

Hematopoietic Stem and Progenitor Cells

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Introduction

Mature blood cells have a finite life-span and must be continuously replaced throughout life. Blood cells are produced by the proliferation and differentiation of a very small population of pluripotent hematopoietic stem cells (HSCs) that also have the ability to replenish themselves by self-renewal (Figure 1). During differentiation, the progeny of HSCs progress through various intermediate maturational stages, generating multi-potential and lineage-committed progenitor cells prior to reaching maturity. Bone marrow (BM) is the major site of hematopoiesis in humans and, under normal conditions, only small numbers of hematopoietic stem and progenitor cells (HSPCs) can be found in the peripheral blood (PB). Treatment with cytokines (in particular granulocyte colony-stimulating factor; G-CSF), some myelosuppressive drugs used in cancer treatment, and compounds that disrupt the interaction between hematopoietic and BM stromal cells can rapidly mobilize large numbers of stem and progenitors into the circulation.

Transplantation of BM or mobilized PB (MPB) cells from related or HLA-matched unrelated donors (so-called allogeneic transplantation) is a potentially life-saving and curative therapy for leukemia and other diseases of the blood and immune system. Autologous transplantation, using the patient's own stem and progenitor cells, has also been found to effectively treat specific disorders, e.g., lymphomas and myelomas, and has been employed as a therapy for other malignancies when allogeneic stem cell transplantation is not possible. Umbilical cord blood (CB) can be collected at birth and cryopreserved, and has become increasingly important as another source of HSPCs for transplantation. The rate of platelet and neutrophil recovery after CB transplantation tends to be slower than after BM or MPB transplantation, due in part to the smaller number of stem and progenitor cells in a typical single-cord graft.

Ongoing research on human hematopoietic cells is directed toward the identification, isolation and characterization of the primitive cell types that mediate rapid and/or sustained hematological recovery after cytoreductive therapy and transplantation. HSPCs are also being investigated in cell-based therapies for non-hematopoietic disorders. This review provides an overview of the current status of HSPC research with a focus on (i) assays used to detect and

enumerate human and mouse stem and progenitor cells, (ii) phenotypic markers and methods used for their identification and isolation, and (iii) culture systems used to amplify stem and progenitor cells or to promote their differentiation in order to produce large numbers of mature blood cells for transfusion.

In Vivo Assays for Hematopoietic Stem Cells

The defining property of a HSC is its ability to reconstitute hematopoiesis following transplantation. This property forms the basis of in vivo assays of HSC function. Transplantation assays performed in mice have proven invaluable for studying murine and human stem cell biology, facilitating an improved understanding of the immunophenotype, homing ability, engraftment properties, cytokine responsiveness and radiation sensitivity of repopulating cells.

Mouse Cells

The hematopoietic potential of mouse HSCs is assayed by injection into mice in which hematopoiesis has been suppressed by irradiation or other methods, and measuring the repopulation of the recipient BM, blood, spleen and/or thymus with donor-derived cells after a period of at least 4 months.^{1,2} Various assay formats have been developed that differ in the choice of donor and host mouse strains, the method to ablate or suppress host hematopoiesis prior to donor cell transplantation, the detection methods used to identify the progeny of donor-derived stem cells, and the endpoints and criteria for "successful" engraftment. In one type of assay, lethally irradiated recipient mice are co-injected with congenic donor-derived "test" cells along with syngeneic (host-type) "competitor" cells to provide short-term radioprotection, ensure survival, and provide a selective pressure to identify stem cells with high competitive repopulating potential. In other assays, host mice are used that have defective endogenous hematopoiesis due to mutations in the c-Kit gene (e.g. W/W^v or W41/W41 mice). After sublethal irradiation these animals can be transplanted with donor "test" cells from wild-type mice without the need for co-transplanted radioprotective cells to promote survival. The most common method for identifying



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the progeny of transplanted HSCs is to use genetic differences between donor and recipient mouse strains. Differential expression of the two isoforms (CD45.1 and CD45.2) of the pan-leukocyte antigen CD45 on donor and host-derived cells readily facilitates determination of the degree of donor engraftment by flow cytometry.³ Alternative methods include the use of transgenic donor mice that express a readily detectable reporter molecule, such as green fluorescent protein.⁴ The frequency of repopulating HSCs in a “test” population can be measured by using a limiting-dilution experimental design. In these assays, groups of recipient mice are transplanted with graded numbers of donor hematopoietic cells. The proportion of reconstituted mice in each group is determined several months later, and Poisson statistics are then used to calculate the frequency of “repopulating units” in the transplanted cell population.⁵ Serial transplantation of BM from primary recipients into secondary, tertiary and even quaternary recipients has been used as an assay for in vivo self-renewal capacity of mouse HSCs. Mice can also be transplanted with individual HSCs that are purified according to their expression of various cell surface antigens and other markers. These single cell transplantation studies have allowed detailed analysis of the engraftment dynamics and differentiation potential of individual HSCs. Recently, cellular barcoding methods have been developed in which individual cells are tagged with unique genetic markers through retroviral gene transfer.⁶ Transplantation of such barcoded cells also enables clonal analysis of individual HSCs, but on a much larger scale than single cell transplantation experiments.

Human Cells

The gold standard assay to experimentally test the in vivo repopulating potential of human HSCs is intravenous or intra-bone injection into sublethally irradiated, genetically immune-deficient mice. Successful engraftment of human stem cells is defined by the detection of a threshold number of human blood cells (typically >0.1% of nucleated cells) in the blood, BM or other mouse organs several weeks to months after transplantation using flow cytometry. As with mouse transplantation assays described above, xenotransplantation assays can be performed under limiting-dilution conditions to determine the frequency of repopulating stem cells in human hematopoietic tissues and purified cell populations. Originally most xenotransplantation assays were performed in the SCID and NOD/SCID mouse strains. In these older studies relatively large numbers of cells were required to overcome immune rejection by residual host macrophages and NK cells. Moreover, human hematopoiesis could only be detected during a relatively short period (6 - 12 weeks) due to the short lifespan of the mice. Due to these

limitations it was not possible to study the kinetics of human cell engraftment or to distinguish between HSC subsets that mediate short-term and long-term reconstitution. Some of these difficulties of xenotransplantation assays have been addressed by the development of mouse strains in which more immunomodulatory cell types have been deleted. The new immunodeficient mouse strains also live longer than the original strains. Specifically, β 2-microglobulin-deficient and interleukin (IL)-2 receptor (R) γ -deficient NOD/SCID mice support high levels of engraftment that can be detected for >20 weeks after transplantation.⁷⁻¹⁰ IL-2R γ deficient mice with functional impairment of endogenous HSCs due to loss-of-function mutations in the c-kit gene are even more permissive for human HSC engraftment and do not require pre-transplant conditioning by irradiation.¹¹ Using these newer mouse strains it is now possible to study the properties of human HSCs in great detail and identify HSC subsets with distinct cell surface marker profiles, lineage potentials and engraftment kinetics.¹²⁻¹⁶

In Vitro Assays for Hematopoietic Stem and Progenitor Cells

Culture assays can be used to examine the ability of hematopoietic stem and progenitor cells to proliferate and differentiate in response to hematopoietic growth factors and to study their interactions with stromal cells of the hematopoietic microenvironment. These assays are used to measure the numbers/frequencies of progenitor cells in various tissues and purified cell preparations, identify cytokines and other compounds that promote or inhibit hematopoiesis, and to determine the effects of manipulations such as cell processing, cryopreservation, ex vivo expansion and genetic modification on the viability and functional properties of the cells. Culture assays can detect hematopoietic cells at different stages of differentiation, from HSCs to lineage-restricted progenitor cells. In the following sections, the principles and applications of two of the best characterized and quantitative culture assays, the colony-forming unit assay and the long-term culture assay, will be discussed.

Colony-Forming Unit Assays

Colony-forming unit (CFU) assays, also referred to as colony-forming cell (CFC) assays, are the most commonly used in vitro assays for hematopoietic progenitor cells. CFU assays are performed by plating a single cell suspension at low cell density in semi-solid, usually methylcellulose-based (e.g. MethoCult™), medium supplemented with appropriate cytokines. These conditions support the proliferation and differentiation of individual progenitor cells, or CFUs, resulting in

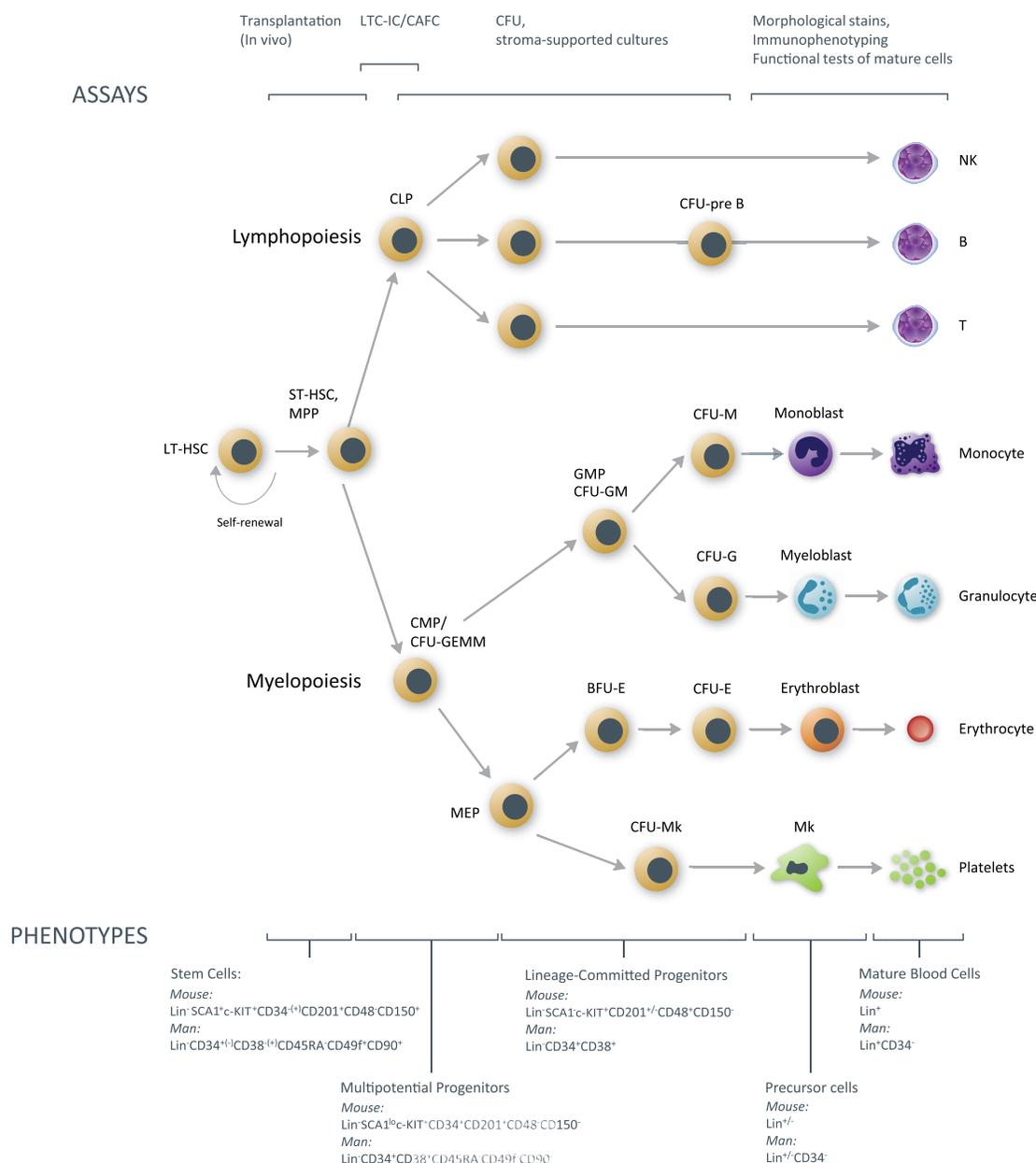


FIGURE 1. HSC Proliferation and Differentiation

Schematic representation of the production of mature blood cells by the proliferation and differentiation of hematopoietic stem cells. Intermediate stages are also depicted. Transplantation assays identify repopulating stem cells. Assays for Long-Term Culture-Initiating Cells (LTC-IC) and Cobblestone Area-Forming Cells (CAFC) identify very primitive progenitor cells that overlap with stem and progenitor cells. Colony-Forming Unit (CFU) assays identify multipotential and lineage-committed progenitor cells. LT-HSC: Long-Term Hematopoietic Stem Cell; ST-HSC: Short-Term Hematopoietic Stem Cell; MPP: Multipotential Progenitor; CMP: Common Myeloid Progenitor; CLP: Common Lymphoid Progenitor; CFU-GEMM: Colony-Forming Unit - Granulocyte/Erythrocyte/Macrophage/ Megakaryocyte; BFU-E: Burst-Forming Unit - Erythroid; CFU-E: Colony-Forming Unit - Erythroid; CFU-Mk: Colony-Forming Unit - Megakaryocyte; CFU-GM: Colony-Forming Unit - Granulocyte/Macrophage; CFU-G: Colony-Forming Unit - Granulocyte; CFU-M: Colony-Forming Unit - Macrophage;. The most definitive markers used to identify the various types of mouse and human hematopoietic cells are shown on the bottom. Additional markers can be used to further distinguish between subsets. Refer to the text for further details. Not shown are the plasmacytoid and myeloid dendritic cell (DC) lineages, which are derived from CLP and CMP, respectively.

the formation of discrete colonies. Colonies derived from different types of progenitor cells are classified and counted based on the number and types of mature cells that they contain using morphological and phenotypic criteria. The CFU assay is most commonly used to detect multipotential and lineage-restricted progenitors of the erythroid, granulocytic and macrophage lineages. Megakaryocyte and B-lymphoid progenitors can also be detected if selective culture conditions for these progenitors are employed. Although purified HSCs can form colonies under appropriate culture conditions, the majority of CFUs detected in BM, blood and other tissues are progenitors with limited self-renewal and in vivo hematopoietic repopulating potential. Nevertheless, the CFU assay can serve as a useful surrogate assay for HSCs in circumstances where long-term transplantation assays are either too expensive or impractical.

Two types of erythroid progenitor cells can be detected using the CFU assay: the colony-forming unit-erythroid (CFU-E) and the burst-forming unit-erythroid (BFU-E). The CFU-E is a more differentiated ("later") progenitor cell than the BFU-E and generates small colonies containing 8 - 200 erythroblasts in one or a few separate clusters after a relatively short culture period (2 - 3 days for mouse CFU-E and 7 - 12 days for human CFU-E). The more primitive ("earlier") BFU-E requires a longer culture period (typically 2 - 3 weeks for human BFU-E) and produces large colonies that may contain multiple separate cell clusters (or "bursts") comprising 200 to many thousands of erythroblasts. Colonies derived from CFU-E and BFU-E may be recognized by their pinkish or red color, which is due to the presence of hemoglobin. Notably, a fraction of colonies derived from more primitive BFU-E (especially from CB) may appear white in color even after 2 weeks of culture. This is because in some cases it can take more than two weeks of culture for hemoglobinization to occur. The survival and proliferation of CFU-E is dependent on the presence of the hormone erythropoietin (EPO) in the culture medium. BFU-E require EPO and one or more other cytokines, particularly Stem Cell Factor (SCF), IL-3, IL-6, and granulocyte/macrophage colony-stimulating factor (GM-CSF) for the initial rounds of cell division and differentiation. The same cytokines, except EPO, also promote colony formation by uni- or bi-potential myeloid progenitor cells, which are classified as CFU-G (granulocyte), CFU-M (macrophage) and CFU-GM depending on the cellular composition of the colonies they give rise to.

Megakaryocytes (Mk) can develop in the same methylcellulose-based media that support erythroid and G/M/GM progenitor cells, and can be identified in large "mixed" colonies derived from immature multipotential CFU-GEMM progenitor cells. Pure

Mk colonies are, however, small and difficult to distinguish from, e.g., macrophage colonies. For this reason CFU-Mk assays are usually not performed using methylcellulose-based media but instead in collagen-based semisolid media, such as MegaCult™. This medium selectively promotes Mk outgrowth and allows the identity of colonies derived from CFU-Mk to be confirmed by staining using immunological and enzymatic staining methods.

Since its introduction over four decades ago,¹⁷ the CFU assay has become the benchmark in vitro functional assay to study hematopoietic progenitor cells. The CFU assay is widely used to study the effects of stimulatory and inhibitory growth factors, and to test the effects of various in vitro manipulations (e.g. cell processing, cryopreservation, gene transduction) on cellular products used in hematopoietic cell transplantation. Although long-term engraftment after transplantation is mediated by more primitive HSCs, the number of CFUs in a graft has been shown to correlate with time to neutrophil and platelet engraftment, and overall survival after transplantation.¹⁸⁻²³ Thus the CFU assay is a useful surrogate assay to predict graft quality and has proven particularly useful in facilitating selection of CB units containing high numbers of viable and functional progenitor cells prior to unrelated allogeneic transplantation.

Recently, several improvements have been made to the CFU assay that simplify colony counting and improve the accuracy and reproducibility of the results. These include the development of MethoCult™ Express medium which enables the total number of viable and functional progenitor cells in a CB unit to be determined after only 7 days of culture, and STEMvision™, a benchtop instrument that acquires high-resolution images of CFU assays performed and automatically identifies, classifies and counts hematopoietic colonies in standard 14-day CFU assays of CB, BM or MPB cells as well as in the faster 7-day assay of CB cells. Together, these new products have enabled further standardization of the CFU assay for routine laboratory use.

Long-Term Cultures

Hematopoietic progenitor cells that are more closely related to HSCs than to CFUs can be identified and enumerated using long-term culture (LTC) assays. The LTC system was originally developed for primitive progenitors of the myeloid (i.e., granulocyte, macrophage, erythroid and megakaryocyte) lineages.^{24, 25} It was subsequently adapted to support the growth of B lymphoid and NK cell progenitors.^{26, 27} LTC assays are performed by culturing hematopoietic cells on an adherent monolayer of primary stromal cells or on immortalized stromal cell lines. Using specialized culture media such as MyeloCult™, this system supports the survival, self-renewal, proliferation

and differentiation of primitive hematopoietic cells, including long-term repopulating HSCs, for many weeks.^{28, 29}

The cells that are detected in LTC assays are called long-term culture-initiating cells (LTC-ICs). LTC-ICs are detected by their ability to generate more differentiated CFUs in these stroma-supported cultures for at least 5 weeks (>4 weeks for mouse cells). This period ensures that any CFUs that were present in the original cell sample become terminally differentiated. Therefore, CFUs that can be detected after 4-5 weeks must have been generated anew from more primitive LTC-ICs. These LTC-IC-derived CFUs are detected by re-plating the contents of individual LTCs in CFU assay media (MethoCult™) and counting colonies ~2 weeks later.²⁹ LTC-IC assays are ideally performed using a limiting-dilution design that enables measurement of the frequency of these progenitor cells. L-Calc™ software (STEMCELL Technologies) and ELDA software (developed by the Walter and Eliza Hall Institute of Medical Research)³⁰ are ideally suited for these analyses. Simpler assay formats that measure the CFU output of bulk long-term cultures can also be used to determine the number of LTC-ICs if the number of CFUs produced per LTC-IC has already been determined from prior studies.

The cobblestone area-forming cell (CAFC) assay is a variant of the LTC-IC assay in which morphological, rather than functional, criteria are used as the assay read-out to identify primitive cells.³¹ Specifically, the assay measures the presence of cells that produce phase dark areas of proliferating cells beneath the stromal layer (called “cobblestone areas”) for several weeks. It is not necessary to replate cells in the CAFC assay, but the morphological assay readout does not provide information on the differentiation potential of the cells. This introduces the possibility that some CAFCs measured in this system may not represent functional LTC-ICs.³²

Isolation of Hematopoietic Stem and Progenitor Cells

Mouse Cells

The first step in the isolation of mouse HSPCs from BM, spleen, fetal liver or other tissues usually consists of removing mature cells that express “lineage” (Lin) antigens specific to terminally differentiated blood cells. These antigens are absent or only weakly expressed on HSPCs. Examples of Lin antigens are CD3 for T cells, B220 for B cells and NK cells, Ly6G/Gr-1 for granulocytes, CD11b/Mac-1 for monocytes and macrophages, and TER-119 for erythroid cells. After removal of

lineage-positive (Lin⁺), HSPCs can be further enriched by positively selecting lineage-negative (Lin⁻) cells that express combinations of cell surface markers that identify HSCs and primitive progenitor cells. Commonly used markers include Thy-1, c-KIT (i.e., the receptor for SCF, also known as CD117) and SCA1.^{3, 33-35, 41} Lin⁻SCA1⁺Thy1^{lo} or Lin⁻SCA1⁺c-KIT⁺ (LSK) cells make up <0.1% of nucleated BM cells but contain most repopulating HSCs. Importantly, SCA1 is only a useful stem cell marker in some mouse strains (e.g., C57BL/6). In other mouse strains (e.g., BALB/c) SCA1 is expressed at low levels on HSCs.³⁶ Similar strain-dependent differences have been demonstrated for the expression of the Thy-1 antigen, which occurs in two forms, Thy-1.1 and Thy-1.2, on HSCs. In mouse strains that express Thy-1.2, HSCs are Thy-1^{lo}. In mouse strains that express Thy-1.1, HSCs are either Thy-1⁻ or Thy-1^{lo}.³⁷

To circumvent these issues, other markers and isolation strategies have been developed that work with most mouse strains. One strategy involves sorting of Lin⁻CD48⁻CD150⁺ (or so-called “SLAM”) cells.^{38, 39}

LSK and SLAM cells are both heterogeneous populations in which HSCs represent at most 10 - 20% of all cells. Further enrichment of HSCs to frequencies as high as 50% has been achieved by selecting LSK or SLAM cells that express high levels of CD201 (endothelial protein C receptor; EPCR), have absent or low expression of CD34, CD135 (Flt3) and CD49b, and that retain only low levels of DNA dyes such as Rhodamine-123 (Rho123) and Hoechst 33342 due to their high expression of the multidrug transporter proteins MDR1 and ABCG2, respectively.^{35, 38-44} Differences in the expression of Sca-1, CD34, Flt3, CD150, CD48, CD49b and other markers (specifically FcγR1, CD127 (IL-7Rα)), and staining with DNA dyes have been used to separate LT-HSCs from ST-HSCs and to distinguish multipotent progenitors: common myeloid progenitors, common lymphoid progenitors, granulocyte and macrophage progenitors, and megakaryocyte and erythroid progenitors (Figure 1).^{38, 43, 45, 46}

The enrichment of mouse LT-HSCs to near complete purity has enabled the identification of distinct HSC subtypes that differ with respect to their engraftment kinetics, self-renewal ability, cytokine responsiveness and the number and lineage distribution of the mature cells they can generate in vivo.^{42, 44, 47-49} LT-HSCs can be divided into myeloid-biased, lymphoid-biased and balanced subsets depending on the relative numbers of myeloid and lymphoid cells that they produce in vivo. Myeloid-biased HSCs express higher levels of CD150 and efflux Hoechst 33342 more efficiently than lymphoid-biased HSCs cells. They also exhibit higher self-renewal ability as demonstrated by

serial transplantation of BM cells from primary recipients into secondary hosts. Myeloid-biased HSCs become the dominant HSC subset during aging.^{44,49,50} However, myeloid-biased HSCs in old mice, or those isolated after serial transplantation in young mice, home less efficiently to the BM, exhibit lower self-renewal ability, and produce fewer mature progeny than those present in young mice. This probably reflects changes in HSC function as a result of aging or the hematological stress induced by transplantation.⁵¹

Human Cells

Arguably the most important marker of primitive human hematopoietic cells is the cell surface protein CD34. CD34 is expressed on the surface of 1 - 5% of nucleated human BM cells, ~1% of CB cells and < 0.1% of normal PB cells. Most human HSCs are CD34⁺, as demonstrated by xenotransplantation assays described above and clinical transplants performed with purified CD34⁺ cells from different hematopoietic tissues.⁵²⁻⁵⁴ In addition to HSCs, the CD34⁺ population includes most LTC-ICs, CFU-GEMM, BFU-E and CFU-GM. CD34 expression decreases with differentiation and the majority of late-stage progenitors (e.g., CFU-E) and end cells are CD34⁻.^{55, 56} Although enumeration of CD34⁺ cells by flow cytometry is a common method to measure the hematopoietic stem and progenitor cell content of grafts used for clinical transplantation, it is important to remember that HSCs and primitive progenitors that read out in xenotransplantation and LTC-IC assays comprise only 0.1 - 1% of CD34⁺ blood or BM cells, while progenitor cells that are detectable in CFU assays comprise about 10 - 20% of CD34⁺ cells. Therefore, CD34 expression alone does not provide an accurate measure of HSCs and immature progenitors, and additional markers are required to identify and isolate the most primitive hematopoietic cells.

As described above for mouse cells, HSPCs do not express Lin antigens that are present on mature erythroid cells, granulocytes, macrophages, NK cells, and B and T lymphocytes. By using cocktails of antibodies to remove these Lin⁺ cells it is possible to enrich HSPCs by 50 to 200-fold.⁵⁷ CD34⁺ cells typically represent between 40 and 90% of Lin⁻ BM, MPB or CB cells, depending on the individual sample, the composition of the lineage antibody cocktail used for negative selection and the isolation method used (e.g., immunomagnetic, immunorotating or FACS isolation methods). A small subset of Lin⁻ HSCs that do not express CD34 has been detected in xenotransplantation assays, in particular after direct intra-BM injection.⁵⁸⁻⁶¹ These cells can generate CD34⁺ cells *in vitro* and *in vivo*. The importance of CD34⁻ HSCs for hematopoiesis and their relationship to the predominant CD34⁺ stem cell population remains unclear. It has been suggested

that some Lin⁻ CD34⁻ cells in adult PB may represent a common progenitor for the hematopoietic and endothelial lineages.⁶²

Human HSPCs can be separated by sub-fractionating Lin⁻CD34⁺ cells using markers that are differentially expressed on primitive and more differentiated cells. The most common markers include CD38 and CD45RA, which are absent or only weakly expressed on primitive cells, and CD90 (Thy-1), which is expressed at higher levels on primitive cells than on differentiated cells.⁶³⁻⁶⁵

CD34⁺Lin⁻ human BM cells that can generate lymphoid and myeloid cells for at least 8 weeks following transplantation into immunodeficient mice are mostly CD38⁻. In contrast, human cells that are capable of only short-term (3 weeks) engraftment of mostly myeloid cells are predominantly CD38⁺.¹² Short-term HSCs that support early platelet and neutrophil engraftment are also different from each other and are phenotypically heterogeneous, i.e., both cell types can be CD38⁺ or CD38⁻, and aldehyde dehydrogenase (ALDH)-positive or -negative.⁶⁶

Together, these and other studies indicate that, similar to mouse HSCs, there are distinct subsets of human HSCs that differ in their phenotype, proliferation and differentiation potential. By combining multiple markers, several groups have achieved a very high level of enrichment of human HSCs from CB. As few as 10 Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ CB cells have been shown to engraft the BM of immunodeficient mice and generate human lymphoid and myeloid cells for at least 12 weeks after transplantation.¹⁴ Purified Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺ BM cells from elderly human donors generated fewer lymphoid but similar numbers of myeloid progeny in culture and in xenotransplantation experiments than phenotypically identical cells from young donors.⁶⁷ This suggests that human HSCs may undergo similar age-related shifts from lymphoid to myeloid-biased differentiation as observed for mouse HSCs. Lin⁻CD34⁺CD38⁻CD45RA⁻ that lack CD90 expression can also contribute to long-term repopulation in immunodeficient mice, but more cells are required to achieve engraftment and the cellular output per transplanted stem cell is lower. This suggests that the frequency of HSCs in the CD90⁻ subset is lower and/or that the cells have less engraftment ability (i.e. represent a less primitive cell subset) than their CD90⁺ counterparts. Using a limiting-dilution transplantation assay to quantitate repopulating stem cells, it was shown that most Lin⁻CD34⁺CD38⁻CD45RA⁻ cells able to engraft immunodeficient mice long-term (30 weeks) express the adhesion molecule CD49f.¹⁶ CD49f⁺ cells engrafted only transiently, indicating that these cells may represent ST-HSCs. In transplantation studies of single purified cells, close to 30% of Lin⁻CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺Rho123^{lo} cells exhibited long-term, multilineage repopulating ability. This is the

highest purity of human HSCs thus far reported.¹⁶

Primitive human hematopoietic cells can also be identified by their expression of the intracellular enzyme ALDH. ALDH converts a non-charged fluorescent substrate (ALDEFLUOR™), which passively crosses cell membranes, into a negatively charged polar fluorescent product, which cannot cross cell membranes and therefore accumulates inside viable, intact ALDH-expressing cells. As a result, cells with high ALDH activity can be detected and isolated by flow cytometry on the basis of their bright fluorescence.⁶⁸ Both long-term and short-term repopulating HSCs and most progenitor cells, with the possible exception of lymphoid progenitors, express high levels of ALDH.^{13,69,70}

A summary of the current literature regarding the expression of phenotypic markers on human and murine HSCs and progenitors is presented in Figure 1.

Culture of Hematopoietic Cells

Hematopoietic cells can be stimulated to proliferate in culture using many different methods. The composition and biological properties of the cells that are produced using these different culture systems will differ considerably depending on the diverse applications for which they are intended to be used. These may include (i) production of LT-HSCs that mediate sustained engraftment after transplantation, (ii) production of ST-HSCs and progenitors to rapidly restore blood counts after transplantation, (iii) generation of large numbers of mature blood cells for infusion following acute blood loss, (iv) use of cells to identify novel regulators of hematopoiesis, (v) testing the toxicity of new drug candidates, and (vi) activating HSCs for retroviral or lentiviral transduction with exogenous genes to correct genetic defects impacting hematopoiesis. Two applications will be discussed here: (1) Ex vivo expansion to increase the limited numbers of HSPCs in CB grafts, and (2) The production of mature blood cells (specifically erythroid cells and Mks/platelets) by expansion and lineage-specific differentiation of HSPCs.

Hematopoietic Stem and Progenitor Cell Expansion

Umbilical CB is an important source of HSCs for both allogeneic and autologous transplantation. However, most CB units do not contain sufficient numbers of primitive cells to mediate successful engraftment of adult recipients. Even in pediatric patients, CB stem cells typically take considerably longer to regenerate clinically meaningful numbers of neutrophils and platelets than HSCs derived from BM or MPB. The development of methods to increase the numbers and/or engraftment potential of repopulating

cells in culture has been explored for several years as a strategy to increase the clinical utility of CB for cellular therapy.

Large (100 to 1000-fold) increases in the numbers of CD34⁺ cells and clonogenic progenitors can be achieved by culturing CD34⁺ cells for several days or weeks in media supplemented with combinations of cytokines such as IL-3, IL-6, SCF, Flt3L, TPO, GM-CSF and G-CSF. It has been more difficult to expand or even maintain more primitive cells with in vivo repopulating ability for even a few days in vitro as most culture conditions do not effectively support HSC self-renewal. The expansion of HSPCs in culture using early-acting cytokines like TPO, SCF and Flt3L has been modest, with the number of recovered stem cells usually not exceeding input numbers by more than 4-fold.⁷¹⁻⁷³ A much larger (up to 10,000-fold) expansion of mouse LT-HSCs has been achieved by enforced expression of transcription factors, such as HoxB4 and NF- κ B, that regulate HSC proliferation and self-renewal.⁷⁴⁻⁷⁶ While these studies demonstrate that large scale expansion of repopulating stem cells is possible, the development of non-genetic expansion methods based on the same principle has been more difficult. Culturing cells with cell-penetrating HOXB4 or NF- κ B recombinant proteins led to a more modest, ~4-fold, expansion of mouse and human HSCs.^{77,78} The weaker effects of the exogenous transcription factors may be related to protein instability and/or their insufficient accumulation inside HSCs. These problems may be addressed by developing proteins that are more resistant to degradation than those used in the original studies.⁷⁹

Stem and progenitor cells have also been co-cultured with primary or immortalized stromal cells that can support their survival and self-renewal in vitro.⁸⁰⁻⁸⁴ Stromal cells produce several secreted or cell membrane-associated factors that have been shown to be necessary for HSC self-renewal in vivo, and which promote HSC expansion in stroma-free cultures when combined with hematopoietic growth factors. Examples of these stromal factors include Notch ligands, angiopoietin-like proteins, and pleiotrophin.⁸⁵⁻⁸⁹ Other molecules also prevent HSC differentiation and promote the survival and possible expansion of repopulating cells in vitro. These include chromatin modifying agents (e.g., valproic acid and nicotinamide), metals (e.g., lithium), antagonists of the aryl hydrocarbon receptor, and small molecules, such as UM171 and UM729.⁹⁰⁻⁹⁴ Short term exposure to some molecules, e.g., prostaglandin-E2, and diprotin A (an inhibitor of dipeptidyl peptidase-4, CD26) can promote HSC engraftment by increasing the efficiency with which HSCs home to the BM after transplantation.^{95,96}

The engraftment potential of ex vivo expanded HSCs has been extensively examined in the clinic. Several studies

have demonstrated contributions of the manipulated cells to hematopoiesis during the first weeks after transplantation.^{88,92,97} In one study, CB CD34⁺ cells were cultured for 17 - 21 days in StemSpan™ SFEM supplemented with SCF, Flt3L, TPO, IL-3, IL-6 and an immobilized fragment of the Notch ligand, Delta-1.⁸⁸ In two other studies, CB cells were, respectively, co-cultured with mesenchymal stromal cells or stimulated with nicotinamide for 2 - 3 weeks, while in a fourth study CB cells were exposed for 2 hours to PGE2.^{95,97,98} In these studies the cultured cells engrafted rapidly, contributed to early neutrophil recovery and promoted durable engraftment of a second non-manipulated CB graft. This demonstrates that clinically useful expansion of ST-HSCs (and/or promotion of their homing and engraftment ability) is achievable. However, sustained engraftment of HSCs from cultured CB cells has not been consistently demonstrated. This may be due either to loss of HSC self-renewal ability in culture or by elimination of the cultured cells in vivo by effector T cells in the co-transplanted non-manipulated CB graft. The latter phenomenon has been observed in patients transplanted with two non-manipulated CB units.⁹⁹

Generation of Mature Blood Cells in Culture

In addition to expansion of HSCs themselves, investigators have directed considerable effort to the in vitro production of committed progenitor cells and mature blood cells of specific lineages, particularly erythroblasts/RBCs and Mk/platelets, by promoting the proliferation and lineage-specific differentiation of stem and progenitor cells in culture. These culture methods are useful for several applications, e.g., to examine the toxicity of new drug candidates on specific hematopoietic lineages, and to generate target cells for reprogramming to develop induced pluripotent stem (iPS) cell lines. Scaled-up versions of such cultures could also be used to generate large numbers of blood cells as an alternative to transfusion with donated blood products. Erythroid progenitor cells can be selectively expanded using optimized serum-free media such as the StemSpan™ line of media, and combinations of growth factors and hormones, usually including SCF, IL-3, EPO and a glucocorticoid receptor (GR) agonist. Erythroid cell expansion can be further increased by chromatin modifying agents (e.g. valproic acid), hypoxia, agents that stabilize the transcription factor HIF-1 α and steroid hormones.^{91,100-103}

Expansion of erythroid cells tends to be highest in cultures initiated with CB cells. This is consistent with the higher proliferative capacity of erythroid progenitors (i.e., BFU-E) from CB, but BM, non-mobilized and mobilized PB cells also give good results.¹⁰⁴ Most erythroid cultures are initiated with purified CD34⁺ cells, but large numbers of erythroid progeny have also been produced in

cultures initiated with non-purified PB and CB cells, and even with CD34-depleted PB cells.^{100, 105, 106}

In the expansion culture conditions described above, cells can proliferate for several weeks and generate thousands of erythroblasts per original progenitor cell, without differentiating into normoblasts and mature RBCs. Removal of IL-3, SCF and GR agonist induces terminal differentiation with loss of proliferative ability, down-regulation of CD71 expression, increased expression of GpA and hemoglobin, and a decrease in cell diameter.¹⁰⁰ Some studies have shown that enucleation and maturation into reticulocytes required co-culture with an immortalized stromal cell line, BM-derived mesenchymal cells, or addition of serum or plasma.^{100,104,107,108} In other studies, erythroid maturation occurred efficiently in stroma-free cultures in serum-free media supplemented with only EPO and insulin.¹⁰⁹ Culture-expanded human erythroblasts can also complete the final steps toward maturation in vivo. Importantly, RBCs that were generated in vitro have been shown to exhibit similar function and lifespan in vivo compared to primary RBCs.¹⁰⁸

Culture systems have also been developed to promote the expansion and differentiation of Mk progenitors from CB, BM, and normal or mobilized PB. The resulting Mk and platelets have been shown in some studies to be functional and able to contribute to platelet engraftment in xenotransplantation assays.¹¹⁰⁻¹¹⁴ TPO is the primary regulator of megakaryocytopoiesis and can be used as a single agent to generate Mks in culture. Other cytokines, such as SCF, Flt3L, IL-1 β , IL-3, IL-6, IL-9, IL-11 and GM-CSF have also been shown to promote expansion of Mk progenitors in combination with TPO, resulting in high yields of Mk and platelets. However, many of these cytokines also promote the expansion and differentiation of granulocyte and macrophage progenitors, and culture conditions that produce the highest Mk expansion may result in low Mk purity due to the presence of large numbers of other cell types. Through systematic testing of different combinations and concentrations of cytokines in serum-free cultures, high purities (~90% CD41⁺ cells) and yields of Mk and mature platelets can be obtained.^{111, 113} Mk progenitor outgrowth can also be stimulated by culturing the cells at 39°C, using 3-D scaffolds, and by co-culturing Mk progenitor cells with adherent stromal cells.^{112, 115, 116} Cell proliferation and Mk yields generally tend to be higher in cultures initiated with CB CD34⁺ cells, but several groups have reported that CB-derived Mk remain less differentiated, have lower ploidy and generate fewer platelets than cells produced from BM and PB.¹¹⁷⁻¹¹⁹ Endoreplication of Mks and platelet formation may be increased by inhibition of pathways involved in cytokinesis, e.g., the Rho/Rock and Src pathways, and by exposure to shear stress.^{120, 121}

Summary And Conclusions

Mouse and human hematopoietic stem and progenitor cells can be identified on the basis of numerous phenotypic markers. The most common of these are cell surface and intracellular proteins, which are detected by antibody staining or assays that measure their functional properties (e.g., the ability to efflux fluorescent dyes or to convert an enzyme substrate to a fluorescent product that is retained inside the cell). Appropriate combinations of these markers can be used to enrich stem and progenitor cells with differing functional properties. HSCs are identified by their ability to regenerate and maintain long-term multilineage hematopoiesis in vivo. Transplantation assays in syngeneic, allogeneic or xenogeneic hosts allow the evaluation of in vivo engraftment properties that are most relevant to clinical transplantation. Progenitor cells have limited in vivo hematopoietic potential and must therefore be identified using in vitro assays. The CFU assay is the most widely used and best standardized in vitro assay for multipotent and lineage-restricted progenitors of most blood cell lineages. Stromal cell-based systems probably more accurately recapitulate the in vivo hematopoietic microenvironment and can also be adapted to support the growth of lymphoid progenitors. Hematopoietic stem and progenitor cells can be cultured under defined conditions designed either to promote self-renewal and increase the number of primitive cells, or to promote lineage-specific expansion and differentiation into mature blood cell types. Both types of cell products are being explored for their utility as cell therapeutics and offer promise for the treatment of inherited and acquired blood cell disorders.

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