B6CBA [42]. This dose enabled 95% of the animals to survive for 12 days with only 0.2 endogenous colony per spleen in the non-transfused irradiated group. Mice, 15-35 weeks of age, were randomly divided over the groups, each containing 10-15 mice. The primary transplant mice were injected with 5×10^3 to 1×10^4 cells low density cells. Secondary recipients were infused with either 1/60 to 1/200 spleen (equalling 8×10^4 to 3×10^5 cells), or with 1/10 to 1/30 femur (equalling 1×10^5 to 6×10^5 cells), per mouse. A control group of 10-15 mice that had not received any cells was included in all experiments. Mice were kept in laminar air flow cabinets during the experiments and were sacrificed at day 12 after injection. Their spleens were excised and fixed in Telleyesniczky's solution. Macroscopically visible colonies were counted.

CAFC assay. Dexter-type murine long-term BM cultures were established in flat-bottomed 96-well microtiter plates for limiting dilution analysis of CAFC as previously described [41,43,44]. Briefly, an adherent stromal layer of cells from BCBA mice ($5\text{-}10 \times 10^5$ cells/well) was grown in 10-14 days at 33 °C, 10% CO_2 in an 100% humidified incubator. At confluency, the cultures were irradiated with 20 Gy ^{137}Cs gamma. For limiting dilution analysis the stroma was overlaid with 12 dilutions of a BM or spleen cell suspension, two-fold apart, using 15 wells per concentration. The stroma could not be overlaid with more than 1/20 femur or 1/40 spleen per well, i.e. $3 \text{ to } 4 \times 10^5$ cells per well, as higher numbers of cells disrupted the stromal layer. Wells were inspected 1 to 2 times per week, from 1 to 5 weeks after inoculation, using a phase-contrast inverted microscope, and were scored positively when at least one hematopoietic clone (cobblestone area, CA, a cluster containing five or more dark-phase cells) was observed. The frequency of CAFC was calculated from the proportion of negative wells on the basis of Poisson statistics [45].

Colony assay. The number of CFU-GM was determined in 1 mL semisolid cultures consisting of 1.2% (wt/vol) methylcellulose (Methocel MC, Fluka Chemie, Buchs, Switzerland) in α-modified Dulbecco's modified Eagle's medium (DMEM; Gibco) at an osmolarity of 280 mOsmol/kg. The medium was supplemented with 20% horse serum (Gibco), 1% BSA, 80 U/mL penicillin, 80 μg/mL streptomycin, 3.2 mmol/L L-glutamine (Merck), 8×10^{-8} mol/L sodium selenite (Merck) and 8×10^{-5} mol/L β-mercaptoethanol (final concentrations). Cultures (4 replicate dishes per group) were stimulated by 10% (vol/vol) pokeweed mitogen-stimulated mouse spleen conditioned medium, and were kept at 37 °C, 5% CO_2 and 100% humidity. Colonies consisting of 50 cells or more were counted at day 8 using an inverted microscope.

Long-term repopulating ability (LTRA). The LTRA was determined by injection of different numbers of male BM cells into sublethally irradiated syngeneic female recipients (10 mice per group). We used 4 cell doses per experimental group, two-fold apart. B6CBA recipients (15-25 weeks of age) received 7.8 Gy total body irradiation one day before transplantation. The dose was chosen to enable survival of all animals, independent of the number or quality of the transfused cells. At 4 months post-transplantation, the percentage of donor-type male peripheral blood leukocytes was determined by *in situ* hybridization using the Y-chromosome specific probe P17-M34/2 [46], as previously described [47].

Statistical analysis. CAFC frequencies were calculated based on the Poisson distribution using the maximum likelihood solution. Other data were tested using a one-way Analysis of Variance or Student's T test.

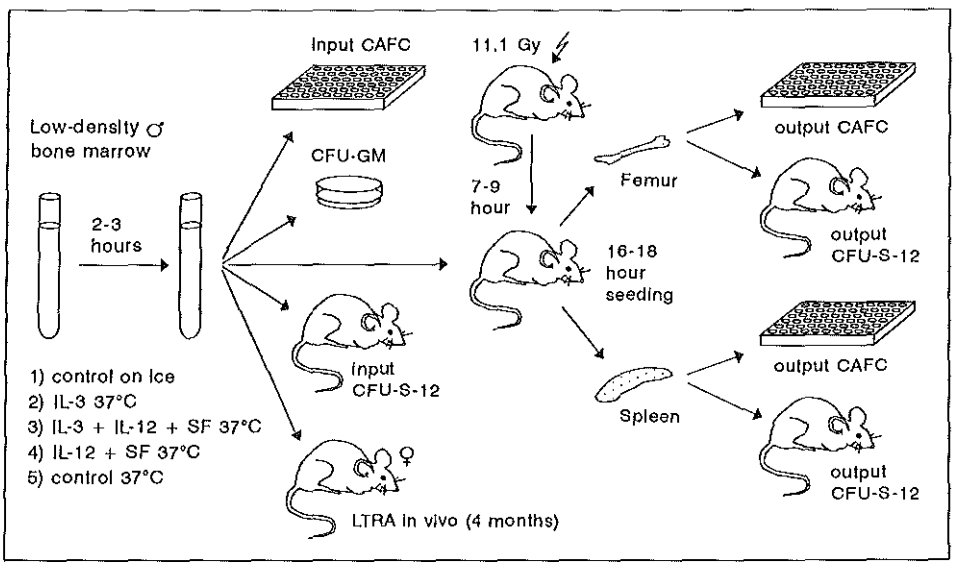

Figure 7.1 Schematic representation of growth factor preincubation, the transplantation strategy and different assays used. Low-density (LD) BM cells were either **(1)** kept on ice, or incubated for 2-3 hours at 37°C in **(2)** 100 U/mL IL-3, **(3)** 100 U/mL IL-3, 10 ng/mL IL-12 and 100 ng/mL steel factor (SF), **(4)** 10 ng/mL IL-12 and 100 ng/mL SF, or **(5)** no additional growth factors. All groups were tested for day-8 CFU-GM, while groups (1) to (3) were tested for their input of CAFC (day-7 through day-28), CFU-S-12, and LTRA cells, and were transplanted into groups of 11.1 Gy irradiated recipients. Output CAFC and CFU-S-12 in spleen and femur were determined after 16-18 hours, as indicated in the *Materials and Methods* section. The seeding of day-6 post-5FU LD-BM cells was determined using only the CAFC assay without growth factor preincubation.

RESULTS

Seeding of CAFC in normal and post-5FU low-density BM. To determine the seeding of more mature and primitive hematopoietic stem cell subsets to BM and spleen, low-density BM cells from normal and 5FU-treated mice were transplanted into 11.1 Gy irradiated recipients (4-5 mice/group). Part of the cells were tested in the CAFC assay to calculate the number of CAFC subsets (CAFC-7 through CAFC-28/35) that were transplanted. After 16-18 hours, the mice were killed and cells from their spleens and femurs were collected and tested for their CAFC content (Figures 7.2A and 7.3A). Significant differences between the seeding efficiencies of early (day-7 through

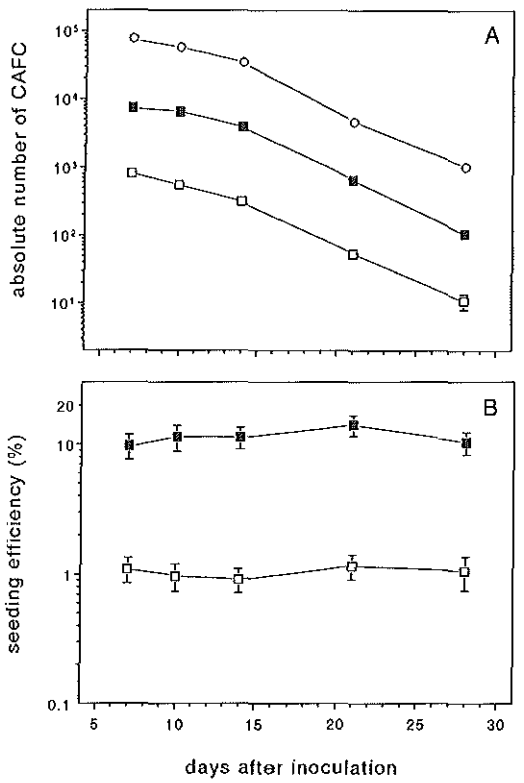

Figure 7.2 Seeding of CAFC subsets from low-density (1.069-1.078 g/mL) BM in spleen and femur at 16-18 hours after transplantation (4-5 recipients). **(A):** (○) number of CAFC injected per mouse, (■) number of CAFC recovered per spleen, (□) number of CAFC recovered per femur. **(B):** Seeding efficiency (mean ± SEM) of CAFC subsets in spleen (■) and femur (□).

14) and late (day-28 through 35) CAFC derived from normal BM (Figure 7.2B), representing the more mature and primitive hematopoietic stem cell subsets, respectively [41,43], were not observed. However, compared with the late CAFC, the seeding of the

more mature progenitor cells (CAFC-7) in post-5FU BM was slightly lower (Figure 7.3B). This may reflect an effect of the 5FU-treatment as it was not observed in normal BM. On average, 9.3% ± 0.5% (mean ± SEM) and 9.5% ± 0.5% of the CAFC-10 through CAFC-28/35, in normal and post-5FU BM, respectively, seeded to the spleen, while 1.1% ± 0.1% and 1.2% ± 0.1% of the CAFC, respectively, were recovered per femur (Figures 7.2B and 7.3B). The seeding efficiencies of normal and post-5FU CAFC were not significantly different (p>0,1).

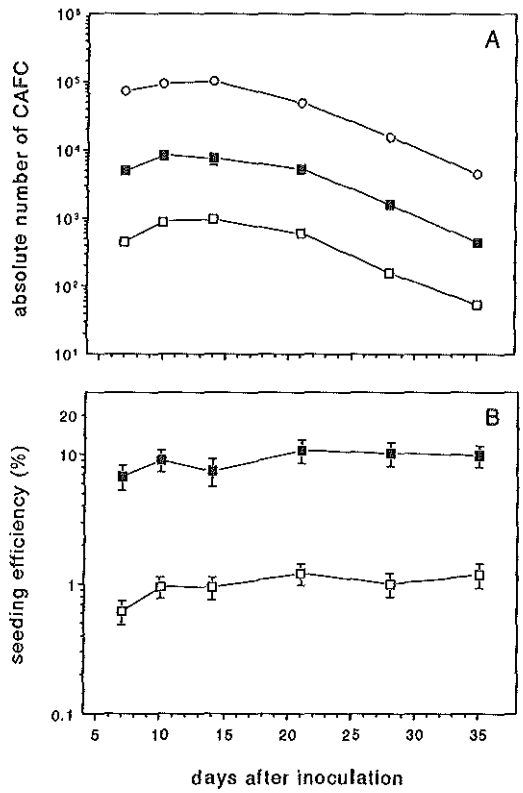

Figure 7.3 Seeding of CAFC subsets from day-6 post-5-fluorouracil low-density (1.069-1.078 g/mL) BM in spleen and femur 16-18 hours after transplantation (4-5 recipients). **(A):** (○) number of CAFC injected per mouse, (■) number of CAFC recovered per spleen, (□) number of CAFC recovered per femur. **(B):** Seeding efficiency (mean ± SEM) of CAFC subsets in spleen (■) and femur (□).

Previously, extensive correlation studies have shown that day-10 CAFC (CAFC-10) enumerate CFU-S-12 numbers, while CAFC-28 through CAFC-35 are related to the long-term repopulating ability of a graft [41,43,48-51]. Therefore, the present results show that the different murine hematopoietic stem cell subsets, ranging from the most primitive long-term repopulating stem cells up to CFU-S-12, in normal as well as in day-6 post-5FU BM, have equal seeding efficiencies.

Seeding of CAFC subsets after growth factor incubation. To test whether a brief preincubation with hematopoietic growth factors would influence the marrow and spleen seeding efficiency of hematopoietic stem and progenitor cells, low-density BM was incubated for 2-3 hours at 37°C with 100 U/mL IL-3, or with a combination of growth factors, and was transplanted into 11.1 Gy irradiated recipients (4-5 mice/group) as indicated in Figure 7.1. The combination of IL-3, IL-12 and SF was included as it has been shown to be more powerful than IL-3 alone in preserving hematopoietic stem cell function in 7-day liquid cultures [52]. The control suspension remained on ice in medium (Dutton with 5% FCS) without additional growth factors. FACScan analysis with propidium iodide showed a viability of 97% in the control, and 87% to 93% in all other groups (data not shown). Compared with the control (group 1), incubation with IL-3 (group 2) or IL-3 + IL-12 + SF (group 3) did not change the number of day-8

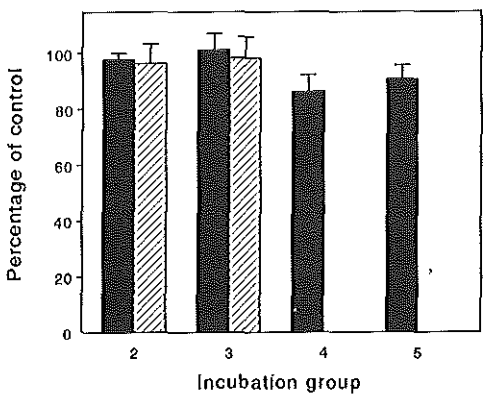

Figure 7.4 Percentage of day-8 CFU-GM (▓) and CFU-S-12 (▒) in low-density BM after 2-3 hour preincubation at 37°C with hematopoietic growth factors, compared to control (group 1) which was kept on ice without added growth factors. (groups as in Figure 7.1). The medium consisted of Dutton containing 5% FCS. Group (2): 100 U/mL IL-3, (3): 100 U/mL IL-3 + 10 ng/mL IL-12 + 100 ng/mL SF, (4): 10 ng/mL IL-12 + SF, (5): no growth factors. Results represent mean values ± SEM from three experiments.

CFU-GM and CFU-S-12, as was shown in the primary assays (Figure 7.4). However, after preincubation in IL-12 + SF (group 4), or in medium without extra growth factors (group 5), the number of CFU-GM was slightly decreased, indicating that IL-3 seems necessary for the survival of progenitors at 37°C. This was roughly reflected by the total cellular viability (>95%, data not shown).

The average of three independent experiments shows that the apparent frequency of primary CAFC was slightly enhanced by preincubation with IL-3 or IL-3 + IL-12 + SF (Figure 7.5A). In spite of this, the CAFC subsets that could be recovered from spleen and marrow after growth factor preincubation, 16-18 hours after transplantation, were at 50% to 70% of control values (Figures 7.5B and C). The overall seeding efficiency of all hematopoietic stem cell subsets tested, calculated from the CAFC frequencies of the graft before transplantation and from BM and spleen suspensions after transplantation, was significantly decreased by growth factor preincubation

(Figures 7.6A and B). A difference between preincubation with IL-3 or IL-3 + IL-12 + SF was not observed.

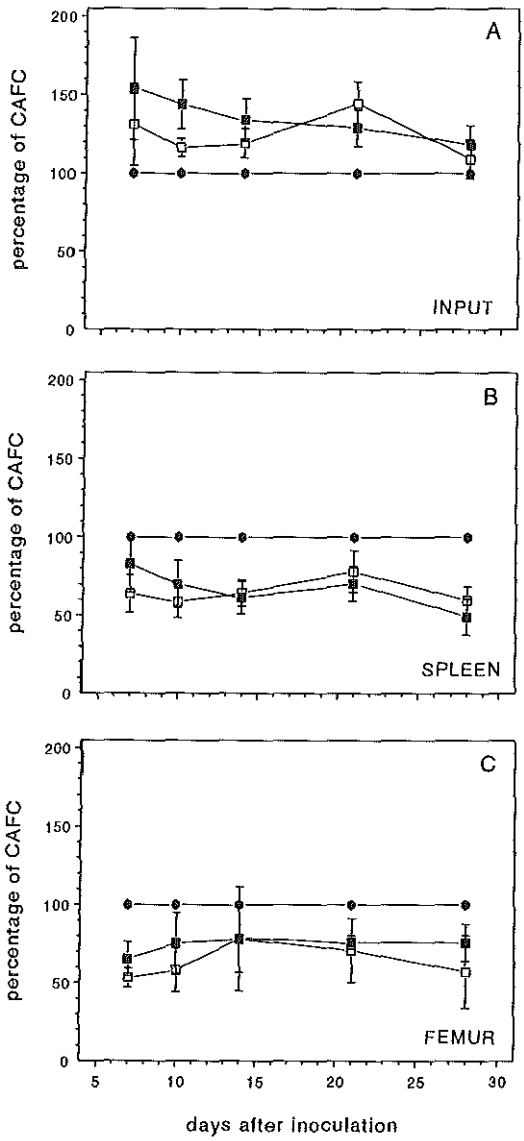

Figure 7.5 **(A):** Percentage of CAFC subsets in low-density BM after preincubation with hematopoietic growth factors (2-3 hours at 37°C) compared to control (kept on ice without growth factors). Numbers of CAFC in the control group (●) are at 100%; (■) 100 U/mL IL-3; (□) 100 U/mL IL-3 + 10 ng/mL IL-12 + 100 ng/mL SF. **(B)** and **(C):** Percentage of splenic and femoral CAFC, 16-18 hours after transplantation of low-density BM into 11,1 Gy irradiated recipients (4-5 per group) after incubation with/without growth factors [legend as in (A)]. Data represent the mean ± SEM from three individual experiments.

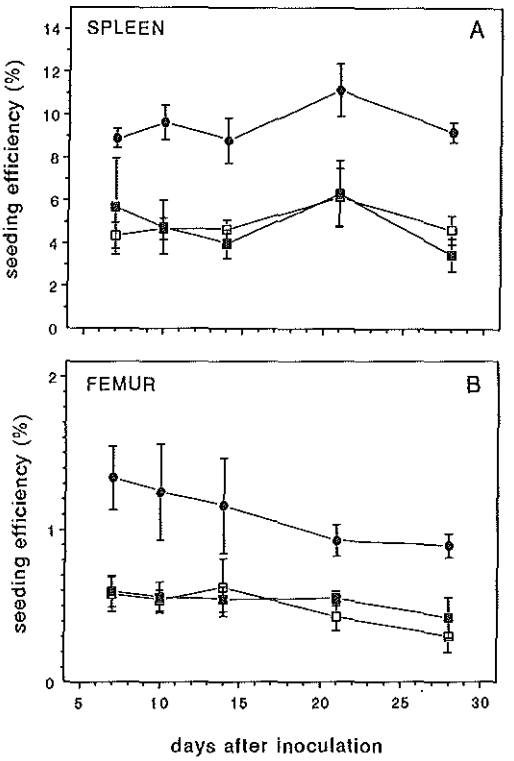

Figure 7.6 Seeding efficiency of CAFC subsets (mean ± SEM) in **(A):** spleen and **(B):** femur, 16-18 hours after transplantation of low-density BM that was incubated with or without hematopoietic growth factors. Control (●) was kept on ice without preincubation, other groups were incubated for 2-3 hours with (■) 100 U/mL IL-3 at 37°C, or (□) 100 U/mL IL-3 + 10 ng/mL IL-12 + 100 ng/mL SF at 37°C. The data from control and incubation groups were analyzed by one-way ANOVA. **(A):** The data from the groups incubated with growth factors differed significantly from the control at all days except at day 7 with p-values ranging from 0.001 to 0.015. At day-7 only the control (●) and IL-3 group (■) differed significantly (p=0.008). **(B):** The control differed significantly from the incubation groups at days 7, 21 and 28 with p values ranging from 0.03 to 0.04.

Seeding of CFU-S-12 after growth factor incubation. The seeding efficiency of CFU-S-12 was also determined on the basis of their number before transplantation and the CFU-S-12 content of femurs and spleen 16-18 hours after transplantation (Table 1). On average, growth factor preincubation also decreased the seeding of CFU-S-12 to the spleen, from 11.4% (group 1) to 7.3% (group-2) or 7.9% (group 3). The lack of statistical difference between group 2 and group 3 versus group 1 in seeding to the femur may be due to the large variation inherent to the spleen-colony assay and retransplantation technique. Spleens and femurs of animals that had been irradiated with

11,1 Gy, but had received no cells, contained no detectable CFU-S-12 nor CAFC in any of the assays performed (not shown).

Long-term *in vivo* repopulating ability. To determine the effect of hematopoietic growth factor preincubation on the stable engraftment ability of stem cells *in vivo*, low-density BM cells from male mice were incubated with and without growth factors (groups 1-3, Figure 7.1) and transplanted in limiting dilution into sublethally irradiated syngeneic female recipients (10 mice per group). At 4 months after transplantation donor-type repopulation was assessed by fluorescent *in situ* hybridization on blood smears of the sex-mismatched chimeric mice using a murine Y chromosome-specific probe (Figure 7.7). The data show that chimerism was lower in the groups preincubated with either IL-3, or IL-3 + IL-12 + SF, when compared with control marrow that was kept on ice throughout the whole incubation.

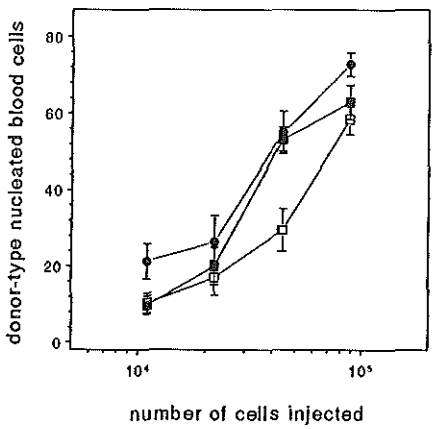

Figure 7.7 Percentage male cells in the peripheral blood of female recipients transplanted with syngeneic male cells incubated with or without hematopoietic growth factors, at 4 months after transplantation. Control (●) was kept on ice without preincubation, other groups were incubated for 2-3 hours at 37°C with (■) IL-3, or (□) IL-3 + IL-12 + SF (concentrations as in Figure 7.6). Chimerism was determined by FISH using a Y chromosome-specific probe. Data represent mean ± SEM of groups of 10 mice (100 cells per slide). As tested by ANOVA, chimerism in the control group (●) differed significantly from chimerism in the recipients of multiple growth factor-incubated BM cells (□), except at the 4,4 x 10^4 cell dose, with p values ranging from 0.007 to 0.076. The control group (●) and IL-3 group (■) differed at the 1,1 x 10^4 and 8,8 x 10^4 cell dose with p=0.09 and p=0.10, respectively.

DISCUSSION

The present study shows that the seeding efficiency of different hematopoietic stem cell subsets, ranging from CFU-S-12 (assessed by the frequency of CAFC-10) to the long-term repopulating stem cells (as assessed by the frequency of CAFC-28/35), can be determined directly without the need for retransplantation studies. The data show that all hematopoietic stem cell subsets tested, in normal as well as in post-5FU low-density BM, have similar seeding efficiencies and that 1.2% of the CAFC could be recovered from a femur while 9.5% seeded to the spleen, which is in agreement with previous estimates using the CFU-S assay [53-56]. As one femur contains about 6% of all BM cells [57], approximately 30% of the intravenously injected hematopoietic stem cells seeded to the two major hematopoietic organs. In addition, we show that a brief 2-3 hour preincubation of BM cells at 37°C in medium containing IL-3, or a combination of IL-3, IL-12 and SF, did not improve but even significantly impaired the seeding of hematopoietic stem cells to both spleen and BM. The *in vivo* engraftment, determined at 4 months after transplantation, was similarly compromised, indicating that the net effect of preincubation of BM cells with hematopoietic growth factors at 37°C is not beneficial for BM transplantation.

In contrast with our findings, it has been suggested that the engraftment of hematopoietic stem cells could be improved by a brief incubation with IL-3 or GM-CSF prior to transplantation [37,39,40]. Fabian *et al.* and Tavassoli *et al.* [37,39] studied the effect of growth factor addition on the engraftment of murine BM cells that were preincubated at 37°C. Unlike in our experiments, these investigators did not include a group that was either non-incubated, or kept on ice. Their data show that numbers of primary CFU-S and CFU-GM were slightly decreased in the 37°C control group compared with the 37°C group that had received IL-3 or GM-CSF [37,39]. In line with their data we observed a loss of CFU-GM after incubation in medium without IL-3. In addition, it has previously been shown that cloned progenitors deprived of growth factors not only undergo apoptosis but also gradually loose their ability to adhere to a stromal layer [58,59]. Consequently, incubation of hematopoietic cells at 37°C without growth factors might influence their capacity to home to hematopoietic organs after intravenous transfer *in vivo*. Therefore, the previously observed beneficial effect of growth factor preincubation may only bear on the survival of stem cells at 37°C rather than demonstrating an increased engraftment ability following preincubation as compared to no incubation. Hence, the difference between their and our findings may be caused by the use of different controls.

In an allogeneic transplantation setting, using T-cell depleted, major histocompatibility complex-disparate donor bone marrow, the effects of growth factor preincubation seem even more complex. A brief ex vivo pre-incubation with GM-CSF facilitated engraftment across extensive histocompatibility barriers [60]. In contrast, using the same transplantation model these investigators found a lower engraftment after

preincubation with IL-3 [61]. In both studies seeding efficiencies were not determined. Because these effects might have been related to Graft-versus-Host disease the results are difficult to interpret.

The effect of ex vivo growth factor preincubation has reportedly also been tested using sheep fetal liver cells that were transplanted *in utero* [40]. A 16 hour incubation of fetal liver cells with phytohemagglutinin-stimulated sheep leukocyte-conditioned medium enhanced their multilineage long-term repopulating ability compared with freshly transplanted cells. However, compared to the BM transplantation performed in the present study, some differences exist with respect to the kinetic status of the stem cells and the specific microenvironment that the cells home to. Unlike normal BM stem cells, fetal liver hematopoietic stem cells have been shown to be in a proliferative state, and have an immediate and absolute requirement for cytokines to protect them from apoptosis [62,63]. Consequently, they might differ in their response to a growth factor preincubation. In addition, when transplanted in fetal sheep at 48 to 52 days of gestation [40], hematopoietic stem cells home exclusively to the fetal liver and spleen, and not to the BM [64,65]. The homing of stem cells in the fetus, and interaction with their specific microenvironment, may be determined by different processes as the ones operative in the adult. It has been documented that BM and fetal liver stromal cells exert different influences on the differentiation of murine B cell progenitors [66]. In addition, differences have been found with respect to the effect of steroids on the amount of hyaluronic acid expressed by human fetal liver and BM stromal cells [67]. As the concentration of hyaluronic acid is directly related to the capacity of the stroma to bind hematopoietic stem cells [67,68], steroids might play a role in the homing of intravenously injected stem cells in the fetus.

In conclusion, the intravenous transplantation of BM cells, as described in the present study, and the in utero transplantation of fetal liver cells, as described previously [40], are difficult to compare as the cells not only have a different sensitivity for hematopoietic growth factors, but also home to different organs with qualitatively different microenvironments. Consequently, the receptors involved in the homing, and the modulation of their expression, may differ between the two models. This is supported by the observation that in the adult homing receptor protein expression is organ specific [33,35].

In summary, the present study shows that all subsets of CAFC have equal bone marrow and spleen seeding efficiencies. These subsets include the CFU-S-12 and the short and long-term *in vivo* repopulating stem cells as has been extensively demonstrated by correlation studies [41,43,48-51]. In addition, the present study shows that a 2-3 hour incubation of mouse BM cells with IL-3 or IL-3 + IL-12 + SF prior to transplantation significantly impairs the seeding efficiency of CFU-S-12 as well as more primitive hematopoietic stem cell subsets. This was accompanied by a loss of long-term *in vivo* engrafting ability as was demonstrated at 4 months after transplantation. The

lack of a beneficial effect of preincubation on the long-term *in vivo* engraftment of mouse BM stem cells questions a general application of preincubation for BM transplantation.

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CHAPTER 8

GENERAL DISCUSSION

8.1 Short and long-term repopulating stem cells subsets

Since the discovery of the CFU-S in 1961 [1] many researchers in the field of experimental hematology have focussed on the purification of the pluripotent hematopoietic stem cell [2-5]. The experimental work described in this thesis supports and extends the notion that phenotypically distinct long and short-term repopulating stem cell subsets exist in the bone marrow of the mouse. Using differences in rhodamine-123 (Rh123) retention and wheat germ agglutinin (WGA) affinity we were able to separate these subsets and demonstrate that the vast majority of CFU-S-12 belong to the subset of short-term repopulating stem cells. The purification procedure for long-term repopulating stem cells could be improved and simplified by the addition of mAb ER-MP12. Like WGA, ER-MP12 positively identifies stem cells and progenitors, and allows separation of these subsets on the basis of their primitivity by differences in antigen expression. In combination, WGA and ER-MP12 prove to be powerful probes that have several advantages when compared to other methods used to highly enrich murine hematopoietic stem cells [4,5]. The method of sorting low density cells on the basis of a low affinity for WGA and intermediate expression of the ER-MP12 antigen (1) enables high enrichment factors for LTRA cells (840-fold when compared to unseparated BMC) without pretreatment of the donor animals with 5-fluorouracil; (2) does not involve the use of intracellular dyes, which could exert toxic effects; and (3) makes the use of a panel of lineage-specific mAbs that are required for the depletion of mature bone marrow cells (BMC) redundant as they are simultaneously excluded in the procedure. This not only significantly simplifies the purification procedure, but also reduces the risk of depleting stem cells that express low levels of lineage markers.

The existence of intrinsically distinct short and long-term repopulating stem cell subsets seems to argue against a stochastic model of hematopoietic stem cell renewal. Moreover, it favors a model of a hierarchically organized stem cell compartment in which successive stem cell clones proliferate and gradually loose their self-renewal capacity as they mature, to finally being replaced by new clones from the original stem cell pool [6-9]. The stochastic model was originally used to explain the differentiation of *in vitro* colony-forming cells [10,11]. Recent studies on the *in vivo* kinetics of retrovirally marked hematopoietic stem cell clones also seemed to demonstrate a random behavior [12-18]. In these studies, the first 4-5 months after bone marrow transplantation were found to be characterized by frequent temporal changes in the number of clones that contributed to hematopoiesis, a condition that was indicated as "clonal instability". It was demonstrated that the longevity of individual stem cell clones could not be predicted. A given stem cell could stay dormant for a long period of time, increase or decrease its proliferative activity, or become completely inactive or even lost. It was suggested that hematopoietic stem cells might form a homogeneous population that exhibits a random and heterogeneous

kinetic behavior [19]. However, clones never reappeared as would have been the case in a true stochastic system, but all disappeared after a shorter or longer period of time. In addition, the fact that short and long-term repopulating stem cell clones can be physically separated (*Chapters 2 and 3* and refs. [20-22]) also argues against such a homogeneous stem cell pool and favors the model of 'clonal succession' which was originally described by Kay in 1965 [6].

In the present report we use the term "long-term repopulating ability" (LTRA) to describe the ability of a cell population to induce a stable chimerism for a period of at least 4 months. The first 4 months after engraftment are dominated by the contribution of multiple short-term repopulating clones such as CFU-S-12 and other more committed progenitors [23-26]. Only after 4-5 months has a stable hematopoietic system been reached [12-18]. Cells defined in this way as long-term repopulating hematopoietic stem cells, however, do not form a homogeneous population. As already mentioned, individual stem cell clones have different life-spans [15,17-19]. Furthermore, LTRA cells were found to be heterogeneous in density as was show by counterflow elutriation [27]. In addition, they were shown to differ in the expression of cell surface markers such as CD4 and AA4.1 [27], and in the expression of the IL-3 receptor-associated antigen [28]. It was suggested that these differences in marker expression and density reflected differences in quiescent and proliferating LTRA cells. Furthermore, mouse bone marrow was shown to contain a subset of LTRA cells that is not readily triggered into cell cycle by repeated 5-fluorouracil injections *in vivo*, suggesting the existence of different levels of quiescence among hematopoietic stem cells [29]. In this light, and given the longer life-span of humans as compared to mice, it will be of interest to investigate whether the current definition of hematopoietic stem cells, when applied to human cells transplanted in SCID mice *in vivo*, or in the LTC-IC [30,31] or CAFC [32] assays *in vitro*, will be sufficient to define and describe the most primitive *in vivo* repopulating stem cells in man.

8.2 The bone marrow prothymocyte

In the experiments described in *Chapter 5* we identified a population of mouse bone marrow cells that is highly enriched for cells with thymus-homing and -repopulating ability, as was tested by intravenous and intrathymic transfer, respectively. However, this subset was not committed to the T-cell lineage. The intriguing question whether thymocytes differentiate directly from multipotent hematopoietic cells that seed the thymus, or whether commitment takes place in the bone marrow before migration, has in fact not yet been answered. The most immature T cell precursors in the thymus, that still have their T-cell antigen receptor genes in a germline state, have been phenotypically identified by a low expression of CD4 and Thy-1, the absence of lineage markers (Lin^-), and expression of the markers Sca-1,

stem cell antigen-2 (Sca-2), Pgp-1 (CD44), heat stable antigen (HSA) and the major histocompatibility class I protein (abbreviated as $CD4^{lo}$ $Sca\text{-}2^{+}$ $Thy\text{-}1^{lo}$ Lin^{-} $Sca\text{-}1^{+}$ $Pgp\text{-}1^{+}$ HSA^{+} $H\text{-}2K^{+}$) [33,34]. Upon intravenous transfer this intrathymic "low-CD4" precursor was shown to be restricted to the lymphoid lineages [35], although upon transfer into an irradiated thymus also dendritic cells were formed [36]. The intrathymic low-CD4 precursor is phenotypically identical to the previously described population of CFU-S-12 and multipotent hematopoietic stem cells, except for the expression of Sca-2 [37]. Recently, a population of $Sca\text{-}2^{+}$ low-CD4 precursors was purified from bone marrow [38]. Although high in thymus-homing and thymus-repopulating ability, these cells were also found to be multipotent and not restricted to the lymphocyte lineages. These observations indicate that there might not be a T-cell restricted precursor in the bone marrow. Based on these data one could speculate that T-cell commitment only takes place in the thymus. Alternatively, bone marrow lymphocyte precursors equipped to home to the thymus could also loose their multilineage character very late, possibly even upon entering the thymus or through contact with thymus epithelial cells. For now, a "prothymocyte" in the bone marrow of the mouse, which is by definition committed to the T-cell lineage, has not yet been phenotypically identified. The question whether or not such a cell exists will remain a subject for further research .

8.3 Determination of hematopoietic stem cell subsets in the CAFC assay

The cobblestone area-forming cell (CAFC) assay, as indicated in the introduction, is a long-term bone marrow culture (LTBMC) that allows a frequency analysis of both short and long-term repopulating hematopoietic stem cell subsets [39,40]. Frequencies of the different subsets are based on the differences in the initiation and duration of clonal expansion on an irradiated stromal layer. Although originally designed for mouse, current data obtained in our department show that the assay can easily be adapted for rat, monkey and man, with only minor technical changes, such as the addition of species-specific growth factors and the use of stromal cell lines. In addition, the availability of drug resistent stromal cell lines makes the assay a valuable tool in assessing the efficacy of gene transfer in different stem cell subsets when applied for frequency analysis under selectable conditions (*Figure 8.1*). Although it cannot be proven directly that the cell capable of inducing a stable engraftment is identical to the CAFC measured at week-4/5 in the murine CAFC-assay, extensive correlation studies with sorted cells of extreme subset ratios (in different murine transplantation models) indicate that the assay is likely to determine frequencies and abilities of the different hematopoietic stem cell subsets, including the *in vivo* engrafting stem cells [39-45]. Frequency estimates of sorted human BMC, as compared to data from other laboratories, indicate that primitive human hematopoietic stem cells are able to generate CA as of 5 weeks after inoculation [30,32,46]. More extensive correlation studies between the frequencies of human CAFC subsets and

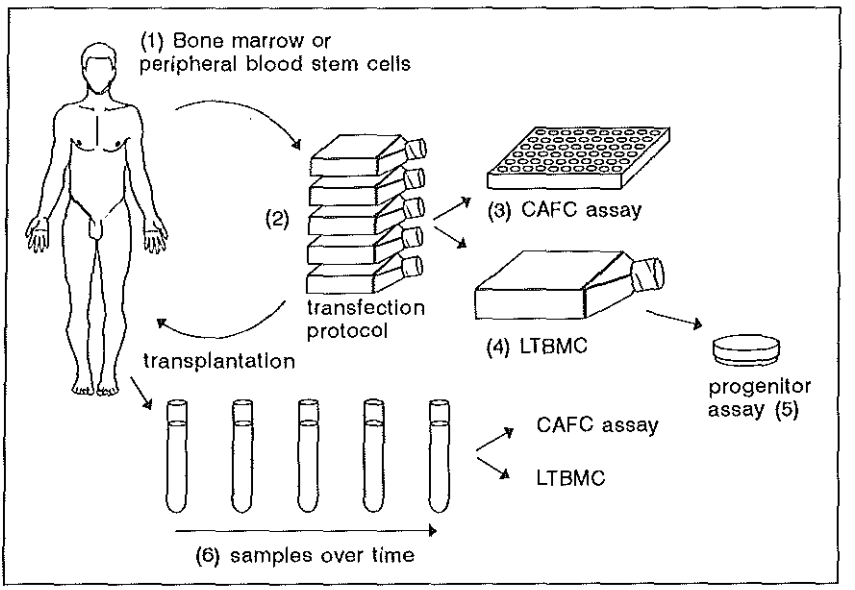

Figure 8.1 Schematic presentation of the application of CAFC and LTBMC in gene therapy. (1) Hematopoietic cells are harvested from either bone marrow or peripheral blood. After retroviral transfection (2) cells are tested for CAFC under normal and selectable conditions (provided that a gene for positive selection is included in the viral DNA) to determine the survival of CAFC subsets and to calculate the transfection efficiency. The proliferative ability of the transfected cells can be tested (4) in the LTBMC. After 5 weeks the cells are harvested from the cultures and tested for progenitors (5). When transfected cells are given back to the patient the CAFC assay and LTBMC can be used routinely in the follow up to monitor the engraftment.

their ability to generate sustained engraftment *in vivo* are currently being carried out at department of Hematology of this University. In addition, the CAFC assay is being tested for future application as a stem cell assay using hematopoietic cells from various sources and from patients with a different background and disease history. A brief summary of the potential future applications of the CAFC assay is given in *Table 8.1*.

8.4 Implications for bone marrow transplantation and gene therapy

Short and long-term repopulating stem cell subsets play different roles in bone marrow transplantation. By definition, the short-term repopulating stem cells are responsible for an early hematopoietic recovery after transplantation, while the long-term repopulating stem cells are responsible for a stable engraftment and long-term survival [23-26]. A change in the frequency and/or viability of different hematopoietic

stem and progenitor cell subsets in the graft may seriously affect the outcome of a transplantation. Manipulations that might influence hematopoietic stem and progenitors include the handling, collection and storage of the BMC; the use of tumor purging and stem cell purification protocols; as well as the systems designed for the transfer of genes into primitive hematopoietic cells. In addition, also changes in the conditioning of the transplant recipient, that result in changes in the survival of endogenous stem cell subsets, may influence the outcome of transplantation since they compete with the stem cells that are transplanted. This will be illustrated in the following.

Mouse studies have shown that the primitive and more mature hematopoietic stem cell subsets, as defined in the CAFC assay and by chimerism studies in glucose phosphate isomerase congenic mice, have different sensitivities for conditioning regimens like gamma radiation and cytotoxic drugs [26,41,47]. Treatment of mice with 5-FU, for instance, reduces progenitors and short-term repopulating cells to less than 1% of their initial number while long-term repopulating cells are virtually unaffected. In contrast, treatment of mice with the cytostatic busulfan reduces the most primitive cells in the bone marrow over a hundred-fold more than the mature progenitors [26]. Another example is the application of low dose rate versus high dose rate total body irradiation and the use of a fractionated instead of single dose radiation, modifications designed to spare the more sensitive tissues in the body such as the lung and the epithelial lining of the digestive tract. It has been shown that these modifications did not benefit the short-term repopulating stem cell subsets, such as CFU-S and early appearing CAFC [41,47]. On the other hand, it did result in a significant sparing of long-term repopulating stem cells as a result of an appreciable level of sublethal damage repair [41,47]. On the downside, the application of low dose rate or fractionated irradiation may seriously affect the outcome of a bone marrow transplantation. It could, for instance, decrease the level of engraftment due to the survival of endogenous hematopoietic stem cells in the transplant recipient. However, the clinical importance of the presence of residual stem cell subsets and the level of chimerism that is required after transplantation depends on the situation. While the induction of partial hematopoietic chimerism can clinically still be relevant, as is the case in thalassemia, the survival of residual hematopoietic cells in leukemia is potentially dangerous as they may include leukemic clones.

Gene therapy has been suggested for many single gene disorders using retroviral tranfection of hematopoietic stem cells [48-50]. However, the preservation of long-term repopulating stem cells *in vitro,* under conditions that allow the transfer of genes into these cells and a stable integration into their genomic DNA, is one of the technical issues that still need to be resolved [51]. Hematopoietic stem cells need to be replicating in order to become transfected [52]. The control of self-renewal and differentiation, however, is still poorly understood. *In vivo,* this process is assumed to be controlled by multiple hematopoietic growth factors and/or inhibitors that are

either secreted by stromal cells or bound to components of the extracellular matrix [53]. Although some combinations of growth factors have shown to support the survival of short-term repopulating stem cells in a serum- and stroma cell-free culture [54], the combination that preserves long-term repopulating cells still remains to be found.

An example of graft manipulation, which requires close monitoring of engrafting stem cells, is the use of long-term bone marrow cultures (LTBMC) to purge bone marrow for autologous bone marrow transplantation in acute myeloid leukemia (AML) [55-57], as well as in chronic myeloid leukemia (CML) [58-61]. The results, however, have been variable. This could be attributed to variation in the relative number of leukemic and normal clones in blood and bone marrow among different patients and to the heterogeneity of the leukemic cell clones themselves with respect to their sensitivity for culture conditions in LTBMC [56,62-66]. A further complication common for purging bone marrow in LTBMC is the loss of long-term engrafting stem cell subsets during culture. Studies with mouse bone marrow have shown that only a limited number of long-term repopulating cells survive in LTBMC [42,67,68]. This seems to contrast with reports on the apparent maintenance, or even expansion, of primitive human hematopoietic cells in human cultures [69,70]. However, stem cells in the latter studies were identified by their phenotype and number of HPP-CFC, but not by frequency analysis of long-term culture-initiating cells [42,67,68]. Colony assays have previously shown to be unable to measure the amplification, maintenance or loss of primitive murine CAFC in liquid cultures [71]. In conclusion, culture conditions in LTBMC significantly impair the survival of leukemic cells and favor the growth of normal progenitors, apparently at the expense of the most primitive hematopoietic stem cells.

Bone marrow can also be manipulated with hematopoietic growth factors prior to transplantation. It has been shown that the expression of a 110 kD cell surface glycoprotein, which is involved in the homing of murine hematopoietic cells to the bone marrow [72-74], could be upregulated by hematopoietic growth factors [75,76]. Based on experimental studies *in vivo*, using mice, sheep and monkey's, it was suggested that a short preincubation of bone marrow with IL-3 and GM-CSF prior to transplantation would enhance its engraftment compared to non-incubated BMC that were kept on ice [75-78]. However, in our hands (*Chapter 7*) preincubation of murine BMC with IL-3 or a combination of IL-3, KL and IL-12, did not benefit its engraftment. In contrast, the seeding of all stem cell subsets (CAFC), CFU-S-12 and the long-term *in vivo* engraftment of the transplant were significantly impaired when compared to non-incubated bone marrow. Our findings therefore argue against a general application of preincubation for transplantation.

These examples illustrate that it would be useful for the clinician to be able to evaluate the effects of manipulation of hematopoietic cells prior to transplantation, or

monitor the progress of the graft after transplantation. This requires assays that enable the analysis of short as well as long-term repopulating stem cell subsets (*Table 8.1*). Under normal conditions, numbers of progenitors and phenotypically identifiable immature cells in the bone marrow or peripheral blood have shown to correlate highly with the long-term performance of a graft. However, these figures loose much of their predictive value under conditions that changed the ratio of short and long-term repopulating stem cell subsets. Mathematically, the most primitive hematopoietic stem cells and their progeny will always correlate as long as they constitute dependent variables, which is the case in a balanced system. In a perturbed hematopoietic system the numbers of progenitors might not adequately reflect the size of the more primitive hematopoietic clone. This can be illustrated with some case reports on graft failure in peripheral blood stem cell transplantation. Despite an initial rapid engraftment and high concentration of CFU-GM, some of the grafts failed, apparently due to a lack of hematopoietic stem cells [79]. To determine the frequencies of both short and long-term repopulating stem cell subsets, and to assess the proliferative ability of these subsets, the human CAFC and LTC-IC assays are currently the best tools available.

Table 8.1 Future potential applications for the human cobblestone area forming cell (CAFC) assay and long-term bone marrow culture (LTBMC)

1	Transplantation	To calculate the size of a transplant (BMC, umbilical cord blood, leukapheresis material) based on the number of CAFC per kg body weight that need to be transplanted.
2	Phenotyping	To determine the phenotype of repopulating stem cell subsets by correlating marker analysis (FACScan) with CAFC content of leukapheresis material after transplantation.
3	Diagnosis	To determine the number of "normal" CAFC in patients with aplastic anemia or acute or chronic myeloid leukemia (AML/CML), combined with cytogenetic analysis of the progenitors in the supernatant.
4	Treatment	To determine the toxicity of a cytostatic treatment or irradiation procedure on hematopoietic stem cell subsets and stromal cells *in vitro* and *in vivo* (CAFC and LTBMC), and to determine the number of CAFC after purging bone marrow for ABMT.
5	Gene therapy	To determine the transfection frequency and the survival of CAFC subsets after transfection. The ability of CAFC to generate CFU-C is determined by replating using the LTBMC.
6	Culture	To determine the frequency of CAFC after culture *in vitro* in the search for conditions that allow an *ex vivo* expansion of primitive hematopoietic stem cells.

The ability to purify hematopoietic stem cells depends strongly on the ability to identify their subsets in specialized assays. Therefore, an assay like the human CAFC assay will no doubt benefit the development of more efficient methods to purify human hematopoietic stem cells. In addition, it will learn us more about the conditions needed to manipulate a bone marrow graft *ex vivo* and to preserve, or possibly even, to extend its long-term engrafting potential.

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SUMMARY

Hematopoietic stem cells form a small but heterogeneous population of bone marrow cells (BMC) that are responsible for the blood cell formation in the body. The most primitive hematopoietic stem cells are characterized by their ability to multiply extensively without apparent differentiation. In addition, these cells can be identified by their capacity to form progeny in all blood cell lineages for an extended period of time. These lineages include the red blood cells, that are primarily responsible for the exchange of oxygen and carbon dioxide; the different types of white blood cells, that are involved in maintaining the bodies' defense mechanisms against infections and foreign materials; and the platelets, that play a key role in the process of coagulation. Mouse studies have shown that a single stem cell can actively participate in blood cell formation for a period as long as 2½ year. When stem cells finally mature they gradually loose their ability to self-renew and ultimately commit to one of the lineages to form mature blood cells. Under normal conditions, however, most stem cells are resting, or quiescent, while only a few contribute actively to blood cell formation. The first assay that was assumed to measure pluripotent self-renewing hematopoietic stem cells dates back to 1961. Bone marrow, when intravenously injected into irradiated mice, was shown to contain cells that were able to form macroscopic nodules on the surface of the spleen at 7 to 12 days after injection. These cells were called spleen colony-forming units (CFU-S). Experiments performed in the subsequent 30 years on the nature of these "stem cells" revealed that they were not the most primitive cells in the bone marrow. In fact, CFU-S were found to represent a heterogeneous and intermediate population. The most primitive hematopoietic cells were functionally defined by the ability to repopulate all hematopoietic lineages for a period of at least 4 months upon intravenous injection. Therefore, these cells are designated as cells with long-term repopulating ability (LTRA). The ability to prevent animals from radiation-inflicted death, which has frequently been used as a definition of stem cells, may not be a property of the most primitive stem cells. This ability most likely results from the combination of more rapidly short-term repopulating (STRA) cells like CFU-S, that can fulfil the immediate need for new blood cells, and LTRA cells that replace the hosts lost stem cells and provide a stable engraftment. The purpose of the work described in this thesis was to phenotypically characterize and highly purify LTRA cells in the bone marrow of the mouse, and physically separate them from the more mature STRA cells.

It has been shown that murine hematopoietic stem and progenitor cells can be distinguished from other bone marrow cells by the fact that they can bind the lectin wheat germ agglutinin (WGA). The experiments described in *Chapter 2* support and extend the previous finding that LTRA cells can be distinguished from CFU-S on the basis of WGA affinity. Using a wide spectrum of *in vitro* and *in vivo* assays, we show that WGA affinity is an inverse function of the primitiveness of the stem cells and that LTRA cells can be physically separated from the majority of day-12 CFU-S. To distinguish the donor cells from the cells of the recipient we used male cells that were

transplanted into female recipients. The level of chimerism in the peripheral blood was determined by *in situ* hybridization using a Y-chromosome-specific probe. In addition, the transplanted bone marrow was also tested for the presence of cobblestone area forming cells (CAFC) in the CAFC assay (*see paragraph 1.2.4*). Comparison of the *in vivo* and *in vitro* data shows that the frequency of the day-28 and day-35 CAFC (CAFC-28 and CAFC-35) in the transplanted bone marrow correlated strongly with the number of cells that had to be injected to induce a level of 40% donor-type chimerism. This indicates that the frequencies of CAFC-28/35 can be used to predict the *in vivo* LTRA of a graft.

Primitive and more mature hematopoietic stem cell subsets differ in their retention of the mitochondrial dye rhodamine-123 (Rh123). Using Rh123 as well as WGA, we obtained a separation of the majority of short and long-term repopulating stem cell subsets (*Chapter 3*). The *in vivo* repopulating ability of the different subsets was determined using a recently developed mouse model for α-thalassemia. Donor derived red blood cells could easily be distinguished against the background of a microcytic anemia. To be able to evaluate the model we also included a sex-mismatch between donor and recipient. The results show that a highly purified population of CFU-S-12 is capable of initiating a short but not a long-term reconstitution *in vivo*. On the other hand, a population highly enriched for more primitive hematopoietic cells was able to induce a stable multilineage chimerism. This population contained very low numbers of CFU-S-12. Therefore, it could be concluded that CFU-S-12 and LTRA cells belong to separate subsets in the bone marrow that can be physically separated and highly enriched. The α-thalassemic chimeric mouse model that was used in this study proved to be extremely useful for the determination of chimerism in large groups of mice. As red blood cell chimerism could be determined directly on the FACScan, it enabled a rapid processing of blood samples without the need for immunochemical staining or molecular biological techniques. In addition, the study showed that red blood cell chimerism in α-thalassemic mice can be used as a reliable index for chimerism at the level of the hematopoietic stem cell.

Monoclonal antibody ER-MP12 has been shown to recognize a novel antigen on mouse hematopoietic cells (*Chapter 4*). The expression of the antigen appeared to be related to the primitiveness of the hematopoietic cells. The data show that the antigen was intermediately expressed by LTRA cells and CAFC-28/35. With differentiation, however, the ER-MP12 antigen expression increased to reach a maximum in the *in vitro* clonable progenitors (CFU-C). During final maturation the antigen disappeared from most lineages except from mature B and T lymphocytes. Antibody ER-MP20, which recognizes the antigen Ly-6C on granulocytes, monocytes and their direct committed progenitors, was also included in the study. The results show that most progenitors and virtually all CFU-S-12 and LTRA cells do not express the ER-MP20 antigen. Using ER-MP12 and ER-MP20 in two-color immunofluorescence analysis

BMC could be separated into six phenotypically distinct subpopulations. The data show that the different mature cells in the bone marrow could be identified on the basis of their differential expression of the ER-MP12 and ER-MP20 antigens.

The six subpopulations of BMC that could be identified in two-color immunofluorescence analysis with antibodies ER-MP12 and ER-MP20 were also tested for T cell progenitors using intravenous and intrathymic transfer into sublethally irradiated animals (*Chapter 5*). The results show that thymus-homing and repopulating cells were almost completely confined to the subset that highly expressed the ER-MP12 antigen but lacked the expression of the ER-MP20 antigen. The antigens did not allow a separation of T cell progenitors from B cell and myeloid progenitors. FACScan analysis revealed that the subset was heterogeneous with respect to the expression of Thy-1, Sca-1, Pgp-1/CD44, B220 and *c-kit*. These results provide a basis for a further separation of T cell progenitors from the other hematopoietic activities.

To investigate whether ER-MP12 could be used to improve the purification of hematopoietic stem cells, we separated our previously defined WGA^{dim} and $Rh123^{dull}$ hematopoietic stem cell subsets on the basis of ER-MP12 antigen expression (*Chapter 6*). The results show that LTRA cells could be highly enriched by sorting low density BMC on the basis of a low affinity for WGA and intermediate expression of the ER-MP12 antigen. In this procedure, mature BMC were simultaneously excluded. In summary, the approach using the combination of ER-MP12 and WGA is simpler than most other purification protocols and is an improvement compared to our previously used methods.

The last chapter (*Chapter 7*) deals with the seeding of the different hematopoietic stem cell subsets to bone marrow and spleen. The seeding efficiencies of these subsets were determined by comparing the number of CAFC and CFU-S-12 in the original transplant with the number of CAFC and CFU-S-12 retrieved from bone marrow and spleen after intravenous injection. The data show that all hematopoietic stem cell subsets had equal seeding efficiencies. It had previously been suggested that a short pre-incubation of bone marrow with IL-3 or GM-CSF could enhance the engraftment when compared to bone marrow that had been kept on ice. To test this hypothesis bone marrow was either pre-incubated at 37°C with IL-3, with a combination of IL-3, IL-12 and SF, or was kept on ice without pre-incubation. However, no beneficial effect of pre-incubation on the seeding could be observed. The seeding efficiencies of all stem cell subsets, as well as the long-term *in vivo* engraftment, were significantly impaired.

SAMENVATTING

Hematopoietische stamcellen vormen een kleine maar heterogene populatie cellen in het beenmerg die samen verantwoordelijk zijn voor de bloedcelvorming in het lichaam. De meest primitieve hematopoietische stamcellen worden gekenmerkt door hun vermogen zich enorm te kunnen vermenigvuldigen zonder te differentiëren. Ook kunnen zij worden gekenmerkt door hun vermogen om gedurende een zeer lange tijd nakomelingen te kunnen genereren in alle typen van bloedcellen. Dit zijn onder andere de rode bloedcellen, die voornamelijk verantwoordelijk zijn voor het transport en de uitwisseling van zuurstof en stikstof; de verschillende typen witte bloedcellen, die betrokken zijn bij de verdediging van het lichaam tegen infecties en het herkennen van lichaamsvreemde stoffen; en de bloedplaatjes die een belangrijke rol spelen bij de bloedstolling. Studies bij de muis hebben laten zien dat een enkele stamcel in staat is om 2½ jaar lang actief bij te dragen aan de bloedcelvorming. Als stamcellen uiteindelijk uitrijpen verliezen ze geleidelijk hun vermogen tot -letterlijk vertaald- zelfvernieuwing, en groeien uit tot volwassen bloedcellen. Onder normale omstandigheden bevinden de meeste stamcellen zich in een fase van rust, terwijl slechts enkele actief bijdragen aan de bloedcelvorming. De eerste techniek waarmee men dacht primitief hematopoietische cellen te kunnen aantonen, stamt uit 1961. Beenmerg, wanneer intraveneus ingespoten in bestraalde muizen, bleek cellen te bevatten die knobbels konden vormen op het oppervlak van de milt, 7 tot 12 dagen na transplantatie. Deze cellen werden functioneel gedefinieerd als milt-kolonie vormende eenheden, of -in het engels- 'spleen colony-forming units' (CFU-S). Onderzoek naar de eigenschappen van deze "stamcellen", verricht in de afgelopen 30 jaar, maakte duidelijk dat dit niet de meest primitieve stamcellen waren. De CFU-S bleek in feite een zeer heterogene groep hematopoietische cellen te vertegenwoordigen. De meest primitieve hematopoietische stamcellen werden uiteindelijk functioneel gedefinieerd door hun vermogen bij te dragen aan de vorming van alle typen bloedcellen gedurende een periode van ten minste 4 maanden na transplantatie. Deze stamcellen worden daarom ook wel aangeduid als cellen met een lange termijn repopulerend vermogen, of 'long-term repopulating ability' (LTRA). Het vermogen om dieren te beschermen tegen de invloed van bestraling, een eigenschap die regelmatig is gebruikt om hematopoietische activiteit te meten, is waarschijnlijk geen eigenschap van stamcellen alleen. Dit vermogen lijkt eerder het resultaat te zijn van de combinatie van korte-termijn repopulerende cellen zoals CFU-S die kunnen voorzien in de onmiddellijke behoefte aan nieuwe bloedcellen, en LTRA cellen die de verloren gegane stamcellen van de ontvanger kunnen vervangen en zorg dragen voor de bloedcelvorming op de lange termijn. Korte-termijn repopulerende cellen worden aangeduid als cellen met 'short-term repopulating ability', ofwel STRA. Het doel van het onderzoek dat is beschreven in dit proefschrift was om LTRA cellen te zuiveren uit het beenmerg van de muis, deze cellen fenotypisch te karakteriseren, en fysisch te scheiden van de meer ontwikkelde STRA cellen.

Het was reeds bekend dat hematopoietische stamcellen en voorlopercellen bij de muis kunnen worden onderscheiden van andere beenmergcellen, doordat ze in staat zijn tarwekiem-lectine, ofwel 'wheat germ agglutinin' (WGA), te binden op hun oppervlak. De experimenten beschreven in *Hoofdstuk 2* laten zien dat LTRA-cellen kunnen worden onderscheiden van CFU-S, gebaseerd op verschillen in WGA binding. Gebruikmakend van verschillende *in vitro* en *in vivo* assays laten we zien dat WGA binding omgekeerd gerelateerd is aan de primitiefheid van de hematopoietische cellen. Tegelijkertijd wordt gedemonstreerd dat de meeste LTRA-cellen fysisch kunnen worden gescheiden van CFU-S-12. Om na transplantatie de cellen van de donor te kunnen onderscheiden van de cellen van de ontvanger maakten we gebruik van mannelijke donoren en vrouwelijke ontvangers. Het aanslaan van het donorbeenmerg werd gemeten in het perifere bloed met behulp van *in situ* hybridisatie, gebruikmakend van een Y-chromosoom-specifieke probe. Het getransplanteerde beenmerg werd eveneens getest op de aanwezigheid van zogenaamde 'cobblestone area forming cells' (CAFC) in de CAFC assay (*zie paragraaf 1.2.4*). Een vergelijking van de *in vivo* en *in vitro* data laat zien dat de frequentie van de dag-28 en dag-35 CAFC (CAFC-28/35) in het getransplanteerde beenmerg sterk correleert met het aantal cellen dat getransplanteerd moest worden voor het verkrijgen van een 40% niveau donor-type chimerisme. Dit betekent dat de frequentie van CAFC-28/35, die gemeten wordt *in vitro*, gehanteerd kan worden om de *in vivo* lange-termijn repopulatie van een transplantaat te voorspellen.

Primitieve en meer volwassen hematopoietische stamcellen verschillen in hun retentie van de mitochondriale kleurstof rhodamine-123 (Rh123). Gebruikmakend van Rh123 en WGA waren we in staat om de overgrote meerderheid van korte en lange-termijn repopulerende stamcelsubsets van elkaar te scheiden (*Hoofdstuk 3*). Voor de repopulatie *in vivo* werd gebruik gemaakt van een recentelijk ontwikkeld muizemodel voor α-thalassemie. Repopulatie van de ontvanger kon worden gemeten door het verschijnen van normale erythrocyten in een bloedbeeld dat getypeerd is door een microcytaire anemie. Door in de transplantaties eveneens een geslachtsverschil aan te brengen tussen donor en ontvanger kon ook het model zelf worden geëvalueerd. De resultaten laten zien dat een gezuiverde populatie CFU-S-12 in staat is om ontvangers te repopuleren, maar slechts voor een korte periode. Een populatie verrijkt voor meer primitieve stamcellen was wel in staat om een stabiel chimerisme te induceren, zelfs met uiterst lage aantallen CFU-S-12. Op basis hiervan kon worden geconcludeerd dat CFU-S-12 en cellen met LTRA deel uitmaken van verschillende subsets, die fysisch van elkaar kunnen worden gescheiden en onafhankelijk van elkaar kunnen worden gezuiverd. Het muizemodel dat werd gebruikt in deze studie bleek zeer geschikt voor het bepalen van chimerisme in grote groepen van dieren. Het chimerisme in de rode bloedcellen kon direct worden gekwantificeerd met behulp van de FACScan zonder immunochemische kleuringen of het gebruik van moleculair biologische technieken. Tenslotte laat de studie zien dat het rode bloedcel chimerisme in α-thalassemische

muizen gebruikt kan worden als een index voor chimerisme op het niveau van de hematopoietische stamcel.

Monoklonaal antilichaam ER-MP12 herkent een nieuw antigeen op het oppervlak van hematopoietische cellen in het beenmerg van de muis (*Hoofdstuk 4*). De expressie van het antigeen blijkt gerelateerd te zijn aan het ontwikkelingsstadium van de hematopoietische cellen. De data laten zien dat het antigeen op een laag niveau tot expressie wordt gebracht door LTRA cellen en CAFC-28/35. Gedurende de differentiatie neemt het niveau van de expressie toe, tot het een maximum bereikt in de *in vitro* kweekbare voorlopercel, de CFU-C. Het antigeen verdwijnt uiteindelijk van het oppervlak van de meeste cellen en wordt alleen nog op een laag niveau tot expressie gebracht door rijpe B en T lymfocyten. In deze studie werd ook gebruik gemaakt van monoklonaal antilichaam ER-MP20. Dit antilichaam is gericht tegen het antigeen Ly-6C dat aanwezig is op granulocyten, monocyten en hun directe voorlopercellen. De resultaten laten zien dat de meeste voorlopercellen en praktisch alle CFU-S-12 en LTRA cellen dit antigeen niet tot expressie brengen. FACScan analyse van beenmerg dat is aangekleurd met zowel ER-MP12 als ER-MP20 laat zien dat zes verschillende populaties kunnen worden onderscheiden. De data laten zien dat de verschillende populaties volwassen cellen in het beenmerg van de muis getypeerd kunnen worden aan de hand van een verschillende expressie van de ER-MP12 en ER-MP20 antigenen. De populatie die de rijpe lymfocyten bevat eveneens de stamcel.

De subpopulaties in het beenmerg die geïdentificeerd konden worden met behulp van ER-MP12 en ER-MP20 werden ook getest op de aanwezigheid van T cel voorlopercellen. Hierbij werd gebruik gemaakt van intraveneuze en intrathymaire injectie in subletaal bestraalde dieren (*Hoofdstuk 5*). De resultaten laten zien dat cellen die in staat zijn naar de thymus te migreren en de thymus te repopuleren alleen aanwezig zijn in de subset die het ER-MP12 antigeen hoog tot expressie brengt en die tegelijkertijd de expressie van het ER-MP20 antigeen mist. Een onderscheid tussen T cel voorlopercellen en andere differentiatie richtingen kon op basis van deze antigenen niet worden gemaakt. FACScan analyse liet zien dat deze subset heterogeen was met betrekking tot de expressie van Thy-1, Sca-1, Pgp-1/CD44, B220 and *c-kit*. Deze heterogeniteit vormt de basis voor een verdere scheiding van T cel voorlopercellen en andere hematopoietische cellen.

Om te onderzoeken of ER-MP12 gebruikt zou kunnen worden om de zuivering van hematopoietische stamcellen te kunnen verbeteren, scheidden we de voorheen gedefinieerde WGA^{dim} en $Rh123^{dull}$ hematopoietische stamcel subsets in een populatie met een hoge, en een populatie met een lage ER-MP12 antigeen expressie (*Hoofdstuk 6*). De resultaten laten zien dat LTRA cellen hoog kunnen worden gezuiverd door beenmergcellen te isoleren met een laag soortelijk gewicht, die het ER-MP12 antigeen laag tot expressie brengen, en die een lage affiniteit bezitten voor

WGA. Deze procedure heeft als voordeel dat volwassen beenmergcellen niet worden meegezuiverd. Er kan worden geconcludeerd dat de combinatie van WGA en ER-MP12 eenvoudiger is dan de meeste vergelijkbare procedures en dat tegelijkertijd een populatie kan worden verkregen met een relatief hoge zuiverheid aan hematopoietische stamcellen. Vergeleken met de zuivering op basis van WGA alleen vormt de toevoeging van ER-MP12 een verbetering.

Het laatste hoofdstuk (*Hoofdstuk 7*) gaat over het terechtkomen van de verschillende hematopoietische stamcel subsets in beenmerg en milt na transplantatie. De zogenaamde 'seeding efficiency' van deze subsets werd bepaald door het aantal CAFC en CFU-S-12 in het beenmerg transplantaat te vergelijken met het aantal CAFC en CFU-S-12 aanwezig in beenmerg en milt na intraveneuze injectie. De data laten zien dat alle hematopoietische stamcel subsets een gelijke 'seeding efficiency' bezitten. In de literatuur was gesuggereerd dat een korte incubatie van beenmerg met IL-3 of GM-CSF een positieve invloed zou hebben op de 'seeding' van stamcellen. Om deze hypothese te testen werd de seeding van beenmerg dat kortdurend was geïncubeerd bij 37 °C met IL-3, of met een combinatie van IL-3, IL-12 en SF, vergeleken met de seeding van beenmerg dat was bewaard bij 4 °C. Echter, een positief effect van pre-incubatie op de 'seeding' werd niet gevonden. De 'seeding efficiency' van alle hematopoietische stamcel subsets, alsmede de lange-termijn *in vivo* repopulatie, waren allen significant verlaagd.

ABBREVIATIONS

5FU	5-fluorouracil
Ag	antigen
AML	acute myeloid leukemia
BFU-E	erythroid burst-forming unit
BMC	bone marrow cell(s)
BSA	bovine serum albumin
CA	cobblestone area(s)
CAFC	cobblestone area-forming cell(s)
CFC-A	colony-forming unit in agar
CFU-Bl	blast colony-forming unit
CFU-C	colony-forming unit in culture
CFU-D	diffusion chamber colony-forming unit
CFU-E	erythroid colony-forming unit
CFU-G	granulocyte colony-forming unit
CFU-GM	granulocyte macrophage colony-forming unit
CFU-M	macrophage colony-forming unit
CFU-Mast	mast cell colony-forming unit
CFU-Meg	megakaryocyte colony-forming unit
CFU-S	spleen colony-forming unit
CML	chronic myeloid leukemia
CSF-1	colony-stimulating factor 1
ER-MP12^{bio}	biotinylated ER-MP12
ER-MP20^{bio}	biotinylated ER-MP20
ER-MP12^{FITC}	fluoresceinated ER-MP12
ER-MP20^{FITC}	fluoresceinated ER-MP20
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
FLUOS	5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester
$FU_{6d}BM$	day-6 post-5-fluorouracil bone marrow
GαRa-PE	goat anti-rat-Ig conjugated to phycoerythrin
HPP-CFC	high proliferative potential colony-forming cell(s)
HS	horse serum
HSA	heat stable antigen
IL	interleukin
KL	*c-kit* ligand, steel factor, mast cell growth factor
$LD/FU_{6d}BM$	low density day-6 post-5-fluorouracil bone marrow
LD/ER-MP20$^-$	low density ER-MP20-negative bone marrow

Lin^-	lineage marker (CD4, CD8, Gr-1, Mac-1, TER-119) negative
LPP-CFC	low proliferative potential colony-forming cell(s)
LTBMC	long-term bone marrow culture(s)
LTC-IC	long-term culture initiating cell(s)
LTRA	long-term repopulating ability
mAb	monoclonal antibody
MACS	magnetic activated cell sorter
M-CSF	macrophage colony-stimulating factor
MDR	multidrug resistance
MGF	mast cell growth factor, *c-kit* ligand, steel factor, stem cell factor
MRA[CFU-C]	marrow repopulating ability defined as the number of secondary CFU-C in the bone marrow after 13 days, per 10^5 cells injected
MRA[CFU-S-12]	marrow repopulating ability defined as the number of secondary day-12 CFU-S in the bone marrow after 13 days, per 10^5 cells injected
NBM	normal (unseparated) bone marrow
NBMC	normal (unseparated) bone marrow cell(s)
PBS	phosphate-buffered saline
PBS-SA	phosphate-buffered saline containing 0.01% (wt/vol) sodium azide
PHSC	pluripotent hematopoietic stem cell(s)
PWM-MSCM	pokeweed mitogen-stimulated mouse spleen conditioned medium
RαRa-FITC	rabbit anti-rat-Ig conjugated to FITC
RBC	red blood cell(s)
Rh123	rhodamine-123
SAV-PE	streptavidin-conjugated phycoerythrin
SAV-TRI	streptavidin-conjugated TRICOLOR
SCF	stem cell factor, steel factor, mast cell growth factor, *c-kit* ligand
SCID	severe combined immunodeficiency
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SF	steel factor, stem cell factor, mast cell growth factor, *c-kit* ligand
STRA	short-term repopulating ability
TBI	total body irradiation
WBC	white blood cell(s)
WGA	wheat germ agglutinin

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CURRICULUM VITAE

The author of this thesis was born on December 8, 1960 in the city of Eindhoven, The Netherlands. After completion of his secondary education (Atheneum-β) at the Van der Puttlyceum in Eindhoven in 1979, he studied Biology at the State University of Utrecht. After obtaining his Bachelor's degree in Medical Biology in 1983 he participated in research in the field of Comparative Endocrinology at the Department of Experimental Zoology, under the supervision of the Professors Dr. H.J.Th. Goos and Dr. P.W.G.J. van Oordt. This was followed in 1985 by a research period in Biochemistry with Prof. Dr. H. van den Bosch, at the Faculty of Chemistry of the same University. His main research subject was conducted at the Department of Clinical Immunology of the Faculty of Medicine in 1986, which was supervised by Prof. Dr. R.E. Ballieux. In addition, he worked several months as a visiting researcher at the Department of Zoology of the University of Edmonton in Canada. He completed his study by obtaining a Teacher's degree in Biology and a Master's degree of Science in August 1987.

During his military service from 1987 to 1989 he worked as a Reserve Officer of the Dutch Armed Forces at the National Institute for Public Health and Environmental Hygiene where he participated in AIDS research under the supervision of Prof. Dr. A.D.M.E. Osterhaus.

The experimental work described in this thesis was initiated in 1989 at the Institute of Cell Biology II (Prof. Dr. O. Vos) and completed at the Department of Hematology (Prof. Dr. B. Löwenberg) at the Erasmus University in Rotterdam. The work was supervised by Dr. R.E. Ploemacher and supported by the Netherlands Organization for Scientific Research.

Since October 1994, he is employed as a postdoctoral fellow with Dr. D.A. Williams at the Herman B Wells Center for Pediatric Research, James Whitcomb Riley Hospital for Children, Indiana University Medical Center, in Indianapolis, Indiana, U.S.A.

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