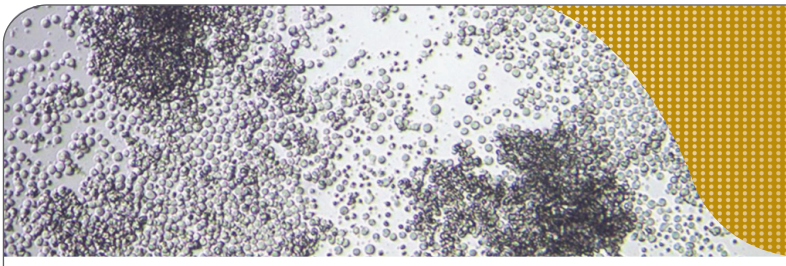

TECHNICAL BULLETIN

CULTURE OF HEMATOPOIETIC STEM AND PROGENITOR CELLS

1. BACKGROUND

Mature hematopoietic cells of both the lymphoid and myeloid lineages are continuously generated from a small self-renewing pool of pluripotent hematopoietic stem cells (HSCs). HSCs are found primarily in the bone marrow (BM) of healthy adults, in umbilical cord blood (CB) and in adult blood after mobilization from the bone marrow with cytokines, such as G-CSF, or other agents. HSCs have the ability to differentiate into all mature hematopoietic cells and to replenish themselves by self-renewal. During differentiation to mature blood cells, the progeny of HSCs will go through intermediate stages prior to reaching maturity, including multi-potential progenitors and lineage-committed progenitors. A number of stimulatory and inhibitory factors present in the hematopoietic microenvironment regulate these events *in vivo*.

The identification of cytokines and other stimuli which support stem cell proliferation and differentiation has led to the development of cell culture methods for *ex vivo* generation of hematopoietic cells for a variety of applications, such as:

1. Generation of large numbers of mature blood cells or lineage-specific cells, for example, erythroid progenitors, mature red blood cells, granulocytes, monocyte/macrophages, dendritic cells and megakaryocytes.
2. Expansion of stem cells capable of reconstituting hematopoiesis after transplantation.
3. Identification of novel regulators of hematopoiesis.
4. Genetic modification, for example, retroviral or lentiviral gene transfer of stem cells and progenitor cells.
5. Characterization of leukemic stem cells and regulation of leukemogenesis.

Large numbers of progenitors and mature blood cells of individual lineages can be generated with high yields by culturing HSC and hematopoietic progenitor cell (HPC) preparations in appropriate culture media, cytokines and other stimuli. However, culture conditions that promote HSC and HPC proliferation and differentiation usually lead to irreversible loss of pluripotent HSCs. Normal HSCs cannot be cultured for more than a few days without losing their stem cell properties (i.e. self-renewal and ability to reconstitute hematopoiesis after transplantation) and entering into the pathway of lineage commitment and terminal differentiation. In this respect, hematopoietic stem cells differ from other types of stem cells, such as embryonic stem cells, which can undergo multiple cell divisions in culture while still retaining their stem cell properties.

In recent years, advances have been made in selecting new cytokines and other cellular regulators that can shift the balance between stem cell self-renewal and differentiation towards the former, and increase the numbers of HSCs and primitive HPCs in culture (Zhang and Lodish provide a recent review[1]). Despite these advances, the development of optimal and reproducible culture conditions for large-scale expansion of genetically and functionally normal HSCs remains the subject of ongoing investigations.

HSC and HPC culture conditions need to be carefully optimized and geared towards the goal of the experiment. Special attention needs to be paid to the choice of culture medium, cytokine combinations and concentrations, composition and purity of the starting population of cells, duration of the cultures, medium replacement and replating schedules, and the choice of assays to characterize the cells after culture.

This technical bulletin provides general guidelines about setting up and analyzing hematopoietic progenitor expansion cultures in serum-free StemSpan® media.

2. HEMATOPOIETIC EXPANSION CULTURES

2.1 SERUM-FREE CULTURE MEDIA

Media used to culture HSCs and HPCs have historically contained serum. However, serum may contain inhibitors of proliferation and/or differentiation, for example, transforming growth factor-β (TGF-β). In addition, serum constituents vary

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in type and concentration between different batches, which can cause considerable variability between experiments. In order to minimize these variables, the use of serum-free media is essential.

StemSpan® SFEM (Catalog #09600/09650) is a serum-free medium optimized for the expansion of hematopoietic progenitor cells in the presence of cytokines. StemSpan® SFEM contains bovine serum albumin (BSA), recombinant human insulin, iron-saturated human transferrin, 2-mercaptoethanol and supplements in Iscove's Modified Dulbecco's Medium (IMDM). StemSpan® SFEM is mainly used for studies with human cells, but it is also frequently used with hematopoietic cells from other species, specifically non-human primates, dogs and mice.

StemSpan® H3000 (Catalog #09800/09850) is an animal protein-free defined medium for expansion of human hematopoietic cells. StemSpan® H3000 contains purified human-derived or recombinant proteins. The performance of StemSpan® H3000 and StemSpan® SFEM is similar in many expansion culture applications, but not identical. StemSpan® H3000 is the medium of choice if the presence of non-human animal proteins in the culture medium is not desired.

In addition to these complete media, STEMCELL Technologies has developed two serum substitutes that can be used in combination with a base medium, cytokines and other supplements of choice to prepare *in vitro* expansion media without the use of serum. Both serum substitutes contain pretested albumin, insulin and iron-saturated transferrrin.

BIT 9500 serum substitute (Catalog #09500) contains BSA and is recommended for the culture of human, non-human primate and mouse hematopoietic cells. HIT serum substitute (Catalog #09550) contains human serum albumin (HSA) and is recommended for the culture of human hematopoietic cells in applications where a medium of defined composition and free of animal proteins is required.

2.2 CYTOKINES

Many cytokines exert specific biological effects on HSCs and HPCs *in vitro*, including supporting cell survival, proliferation and differentiation. Although individual cytokines demonstrate one or more of these properties, most show additive or synergistic effects when used in combination with other cytokines. In general, the proliferation and differentiation of a purified HSC and HPC preparation is stimulated most effectively with combinations of cytokines that promote the survival and proliferation of HSCs and primitive progenitors, as well as the proliferation and differentiation of lineage-committed progenitors. The most commonly used cytokines include stem cell factor (SCF), Flt-3 ligand (FLT-3L), thrombopoietin (TPO), interleukin-3 (IL-3), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO).

The types and yields of cells generated in culture is determined in part by the combination of cytokines used. The cytokine concentrations are also important as low or high concentrations of the same cytokine can have significant effects on the balance between HSC and HPC proliferation and differentiation.[2] For some applications, a switch between different cytokine combinations during the culture period may be used to selectively promote the proliferation and differentiation of a specific progenitor subset and generate pure populations of a specific mature cell type. An example is the generation of mature red blood cells in StemSpan® SFEM cultures. Progenitors are first stimulated with SCF, EPO and other compounds (specifically dexamethasone) to generate large numbers of erythroid progenitors. Then high concentrations of EPO and a dexamethasone antagonist induce terminal erythroid differentiation.[3]

In order to assist researchers in establishing hematopoietic cell expansion cultures, STEMCELL Technologies has developed three cytokine combinations that have been formulated for common applications with human cells. Note that these cytokine cocktails cannot be used with mouse cells as some of the human cytokines in the cocktails, most notably SCF and IL-3, do not cross-react with mouse cells.

StemSpan® CC100 Cytokine Cocktail (Catalog #02690) contains a combination of cytokines that act on both primitive and more differentiated progenitors of multiple lineages. The cytokines are FLT-3 Ligand, SCF, IL-3 and IL-6. This cytokine combination can be used to achieve a rapid and strong proliferative response of hematopoietic progenitor cells and to generate large numbers of cells, including $CD34^+$ cells, colony-forming cells (CFCs) and more mature hematopoietic cells during culture periods of one to two weeks. Figure 1 shows an example of the expansion of $CD34^+$ CB cells in StemSpan® SFEM and StemSpan® H3000 during eight days of culture in the presence of StemSpan® CC100.

StemSpan® CC110 Cytokine Cocktail (Catalog #02697) contains SCF, TPO and FLT-3 Ligand. This combination of cytokines induces the proliferation of HSCs and immature progenitors. This cytokine cocktail is primarily designed for use in short-term cultures (i.e. two to seven days) to activate stem cell and immature progenitor cell cycling. StemSpan® CC110 lacks cytokines, such as IL-3 and IL-6, that promote survival and differentiation of later progenitors. Therefore, StemSpan® CC110 is not recommended for applications in which progenitors at successive stages of differentiation need to be cultured for longer periods and cell yields with StemSpan® CC110 are usually lower than in cultures with StemSpan® CC100. If desired, StemSpan® CC110 and StemSpan® CC100 can be supplemented with

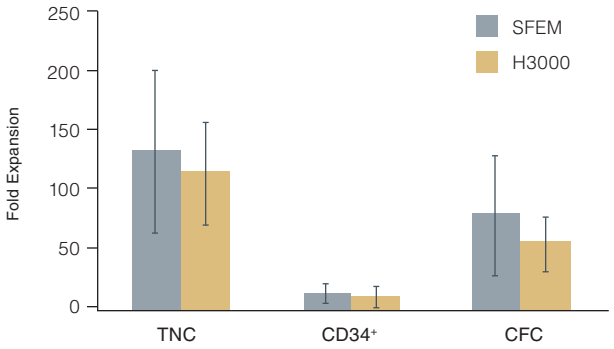

FIGURE 1: Human cord blood CD34+ cells (2000 cells per mL of medium) were cultured for 8 days in StemSpan® SFEM or StemSpan® H3000 media supplemented with the StemSpan® CC100 Cytokine Cocktail. Total cell numbers were counted using a haemocyometer, CD34+ cells were measured using flow cytometry and colony-forming cells (CFCs) were cultured using MethoCult® H4435 Enriched. Results are expressed as average fold expansion (± 1 standard deviation) of 5 experiments. Note that results of cultures in StemSpan® SFEM and StemSpan® H3000 were similar, but also that the magnitude of expansion was highly variable between donors and experiments. Such variability is inherent to hematopoietic expansion cultures as expansion is dependent on the progenitor frequency, which can be highly variable between individual cord blood samples.

cytokines that selectively promote the production of mature cells of specific lineages, for example, EPO for erythroid cells and GM-CSF and/or G-CSF for monocytes/macrophages and granulocytes.

StemSpan® CC220 Cytokine Cocktail (Catalog #02696) has been designed to promote the expansion and differentiation of megakaryocyte progenitors and to generate megakaryocytes and platelets in cultures of CD34+ cells, with minimal expansion of cells from other lineages. Results of typical experiments are shown in Figure 2. The frequency of CD41+ megakaryocytes can increase from less than 10% before culture to over 90% after stimulation with StemSpan® CC220, with massive production of phenotypically mature platelets.

2.3 OTHER CULTURE SUPPLEMENTS

Low density lipoproteins (LDLs) (Catalog #02698) are important for transport of cholesterol, which is required for membrane biogenesis.[4] LDLs present cholesterol to the cells in a soluble form by LDL-receptor-mediated uptake. LDLs are more effective than free lipids and fatty acids, which have low solubility in aqueous solutions and may not be efficiently taken up by the cells. Cells also obtain cholesterol in other forms, for example, by being adsorbed to albumin or through *de novo* synthesis.

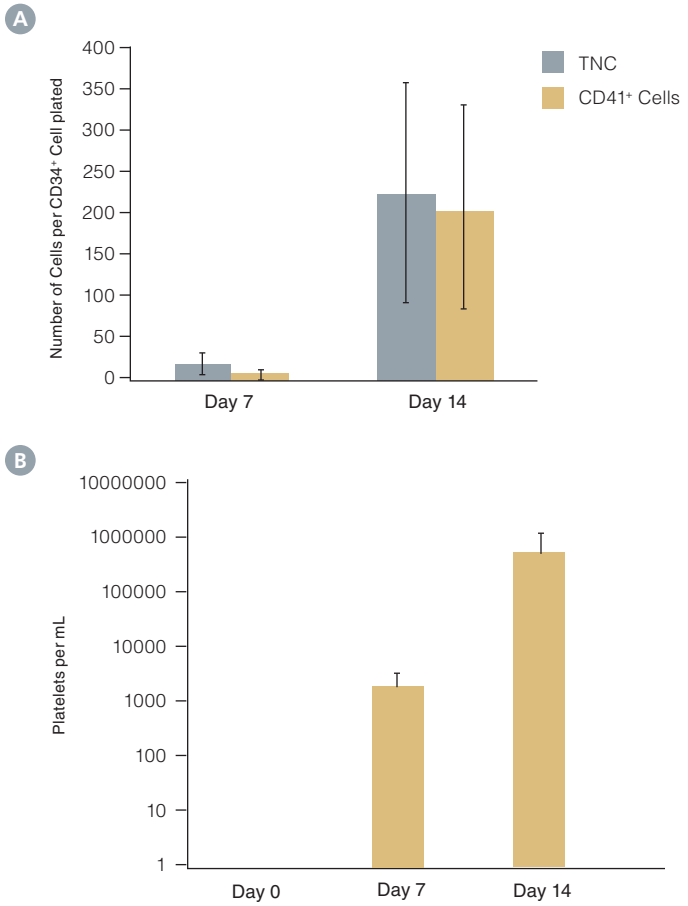

FIGURE 2: Megakaryocyte expansion in StemSpan® SFEM medium supplemented with StemSpan® CC220 Cytokine Cocktail. Human cord blood CD34+ cells (2 x 10^4 cells per mL of medium) were cultured for 7 days; the cells were then counted and replated in fresh medium and cultured for another 7 days. Number of Total Nucleated Cells (TNC) and CD41+ megakaryocytes per CD34+ cell plated (A) and of CD41+ platelets per mL of cell suspension (B) after 7 and 14 days of culture. Results show the average (±1 standard deviation) of 6 experiments.

For these reasons hematopoietic cells can grow in serum-free cultures in the absence of an exogenous source of lipids. However, for many applications, supplementation of the cultures with LDL at concentrations between 10 and 40 µg/mL can significantly improve HPC proliferation, especially when cells are cultured at a low density.

One application for which supplementation with LDLs is recommended is the expansion of megakaryocyte progenitors and production of megakaryocytes from CD34+ CB cells in StemSpan® SFEM or StemSpan® H3000 media using the

StemSpan® CC220 cytokine cocktail. Figure 3 shows that supplementing cultures of CB CD34+ cells in StemSpan® H3000 medium increases megakaryocyte output in a dose-dependent manner. In this example, the number of CD41+ cells produced after 14 days of cultures containing LDL at 40 µg/ml was approximately five-fold higher than in cultures without LDL.

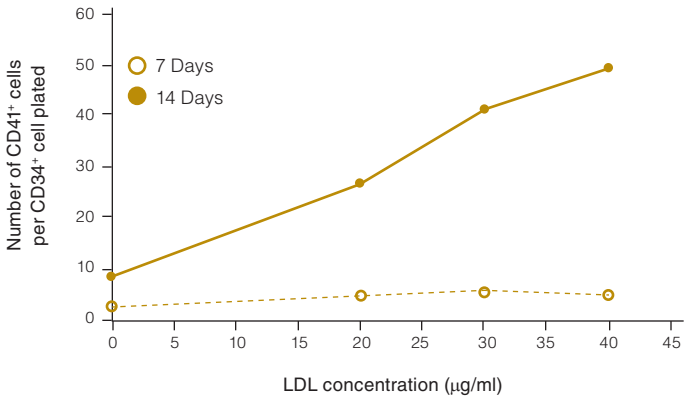

FIGURE 3: Effect of low density lipoproteins (LDLs) on megakaryocyte expansion in StemSpan® H3000 supplemented with StemSpan® CC220 cytokine cocktail.

3. CULTURE CONDITIONS

3.1 CELL PREPARATION

Starting cultures with unfractionated cells or partially processed cells (e.g. mononuclear cells (MNCs) isolated by sedimentation over Ficoll-Paque™ PLUS*) is possible but not recommended as the large numbers of mature and non-hematopoietic cells present in cultures of unfractionated cells can potentially stimulate HSC and HPC through paracrine mechanisms or inhibit growth by secretion of inhibitory cytokines, competition for nutrients and accumulation of toxic waste products. These effects are variable and unpredictable and can make data interpretation difficult. For these reasons the use of purified CD34+, lineage-depleted cells (Lin-) or more highly purified cells obtained by cell sorting is preferable. Purified cells can also be plated at higher cell densities and this may provide a stronger and more reproducible proliferative response, provided other culture conditions have been optimized. For more details about STEMCELL Technologies' products for isolation of human and mouse hematopoietic stem and progenitor cells prior to culture, please contact us or visit our website at www.stemcell.com/product_catalog/huprog.aspx (human) or www.stemcell.com/product_catalog/mprogenitors.aspx (mouse).

*Ficoll-Paque™ PLUS is a trademark of GE Healthcare Ltd.

3.2 CELL PLATING DENSITIES

The optimal number of cells plated is dependent on the types and purity of the progenitors, the types and concentrations of cytokines and other stimuli, the culture conditions (i.e. culture time and refeeding or replating schedules) and the purpose of the experiment. Suggested minimum plating densities for CD34+ or Lin- cells are in the order of 2000 – 3000 cells per mL. For more highly purified progenitors, for example, primitive progenitors isolated with the StemSep® Primitive Progenitor Enrichment Cocktail (Catalog #14057/14067), EasySep® CD34+ Positive Selection Kit (Catalog #18056/18056R) or subsets of CD34+ cells obtained by cell sorting, lower cell densities, in the order of a few hundred cells per mL, can be used. Highly purified stem and progenitor cells, for example, subsets of Lin- Sca-1+Kit+ mouse BM cells or CD34+CD38- human cells, can be cultured at very low densites, or even at one cell per well, provided that culture conditions have been optimized.[5]

For some applications it may be useful to plate cells at high plating densities, in the range of 10^4 – 10^5 cells per mL. This may be the case if the progenitor frequency in the cell sample is very low, the cells are cultured for only a few days (e.g. during gene transfer procedures), and/or the cultures are replated or refed with fresh medium and cytokines before the cultures become overgrown.

3.3 REFEEDING AND REPLATING

Frequent inspection of cultures and monitoring of cell number and viability is important to assess whether refeeding or replating is necessary. The growth rate is dependent on the type and purity of the cells, as well as the cytokines and other stimuli to which they are exposed. If cultures are started with highly purified HSCs, for example, $CD45^{mid}lin^-Rhodamine^-$Side Population mouse BM cells, which are mostly quiescent *in vivo*, it may take 24 hours or more before the cells initiate their first cell division in culture, but the second and later cell divisions occur much faster.[6]

In our experience, human CB CD34+ cells grow faster with StemSpan® CC100 than with StemSpan® CC110 and may need to be refed or replated earlier. Overall expansion rates with StemSpan® CC220 are much slower than with either StemSpan® CC100 or StemSpan® CC110 as only the megakaryocyte progenitors are optimally stimulated by this cytokine combination.

It may be useful to keep the cell concentration below 10^5 cells per mL during the entire culture period to maintain optimal growth and viability. Replating can either be done by harvesting and centrifuging the cells and resuspending them in fresh culture medium at a lower density, or by simply diluting the cell

suspension in a larger volume with fresh medium and cytokines and transferring the cells to a larger flask or to several flasks. The latter approach is easier and avoids cell loss as a result of harvesting and centrifugation, but may not be the best method if the growth rate is very high. Trypsinization of hematopoietic cells is usually not necessary as hematopoietic stem cells and progenitor cells are non-adherent, unless the cells are grown on stromal feeder layers or in wells coated with extracellular matrix proteins that promote adherence.

The refeeding frequency and cell density may need to be determined on a case-by-case basis, as growth rates and cell yields can vary significantly between different experiments and cell preparations, even if all other experimental conditions are identical. If the growth rate is low and the cell density stays well below 10^5 cells per mL, it may still be beneficial to refresh part of the culture medium or add fresh cytokines every three or four days as cytokines have limited stability at 37°C and their levels may decline steadily as a result of breakdown and consumption by the cells. Simply resuspending the cells by gently pipetting up and down in the wells or culture flask may also help the overall expansion of total cells.

3.4 CULTURE PERIOD

The optimal culture period is highly dependent on the type, purity and origin of the progenitor cells before culture, the culture conditions and type and number of cells required at the end of the culture. As many cytokines induce proliferation and differentiation, stem cells and progenitors will be lost after a few days of culture with most cytokine combinations. Even if immature progenitor numbers remain constant, their frequency will gradually decline by accumulation of their expanding progeny. As shown in Figure 1, total cell numbers can increase 100-fold after eight days of culturing CB $CD34^+$ cells in the presence of StemSpan® CC100. With BM or mobilized peripheral blood (MPB) similar results can be obtained. Most of the cultured cells consist of neutrophils and monocytes and their immediate precursors. The frequency of immature, $CD34^+$ cells and CFCs is typically less than 1% of the expanded cells, while more primitive cells, in particular long-term culture initiating cells (LTC-ICs) and HSCs identifiable in transplantation assays, are even less frequent and will often become undetectable after eight days of culture.

For the generation of specific cell types, cultures can be maintained for several weeks provided the cells are refed and replated appropriately as discussed above. For example, the optimal culture period for expansion of megakaryocyte progenitors in StemSpan® SFEM or StemSpan® H3000, supplemented with LDL and StemSpan® CC220, is 14 days with replating in fresh medium, LDL and cytokine cocktail on day 7 (Figure 2). Cultures of erythroid progenitors in StemSpan® SFEM supplemented with SCF, EPO, dexamethasone and lipids have been maintained for over 40 days with daily partial medium changes and maintenance of the cell concentration at 2×10^6 cells per mL. Under these conditions, over a million fold expansion of erythroblast numbers was achieved.[3]

For gene transfer applications, culture periods before or during gene transfer are generally kept short, usually four days or less for retroviral vectors and one day or less for lentiviral vectors. This is because the purpose is usually not to generate large numbers of differentiated cells, but to transduce as many stem cells and progenitors as possible without inducing their differentiation.[7,8]

4. ASSAYS TO MEASURE STEM CELL AND PROGENITOR CELL EXPANSION

Careful analysis of the numbers and types of cells produced, preferably at different time points, is essential to assess the effects of culture conditions on stem cell survival, proliferation and differentiation. Depending on the purpose of the expansion cultures, several of the assays discussed below should be considered, in addition to counting total and viable cells, and other analytical methods to assess cell morphology and specific functions.

4.1 IMMUNOPHENOTYPING

Immunophenotyping by flow cytometry is usually performed to assess the frequency of progenitors and of mature cells of different lineages. Useful panels to assess lineage-specific human cell expansion include CD71 and glycophorin A (CD235a) for erythroid cells; CD14, CD15, CD11b and CD66b for cells of monocyte and granulocyte lineages; and CD41a for megakaryocytic cells. The number of human $CD34^+$ cells after culture can give information on the presence of immature cells in the culture and on gradual changes during the course of the culture.

Immunophenotyping alone is not sufficient to identify and characterize primitive hematopoietic cells in expansion cultures. Hematopoietic cell surface antigen expression patterns change during culture and the marker combinations used to identify and isolate primitive cells before culture may not identify primitive cells after culture. For example, non-cultured human $CD34^+CD38^-$ cells are enriched for primitive cells (including NOD/SCID repopulating cells and LTC-IC), but most $CD34^+CD38^-$ cells detected after culture have lost their repopulating ability and do not represent primitive cells.[9] Other phenotypic characteristics (e.g. $CD34^+CD90^+$ or $CD34^+CD38^-CD33^+CD90^+CD45RA^-$) may be more informative for measuring the presence of primitive human hematopoietic cells after culture.[9,10]

Similarly, non-cultured mouse stem cells can be purified to almost homogeneity using combinations of the $Lin^-Sca1^+Kit^+$ (LSK), $CD48^-CD150^+$ (SLAM) and $Lin^-CD150^+EPCR^+$ phenotypes. Cells with repopulating ability in seven-day cultures of $CD34^-$LSK cells have been shown to be enriched in a $CD48^-$LSK subset, while cultured $CD48^+$LSK and $Sca1^-$ cells did not engraft.[11] This suggests that this marker combination may be useful to identify mouse stem cells after culture.

Because of the uncertainties regarding the choice of markers that define stem cells after culture, evaluation of stem cell expansion on the basis of immunophenotyping or gene expression assays are only valid if confirmed by examination of the functional properties of the cultured cells using *in vitro* assays and/or transplantation assays.

4.2 COLONY-FORMING CELL ASSAYS

Colony-forming cell (CFC) assays are short-term clonal assays that can be used to measure changes in the numbers of different subsets of progenitor cells in hematopoietic expansion cultures. They are also used as a read-out in the LTC-IC and marrow repopulating ability (MRA) assays for more primitive progenitors described below. Myeloid CFC assays measure the frequency of committed progenitors of erythroid, monocyte, granulocyte and megakaryocyte lineages by their ability to form colonies of morphologically recognizable cells after approximately two weeks of culture in semi-solid medium, such as MethoCult® and MegaCult®-C. More primitive multi-potent progenitors, usually referred to as CFU-GEMM, that can form colonies containing erythroid and non-erythroid (granulocycytes, monocytes and/or megakaryocytes) cells can also be detected in standard CFC assays. However, most progenitors that can be detected in CFC assays are not thought to participate in long-term *in vivo* hematopoiesis after transplantation.[12] Therefore CFC assays are not sufficient to measure HSC expansion *per se*, but they are very useful to monitor the functional capacities of hematopoietic cell populations before, during and after expansion cultures and to examine the effects of cytokines or culture protocols on expansion of different hematopoietic lineages. Several publications provide details about human and mouse CFC assays.[13,14]

4.3 LONG-TERM CULTURE ASSAYS

The long-term culture-initiating cell (LTC-IC) assay and the similar cobblestone area-forming cell (CAFC) assay, both performed on stromal feeder layers, can measure the frequency of primitive progenitor cells that are more differentiated than repopulating stem cells but more primitive than progenitor cells detected in CFC assays. In order to provide information about the frequency of primitive progenitors, LTC-IC and CAFC assays have longer incubation periods to ensure that more mature hematopoietic progenitors (e.g. CFCs) present in the input cells are not detected. Culture periods of five weeks are standard but extension of the culture period to eight weeks or longer has been used as a strategy to identify more primitive subsets that more closely resemble repopulating stem cells than the LTC-IC measured after five weeks.[15]

LTC-IC assays and the transplantation assays described below are currently the standard assays that are required to demonstrate whether expansion (or maintenance) of stem cells or primitive progenitors occurs in culture. Details about performing, analyzing and interpreting LTC-IC and CAFC assays can be found in the following references: 14, 16 and 17. For more information about MyeloCult® media formulated for use in LTC-IC and CAFC assays, please visit our website at www.stemcell.com/product_catalog/myelo.aspx.

4.4 TRANSPLANTATION ASSAYS

4.4.1 MOUSE CELLS

The presence of hematopoietic stem cells in non-cultured or cultured cell preparations can only be tested experimentally by transplanting the cells into lethally or sublethally irradiated mice and measuring the ability of these cells to reconstitute hematopoiesis. Hematopoiesis derived from donor cells can be measured by employing genetic differences between donor and recipient mouse strains, such as the expression of different CD45 isoforms (CD45.1 and CD45.2), which can be detected by flow cytometry after staining blood, spleen or BM samples with appropriate antibodies.[18] Co-staining with lineage-specific antibodies is used to measure the contribution of donor cells to different blood cell lineages.

By examining serial peripheral blood samples of the transplanted mice over weeks and months it is possible to get information on the ability of the transplanted cells to perform short-term engraftment and long-term multilineage hematopoiesis. Some cultured cells may be able to reconstitute donor hematopoiesis during a short period (e.g. several weeks) and disappear later, and/or only reconstitute a single lineage (e.g. only granulocytes). A cell population can only be considered to contain true pluripotent stem cells if donor cells can be detected for prolonged periods of time, usually at least five months after transplantation and in different blood cell lineages, specifically granulocytes and/or monocytes and lymphocytes.

The donor cells should also contribute to the erythroid and megakaryocytic lineages, but RBC and platelet chimerism cannot be easily measured in the CD45.1/CD45.2 transplantation model as mature RBCs and platelets lack CD45 expression. If

measuring the contribution of transplanted cells to RBC and/or platelet reconstitution is important, other transplantation models could be used. These include transplantation with cells from transgenic EGFP-expressing donor mice, which can be detected on the basis of their fluorescence.[19] RBC derived from normal stem cells can also be measured. If mutant strains carrying the W or alpha-thalassemia mutations are used as recipients of HSCs from normal mice RBC chimerism can be measured on the basis of size differences between donor-derived normal RBCs and mutant recipient RBCs using the forward scatter parameter of a standard flow cytometer.[20]

Measuring differences between the frequency of donor cells in mice transplanted with equal numbers of cells is the simplest method to compare the engraftment ability of two cell populations, for example, cultured and non-cultured cells, or cultured cells exposed to different culture conditions. A more accurate method is to transplant different doses of the various cell preparations into groups of mice and obtain information about relative differences in engraftment ability from the number of cells in each group required to achieve a certain level of chimerism in the recipients. The frequency of repopulating cells can be measured by setting up these assays in a limiting dilution format and determining the fraction of non-engrafting mice in each cell dose group using Poisson statistics and the method of maximum likelihood. This analysis can be done using computer programs, such as L-Calc™ (Catalog #28600). A free software download is available on our website at www.stemcell.com/product_catalog/myelo.aspx.

Donor cells usually have to compete against constant numbers of surviving stem cells in sublethally irradiated recipients or against recipient-type stem cells which were co-transplanted with the donor cells to ensure survival of lethally irradiated recipient mice. For this reason, the donor stem cells that are able to engraft in this assay are referred to as competitive repopulating units (CRU) and the assay itself is often referred to as the CRU assay. Detailed descriptions of the CRU assay can be found in references 14 and 21.

Performing CRU assays to test stem cell expansion protocols requires large numbers of mice and takes six months or longer to complete. This may not always be feasible if many different cell preparations need to be tested or little time is available. Two of the older *in vivo* assays might be considered as faster alternatives to study the *in vivo* repopulating ability of hematopoietic cells. These are the colony-forming unit-spleen (CFU-S) assay[22] and the marrow-repopulating ability (MRA) assay.[23]

In the CFU-S assay, cells that home to the spleen of the recipient mice give large mixed colonies of hematopoietic cells that form nodules which can be counted after 8 - 12 days. In the MRA assay, which is often combined with the CFU-S assay, BM cells from the recipient mice are harvested at approximately 12 days after tranplantation and assayed for the presence of donor-type CFCs in standard CFC assays, or for the presence CFU-S by transplantation into secondary recipients. The CFU-S assay identifies cells that can contribute to rapid and short-term engraftment after transplantation, but does not detect the most primitive stem cells subsets that are needed for sustained long-term *in vivo* hematopoiesis (i.e. long-term repopulating cells). The MRA-assay appears to detect more primitive cells, more similar to those detected in long-term repopopulation assays[24,25] and might be useful as a quicker alternative to long-term transplantation assays to detect stem cell expansion in cultured cell preparations.

4.4.2 HUMAN CELLS

The engraftment ability of human stem cells can be measured by transplantation into immunodeficient mouse strains such as sublethally irradiated NOD/SCID mice. Similar to the mouse CRU assay, these assays can also be performed in a limiting dilution format and provide information about the frequency of repopulating cells (referred to as NOD/SCID repopulating cells). NOD/SCID assays are usually terminated at 5 to 8 weeks after transplantation and it has not yet been fully established how well they can measure the long-term repopulating ability of human stem cells, in particular after expansion culture. Data from parallel transplantations of genetically marked baboon hematopoietic cells into NOD/SCID mice and baboons suggest that the NOD/SCID mouse assay may only detect short-term repopulating cells.[26] Despite these limitations, the NOD/SCID assay is currently the best functional assay to measure the effects of expansion cultures and other manipulations on human repopulating hematopoietic stem cells.[27,28]

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