

[7] Hematopoietic Stem Cells

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Abstract

Hematopoietic stem cells (HSCs) have the capacity to self-renew and the potential to differentiate into all of the mature blood cell types. The ability to prospectively identify and isolate HSCs has been the subject of extensive investigation since the first transplantation studies implying their existence almost 50 years ago. Despite significant advances in enrichment protocols, the continuous *in vitro* propagation of human HSCs has not yet been achieved. This chapter describes current procedures used to phenotypically and functionally characterize candidate human HSCs and initial efforts to derive permanent human HSC lines.

Introduction

Hematopoietic stem cells (HSCs) are multipotent precursors that have self-renewal capacity and the ability to regenerate all the different cell types that comprise the blood-forming system (Bonnet, 2002; McCulloch and Till, 2005). Transplantation of HSCs forms the basis of consolidation therapy in cancer treatments and is used to cure or ameliorate a number of hematologic and genetic disorders (Shizuru *et al.*, 2005; Steward and Jarisch, 2005). With certain caveats (McCormack and Rabbitts, 2004), HSCs are also an attractive target cell population for gene therapies because they are readily accessible for *ex vivo* genetic modification and allow for the possibility of sustained transgene expression in circulating peripheral blood cells throughout the lifetime of an individual (Hawley, 2001; Moayeri *et al.*, 2005).

Historically, mouse HSCs were identified retrospectively by utilizing clonal *in vivo* assays wherein labeled cells (e.g., genetically tagged with reporter genes) were assessed for potential to functionally reconstitute hematopoiesis after injection into conditioned hosts, with self-renewal capacity demonstrated by serial transfer into secondary recipients (Abramson *et al.*, 1977; Capel *et al.*, 1990; Jordan and Lemischka, 1990; Keller *et al.*, 1985). Limiting dilution analysis of total bone marrow preparations allowed quantitative estimation of HSC frequencies ranging from 1 in 10,000 to 1 in 100,000 cells (Harrison, 1980; Harrison *et al.*, 1993; Szilvassy *et al.*, 1990). A major advance in the field of HSC biology was the prospective isolation of enriched populations of mouse HSCs on the basis of cell surface phenotype

(Spangrude *et al.*, 1988). With the exception of clinical gene-marking trials (Stewart *et al.*, 1999), analogous HSC transplantation experiments cannot be performed in humans. For this reason, xenogeneic transplant models have been developed as surrogate assays to evaluate human hematopoietic precursors for *in vivo* repopulating potential. These assays have helped to elucidate the composition of the human HSC compartment (Bhatia *et al.*, 1998; Gallacher *et al.*, 2000; Glimm *et al.*, 2001; Guenechea *et al.*, 2001; Mazurier *et al.*, 2003; Wang *et al.*, 2003; Zanjani *et al.*, 1998) and have provided paradigms for translation to clinical applications (Baum *et al.*, 1992; Civin *et al.*, 1996b; Lang *et al.*, 2004; Shizuru *et al.*, 2005; Shmelkov *et al.*, 2005; Shpall *et al.*, 1994; Yin *et al.*, 1997).

In this chapter, we discuss the phenotypic and functional characteristics of mouse and human HSCs, and describe protocols for the isolation and assay of candidate human HSCs. A procedure to derive factor-dependent human hematopoietic progenitor cell lines is also provided.

Identification and Enrichment of HSCs

Cell Surface Markers

All HSC activity in adult mouse bone marrow is contained in a population of cells characterized by expression of the c-Kit tyrosine kinase receptor (the receptor for stem cell factor, SCF), stem cell antigen-1 (Sca-1, Ly-6A/E), low levels of the Thy-1.1 cell surface antigen (Thy-1.1^{lo}), and no or low levels of expression of many cell surface antigens found on differentiated cells belonging to various lineages (referred to as lineage-negative or Lin⁻) (Shizuru *et al.*, 2005). Mouse HSCs variably express the sialomucin CD34, depending on developmental stage and cell cycle status (Ito *et al.*, 2000; Matsuoka *et al.*, 2001; Osawa *et al.*, 1996; Sato *et al.*, 1999). Studies have identified a number of additional cell surface antigens that mark mouse HSCs, including the following: the TIE family of receptor tyrosine kinases (Arai *et al.*, 2004; Iwama *et al.*, 1993); endoglin, an ancillary transforming growth factor- β receptor (Chen *et al.*, 2002); endomucin, a CD34-like sialomucin (Matsubara *et al.*, 2005); CD150, the founding member of the SLAM family of receptors (Kiel *et al.*, 2005; Yilmaz *et al.*, 2006); CD201, the endothelial protein C receptor (Balazs *et al.*, 2006); and prion protein (Zhang *et al.*, 2006b). The receptor for thrombopoietin (TPO), c-Mpl, is also expressed on $\sim 70\%$ of mouse c-Kit⁺Lin⁻Sca-1⁺ HSCs (Solar *et al.*, 1998).

In humans, clinical protocols involving enrichment for HSCs generally utilize cells expressing CD34 (Civin *et al.*, 1996b; Shizuru *et al.*, 2005; Shpall *et al.*, 1994), which is expressed on $\sim 0.2\text{--}3\%$ of the nucleated cells in cord

blood, bone marrow, and mobilized peripheral blood (Civin *et al.*, 1984; Krause *et al.*, 1996; Sutherland *et al.*, 1996). Experimentally, further isolation and characterization of Lin⁻CD34⁺ subpopulations have defined more primitive precursors with hematopoietic repopulating activity that express combinations of the CD59 surface antigen related to Sca-1, the vascular endothelial growth factor receptor-2 (VEGFR2 or KDR), and low levels of c-Kit (CD117), Thy-1 (CD90), and the CD38 surface antigen (Baum *et al.*, 1992; Civin *et al.*, 1996a; Gunji *et al.*, 1993; Hill *et al.*, 1996; Kawashima *et al.*, 1996; Larochele *et al.*, 1996; Ziegler *et al.*, 1999). As in the mouse, the TIE family of receptor tyrosine kinases and the TPO receptor c-Mpl also appear to further enrich for human HSCs, being expressed on ~80% and ~70% of CD34⁺CD38⁻ cells, respectively (Hashiyama *et al.*, 1996; Ninos *et al.*, 2006; Solar *et al.*, 1998).

It has become appreciated that the CD133 cell surface antigen is another important human HSC marker (de Wynter *et al.*, 1998; Gallacher *et al.*, 2000; Hess *et al.*, 2006; Lang *et al.*, 2004; Shmelkov *et al.*, 2005; Yin *et al.*, 1997). CD133, the human homolog of mouse Prominin-1 (Shmelkov *et al.*, 2005), was first identified as a selective human HSC surface molecule, using a monoclonal antibody recognizing a particular glycosylated form of Prominin-1 designated as AC133 (Yin *et al.*, 1997). Selection for CD133⁺ hematopoietic precursors yields >90% CD34⁺ cells containing all the human hematopoietic repopulating activity. Notably, the extremely rare CD34⁻ candidate HSCs that had previously been identified (Bhatia *et al.*, 1998; Gao *et al.*, 2001; Wang *et al.*, 2003; Zanjani *et al.*, 1998) reside within the CD133 fraction (Gallacher *et al.*, 2000).

Enriched populations of human HSCs are routinely obtained by positive selection for CD34/CD133 and/or by depletion of lineage-committed cells, using monoclonal antibodies recognizing differentiation markers (such as CD2, CD3, CD14, CD16, CD19, CD24, CD41, CD56, CD66b, and CD235a) in the context of immunomagnetic or fluorescence-activated cell-sorting methodologies. In this regard, it is important to bear in mind that physical manipulation of HSCs during the enrichment procedure may not be without effect on cell physiology (Kimura *et al.*, 2004). For example, it is conceivable that binding of antibodies to CD34/CD133 may trigger intracellular signaling pathways that could modulate HSC function. Interestingly, one study suggests that the majority of cells within the CD34⁺CD38⁻Lin⁻ HSC compartment express the myeloid-associated lineage markers CD13, CD33, and CD123 [the low-affinity binding subunit of the interleukin (IL)-3 receptor] (Taussig *et al.*, 2005), indicating that some caution is warranted when selecting a cocktail of monoclonal antibodies for lineage marker-depletion enrichment of human HSCs.

Fluorescent Dye Staining

Hoechst 33342 and Rhodamine 123

Other strategies that have been utilized to identify and enrich for HSCs are based on the staining patterns of fluorescent dyes (Bertoncello *et al.*, 1985; Goodell *et al.*, 1996; Jones *et al.*, 1995; Leemhuis *et al.*, 1996; Storms *et al.*, 1999; Visser *et al.*, 1981; Wolf *et al.*, 1993). Rhodamine 123 (which preferentially accumulates in active mitochondria) and Hoechst 33342 (a *bis*-benzimidazole that binds to adenine–thymine-rich regions of the minor groove of DNA) are two fluorescent vital dyes that have been routinely used to characterize hematopoietic precursor populations (Bertoncello *et al.*, 1985; Leemhuis *et al.*, 1996; McAlister *et al.*, 1990; Visser *et al.*, 1981; Wolf *et al.*, 1993). Rhodamine 123 staining of mouse bone marrow cells demonstrated that HSCs with long-term repopulating potential stained dimly whereas more brightly staining hematopoietic precursors could provide only short-term repopulation (Bertoncello *et al.*, 1988, 1991; Spangrude and Johnson, 1990; Zijlmans *et al.*, 1995). Moreover, subpopulations of mouse bone marrow cells that stained most weakly with both dyes were shown to be highly enriched for long-term repopulating HSCs (Bertoncello and Williams, 2004; Leemhuis *et al.*, 1996; Wolf *et al.*, 1993). Decreased staining with these dyes generally reflects a metabolically and mitotically inactive state (Arndt-Jovin and Jovin, 1977; Johnson *et al.*, 1980; Spangrude and Johnson, 1990). However, it is now appreciated that decreased staining of HSCs with rhodamine 123 and Hoechst 33342 is also due to efflux mediated by at least two members of the ATP-binding cassette (ABC) family of transporters, ABCB1 (also referred to as MDR1 or P-glycoprotein) and ABCG2 (also referred to as BCRP, MXR, or ABCP) (Chaudhary and Roninson, 1991; Juliano and Ling, 1976; Scharenberg *et al.*, 2002; Zhou *et al.*, 2001, 2002).

Side Population Assay

A novel method that simultaneously monitors the low fluorescence intensity of Hoechst 33342 staining at ~ 450 nm and at >675 nm after ultraviolet excitation identifies a rare ($<0.1\%$) subpopulation of mouse bone marrow cells, referred to as “side population” (SP) cells, which contains the vast majority of long-term hematopoietic repopulating activity (Goodell *et al.*, 1996). The ABC transporter *Bcrp1* (the mouse ortholog of human ABCG2) expressed in mouse bone marrow cells is the major determinant of the mouse SP profile (Zhou *et al.*, 2001, 2002). Subsequent multiparameter flow cytometric analysis of mouse bone marrow SP cells showed that approximately one third exhibited the $c\text{-Kit}^+\text{Thy-1.1}^{\text{lo}}\text{Lin}^-$ Sca-1^+ phenotype whereas approximately one half expressed CD34

(Pearce *et al.*, 2004). In another study, mouse bone marrow cells with the strongest dye efflux activity, which exhibited the highest hematopoietic repopulating activity, were shown to have a c-Kit⁺Lin⁻Sca-1⁺CD34⁻ phenotype (Matsuzaki *et al.*, 2004).

The SP assay has also been applied to human hematopoietic tissues (Eaker *et al.*, 2004; Goodell *et al.*, 1997; Naylor *et al.*, 2005; Preffer *et al.*, 2002; Scharenberg *et al.*, 2002; Storms *et al.*, 2000; Uchida *et al.*, 2001). Unlike mouse bone marrow SP cells, human hematopoietic SP cells constitute a much more phenotypically and functionally heterogeneous precursor population (Naylor *et al.*, 2005; Preffer *et al.*, 2002; Storms *et al.*, 2000; Uchida *et al.*, 2001). CD34⁻ SP cells have been identified in several studies, but to date repopulating ability of human hematopoietic SP cells has been demonstrated only for CD34⁺ subpopulations (Eaker *et al.*, 2004; Scharenberg *et al.*, 2002; Uchida *et al.*, 2001).

Fluorescent Substrates for Cytosolic Aldehyde Dehydrogenase Activity

Cytosolic aldehyde dehydrogenase (ALDH), an enzyme responsible for oxidizing a variety of intracellular aldehydes, is expressed at high levels in HSCs, conferring resistance to the alkylating agents cyclophosphamide and 4-hydroxyperoxycyclophosphamide (Gordon *et al.*, 1985; Kastan *et al.*, 1990; Sahovic *et al.*, 1988). Fluorescent substrates for ALDH have been developed and shown to be useful for isolating mouse and human HSCs (Fallon *et al.*, 2003; Hess *et al.*, 2004, 2006; Jones *et al.*, 1995, 1996; Storms *et al.*, 1999). In proof-of-principle studies (Jones *et al.*, 1995, 1996), dansyl-aminoacetaldehyde (DAAA) was used as an ALDH substrate. DAAA can diffuse freely across the cell membrane because it is uncharged. Cells expressing ALDH oxidize DAAA to dansyl-glycine, which is retained intracellularly by virtue of a charged carboxylate group at physiologic pH, and ALDH⁺ cells are identified by dansyl fluorescence on excitation with ultraviolet light. More recently, a newer fluorescent substrate for ALDH, termed BODIPY-aminoacetaldehyde (BAAA), was synthesized, which uses a nontoxic visible light-excitable fluorophore BODIPY (Storms *et al.*, 1999). Similar to DAAA, BAAA is uncharged and diffuses freely across the cell membrane, becoming converted to BODIPY-aminoacetate (BAA), which is retained intracellularly because of its net negative charge in the presence of an inhibitor of the ABC transporter ABCB1 (Storms *et al.*, 1999).

Mouse hematopoietic precursors enriched for high expression of ALDH by staining with BAAA or DAAA may represent a novel class of HSCs, which express undetectable or low levels of the c-Kit, Thy-1, Sca-1, and CD34 HSC markers, but which produce long-term albeit delayed multilineage engraftment (Armstrong *et al.*, 2004; Jones *et al.*, 1996). Flow cytometric

analysis of human cord blood cells stained with BAAA identified a population of cells (at a frequency of $\sim 1\%$) with bright fluorescence intensity (ALDH^{br}) and low orthogonal light “side” scattering (SSC^{lo}), comprising $\sim 74\%$ CD34⁺ cells and $\sim 46\%$ CD34⁺CD38^{lo/-} cells, which was largely depleted of cells with mature T cell, natural killer cell, myeloid, erythroid, and platelet lineage markers (Storms *et al.*, 1999). The SSC^{lo}ALDH^{br} population still contained a small number of B cells, however ($\sim 12\%$) (Storms *et al.*, 1999). In another study, Lin⁻ depletion combined with selection for ALDH^{br} cells by BAAA staining demonstrated enrichment for hematopoietic precursors coexpressing CD133 and CD34 ($\sim 73\%$) and all the hematopoietic repopulating activity in human cord blood preparations (Hess *et al.*, 2004). A follow-up study by the same group reported that prospective selection of ALDH^{br}Lin⁻ human cord blood cells for CD133 expression yields a population of primitive precursors that are primarily CD34⁺ ($\sim 95\%$) and that contains all long-term hematopoietic repopulating activity (Hess *et al.*, 2006).

A two-step enrichment strategy for human HSCs combining positive selection for CD133⁺ cells and an assay for high-level ALDH expression (SSC^{lo}ALDH^{br} cells) is described here.

Protocol for Isolation of Candidate Human HSCs

1. Obtain human cord blood, bone marrow, or mobilized peripheral blood cells after informed consent in conformity with a human subjects protocol approved by an institutional review board, or purchase from a commercial source [e.g., AllCells (Berkeley, CA), Cambrex (East Rutherford, NJ), or StemCell Technologies Vancouver, BC, Canada)]. For human cord blood cells, dilute anticoagulated cord blood 1:3 with phosphate-buffered saline (PBS) containing 0.6% anticoagulant citrate dextrose solution A (ACD-A, cat. no. C3821; Sigma-Aldrich, St. Louis, MO). Layer 35 ml of diluted cord blood over 12 ml of Ficoll-Paque PLUS (cat. no. 17-1440-02; GE Healthcare Life Sciences, Piscataway, NJ). Centrifuge at 375g for 30 min at room temperature (22°). Collect cells at the interface, dilute with PBS containing 0.6% ACD-A, and centrifuge at 375g for 10 min at 22°. Resuspend cells in erythrocyte lysing solution (0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA, pH 7.2–7.4) and incubate for 10 min at 22°. Centrifuge at 375g for 10 min at 22° and wash once in PBS.

2. Subsequent enrichment for cells expressing CD133 can be performed with a CD133 MicroBead kit (cat. no. 130-050-801; Miltenyi Biotec, Bergisch Gladbach, Germany), utilizing superparamagnetic beads conjugated to a monoclonal mouse anti-human CD133/1 antibody and a VarioMACS separator (cat. no. 130-090-282; Miltenyi Biotec). Follow the manufacturer’s recommendations and obtain $\sim 1 \times 10^6$ CD133⁺ cells/ml

with >95% purity (if necessary, repeat the enrichment with a second MACS cell separation column).

3. Prepare aliquots of $\sim 5 \times 10^4$ CD133⁺ cells/50 μ l in PBS containing 2% fetal bovine serum (FBS) for staining individually with fluorochrome-conjugated anti-CD133/2 (which recognizes a different epitope than CD133/1), anti-CD34 and anti-CD38 monoclonal antibodies, as compensation controls: unstained; anti-CD133/2–phycoerythrin (PE) (cat. no. 130-090-853; Miltenyi Biotec); anti-CD34–peridinin chlorophyll protein (PerCP) (cat. no. 340430; BD Biosciences Pharmingen, San Diego, CA); and anti-CD38–allophycocyanin (APC) (cat. no. 555462; BD Biosciences Pharmingen). Incubate at 4° for 20 min. Add 2 ml of PBS containing 2% FBS. Centrifuge at 375g for 10 min at 4°. Decant the supernatant and drain. Resuspend in 300 μ l of PBS containing 2% FBS. Keep on ice until analysis.

4. Identification of CD133⁺ cells expressing high levels of ALDH activity can be facilitated by using a commercial kit for BAAA staining (ALDEFLUOR kit, cat. no. 01700; StemCell Technologies). Centrifuge 1×10^6 CD133⁺ cells at 375g for 10 min at 25°. Decant the supernatant and drain. Resuspend CD133⁺ cells in 1 ml of proprietary ALDEFLUOR assay buffer (containing an inhibitor of ABCB1 transporter efflux activity) with the ALDEFLUOR reagent (BAAA-DA; BODIPY-aminoacetaldehyde diethyl acetal) according to the manufacturer's instructions. BAAA-DA is dissolved in dimethylsulfoxide and exposed to hydrochloric acid to convert it to the ALDH substrate BAAA. As BAAA diffuses freely across the cell membrane, all the viable cells will be fluorescent. However, cells with high ALDH activity metabolize the substrate into BAA (BODIPY-aminoacetate) containing a charged carboxylate group and become intensely fluorescent. Including an inhibitor of ABCB1 transporter efflux activity throughout the assay ensures retention of the fluorescent BAA compound within the cell. Cells incubated in the presence of diethylaminobenzaldehyde (DEAB), a potent ALDH inhibitor, provide a control for background BAA fluorescence.

5. On completion of the assay (30–60 min), stain aliquots of the cells with combinations of anti-CD133/2–PE, anti-CD34–PerCP, and anti-CD38–APC at 2–8° as described previously. After 20 min, centrifuge all samples at 375g for 10 min at 4°. Decant the supernatant and drain. Resuspend samples in ALDEFLUOR assay buffer.

6. Analyze the samples with a flow cytometer equipped for excitation wavelengths of 488 and 633 nm. Detect scatter and fluorescence signals with 488/10 bandpass (BP) filters for SSC and forward scatter signals, 530/30 BP for BAA fluorescence, 576/26 BP for anti-CD133–PE, 675/20 BP for anti-CD34–PerCP, and 660/20 BP for anti-CD38–APC signals.

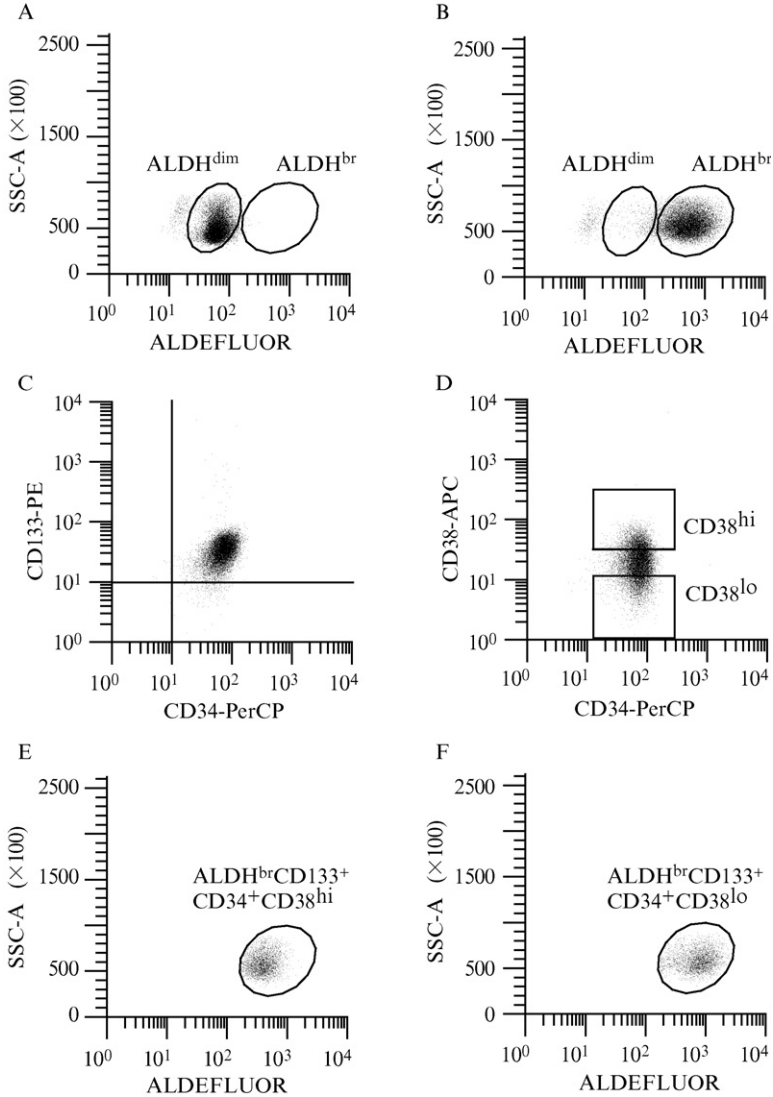


FIG. 1. Flow cytometric characterization of human CD133⁺ cord blood cells expressing high levels of ALDH activity. (A) Negative control: Human cord blood cells highly enriched for CD133 expression (>95% CD133⁺), exhibiting low side scatter (SSC^{lo}), and stained with BAAA (using the ALDEFLUOR reagent) in the presence of DEAB, a potent ALDH inhibitor, show background levels of BAA fluorescence (ALDH^{dim}). Events to the left of the ALDH^{dim} gate represent dead cells with no BAA fluorescence. (B) Human cord blood cells highly enriched for CD133 expression (>95% CD133⁺), exhibiting low side scatter (SSC^{lo}) and stained with BAAA (using the ALDEFLUOR reagent) in the absence of DEAB, show

7. Fluorescence-activated cell sorting of CD133⁺ cells expressing the highest levels of ALDH activity enriches for candidate human HSCs with a predominantly SSC^{lo}ALDH^{br}CD133⁺CD34⁺CD38^{lo} phenotype (Fig. 1). *Note:* Because dead and dying cells without intact cellular membranes cannot retain the fluorescent BAA derivative, only viable cells are identified by this method.

Functional Characterization of Candidate HSCs

Surrogate In Vivo Assays

Heterogeneity of the human HSC compartment and continued questions regarding cell surface phenotype necessitated the use of *in vivo* assays of HSC function (Baum *et al.*, 1992; Bhatia *et al.*, 1998; Dao *et al.*, 2003; Dorrell *et al.*, 2000; Gallacher *et al.*, 2000; Glimm *et al.*, 2001; Guenechea *et al.*, 2001; Mazurier *et al.*, 2003; Sieburg *et al.*, 2006; Wang *et al.*, 2003; Zanjani *et al.*, 1998).

Several xenogeneic transplant models have been developed as surrogate assays of human hematopoietic repopulating cells. The preimmune fetal sheep transplant assay has emerged as a useful large animal model (Civin *et al.*, 1996a; Zanjani *et al.*, 1996). However, the majority of functional assays of human HSC activity involve transplantation into immunodeficient mice with various degrees of residual natural immunity (Bhatia *et al.*, 1997b; Bock *et al.*, 1995; Cashman *et al.*, 1997; Cheng *et al.*, 1998; Gimeno *et al.*, 2004; Glimm *et al.*, 2001; Goldman *et al.*, 1998; Guenechea *et al.*, 2001; Hiramatsu *et al.*, 2003; Hogan *et al.*, 1997; Ishikawa *et al.*, 2002; Ito *et al.*, 2002; Kamel-Reid and Dick, 1988; Kollet *et al.*, 2000; Kyoizumi *et al.*, 1992; Lapidot *et al.*, 1992; Larochelle *et al.*, 1996; Lowry *et al.*, 1996; Mazurier *et al.*, 1999; McCune *et al.*, 1991; Meyerrose *et al.*, 2003; Nolta *et al.*, 1994; Pflumio *et al.*, 1996; Shultz *et al.*, 2005; Traggiai *et al.*, 2004; Vormoor *et al.*, 1994; Wang *et al.*, 1997). The most widely used of these small animal models is the

that almost all the cells expressed high levels of ALDH activity (ALDH^{br}). (C) The vast majority of ALDH^{br}CD133⁺ cells coexpress the CD34 HSC surface antigen. (D–F) Flow cytometric analysis indicates that cells within the more primitive CD133⁺CD34⁺CD38^{lo} subpopulation express higher levels of ALDH activity than do cells within the CD133⁺CD34⁺CD38^{hi} subpopulation. (D) Gating strategy for CD133⁺CD34⁺CD38^{hi} and CD133⁺CD34⁺CD38^{lo} subpopulations. (E) CD133⁺CD34⁺CD38^{hi} cells are enriched for cells with the lowest levels of BAA fluorescence within the ALDH^{br} gate. (F) CD133⁺CD34⁺CD38^{lo} cells are enriched for cells with the highest levels of BAA fluorescence within the ALDH^{br} gate. Flow cytometry data were acquired with a FACSAria instrument (BD Biosciences Immunocytometry Systems, San Jose, CA) and analyzed with WinList 3D version 6.0 prerelease software (Verity Software House, Topsham, ME).

NOD.CB17-*Prkdc*^{scid} mouse—nonobese diabetic (NOD) mice crossed with severe combined immunodeficient (SCID) mice (Bhatia *et al.*, 1997b; Cashman *et al.*, 1997; Hogan *et al.*, 1997; Larochelle *et al.*, 1996; Lowry *et al.*, 1996; Pflumio *et al.*, 1996; Shultz *et al.*, 1995; Wang *et al.*, 1997). NOD/SCID mice support human cell engraftment because of defective rearrangement of T cell receptor and immunoglobulin (Ig) genes, resulting in defects of functional T and B cells; they also have low levels of natural killer cell cytotoxic activity, functionally immature macrophages, and an absence of hemolytic complement. Candidate HSCs collectively termed SCID-repopulating cells (SRCs) are scored positive for engraftment if $\sim 1\%$ CD45⁺ human cells or $>0.1\%$ human DNA can be detected in the bone marrow of NOD/SCID recipients at or greater than 6 weeks posttransplantation. Under most conditions, the NOD/SCID xenograft assay does not require administration of exogenous human cytokines; however, a sublethal conditioning regimen of 250–400 cGy of irradiation is necessary, and cytokine administration or coadministration of accessory cells facilitates engraftment at limiting doses (Bonnet *et al.*, 1999). Under these conditions, the frequency of SRCs in human cord blood cells was determined to be 1 in 9.3×10^5 mononuclear cells (Wang *et al.*, 1997) and 1 in 617 CD34⁺CD38⁻Lin⁻ cells (Bhatia *et al.*, 1997b). Although both lymphoid and myeloid cell populations are found, a shortcoming of the NOD/SCID xenograft assay is the general lack of T cell development, and differentiation of human hematopoietic precursors is limited mainly to immature cells belonging to the B cell and, to a lesser degree, myeloid lineages. Other disadvantages of the NOD/SCID mouse model include its high sensitivity to irradiation and relatively short life span ($\sim 80\%$ of female and $\sim 50\%$ of male NOD/SCID mice develop lethal thymic lymphomas by 20 weeks of age).

Attempts to obtain an improved host for human HSC transplantation led to the development of a strain of immunodeficient mouse in which the residual low natural killer activity present in the NOD/SCID mouse was eliminated by backcrossing the β_2 -microglobulin null allele onto the NOD/SCID background (NOD/SCID/B2m^{-/-}) (Kollet *et al.*, 2000). NOD/SCID/B2m^{-/-} mice support a more than 11-fold higher level of SRC frequency than NOD/SCID mice, with transplantation of $\sim 8 \times 10^4$ human cord blood mononuclear cells resulting in multilineage differentiation in the mouse bone marrow (Kollet *et al.*, 2000). The enhanced SRC frequency in NOD/SCID/B2m^{-/-} mice is due to short-term repopulation by myeloid-restricted CD34⁺CD38⁺ cells and a predominantly CD34⁺CD38⁻ population that has broader lymphomyeloid differentiation potential but that does not efficiently engraft NOD/SCID mice (Glimm *et al.*, 2001). A limitation of NOD/SCID/B2m^{-/-} mice is a relatively short life span due to earlier onset and increased incidence of thymic lymphomas (the mean life span of

NOD/SCID/B2m^{-/-} mice is ~11 weeks shorter than that of NOD/SCID mice) (Christianson *et al.*, 1997). New NOD/SCID models for human HSC engraftment have been reported that lack a functional X-linked common cytokine receptor γ -chain gene (NOD/SCID/ γ_c^-) (Ito *et al.*, 2002; Shultz *et al.*, 2005; Yahata *et al.*, 2002). NOD/SCID/ γ_c^- mice support ~6-fold higher percentages of human hematopoietic cells in the host bone marrow than do NOD/SCID mice, with precursors developing into mature human CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, Ig⁺ B cells, natural killer cells, myeloid cells, and plasmacytoid dendritic cells. Notably, NOD/SCID/ γ_c^- mice survive beyond 16 months of age and even after sublethal irradiation resist lymphoma development.

Other immunodeficient mouse models have been created by crossing mice with a deficient recombinase activating gene 2 (*Rag2*) with mice harboring the γ_c cytokine receptor gene deletion (Gimeno *et al.*, 2004; Goldman *et al.*, 1998; Mazurier *et al.*, 1999; Traggiai *et al.*, 2004; Weijer *et al.*, 2002). *Rag2*^{-/-} γ_c^- mice are characterized by an absence of all T cell, B cell, and natural killer cell function and show no spontaneous lymphoma development. However, efficient human multilineage hematopoietic engraftment in *Rag2*^{-/-} γ_c^- mice with a mixed H-2 major histocompatibility locus background requires exogenous human cytokines (Mazurier *et al.*, 1999).

As noted earlier, the TPO receptor c-Mpl is a selective marker of mouse and human HSCs (Hashiyama *et al.*, 1996; Ninos *et al.*, 2006; Solar *et al.*, 1998). Consistent with this observation, TPO has been demonstrated to be an important HSC supportive factor (Alexander *et al.*, 1996; Fox *et al.*, 2002; Kaushansky, 2003a; Petzer *et al.*, 1996; Solar *et al.*, 1998) in addition to being the physiologic regulator of megakaryocytopoiesis and thrombopoiesis (Kaushansky, 2003b). One report suggested that human TPO is a major limiting factor for multilineage outgrowth of human hematopoietic cells in NOD/SCID mice (Verstegen *et al.*, 2003). To assess the effects of human TPO on hematopoietic engraftment of candidate human HSCs in *Rag2*^{-/-} γ_c^- mice, we generated human TPO-producing *Rag2*^{-/-} γ_c^- mice by lentiviral vector-mediated transgenesis (Lois *et al.*, 2002; Ma *et al.*, 2003; Pfeifer *et al.*, 2002; Punzon *et al.*, 2004). A self-inactivating (SIN) HIV-1-based lentiviral vector, SIN^F-EF-hTPO-W, was developed that expresses the human TPO cDNA from an internal human elongation factor 1 α (EF1 α) promoter (Ramezani and Hawley, 2002a, 2003; Ramezani *et al.*, 2000, 2003). Concentrated vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped lentiviral vector particles (10⁸ transducing units/ml) were microinjected into the perivitelline space of single-cell H-2^b *Rag2*^{-/-} γ_c^- embryos and implanted into pseudopregnant recipient H-2^b *Rag2*^{-/-} γ_c^- mice (Lois *et al.*, 2002; Punzon *et al.*, 2004; Ramezani and Hawley, 2002b). Polymerase chain reaction analysis of

genomic tail DNA, using a forward primer located within the EF1 α promoter and a reverse primer located within the human TPO cDNA, was used to detect founder animals carrying the integrated transgene (H-2^b Rag2^{-/-} γ_c^- -hTPO mice). Serum levels of human TPO in the founder mice ranged between 100 and 500 pg/ml (R. Behnam, M. B. Chase, S. Soukharev, A. Ramezani, and R. G. Hawley, unpublished data). Human CD34⁺ hematopoietic cells were isolated from cord blood as described later and intravenously injected into sublethally irradiated (350 cGy) H-2^b Rag2^{-/-} γ_c^- -hTPO and control H-2^b Rag2^{-/-} γ_c^- mice. As a potential preclinical predictor of the rate of platelet recovery after transplantation and thus an indication of the quality of hematopoietic engraftment (Angelopoulou *et al.*, 2004; Bruno *et al.*, 2004; Perez *et al.*, 2001; Yasui *et al.*, 2003), human platelets were evaluated in peripheral blood from weeks 1 to 8 after transplantation. Human platelets were detected in the peripheral blood of H-2^b Rag2^{-/-} γ_c^- -hTPO but not in control H-2^b Rag2^{-/-} γ_c^- mice by week 3 ($1.2 \pm 0.8\%$), reaching $8 \pm 2\%$ by week 8 (Fig. 2). Flow cytometric analysis of nucleated peripheral blood cells revealed that all the H-2^b Rag2^{-/-} γ_c^- -hTPO mice (15 of 15) but none of the control H-2^b Rag2^{-/-} γ_c^- mice (0 of 6) engrafted with human hematopoietic cells ($17 \pm 7\%$ CD45⁺ human cells at 6 weeks posttransplantation Fig. 3A). Slightly higher engraftment levels were obtained in mice that received coadministration of CD34⁻Lin⁺ accessory cells (17 vs. 13%). Of the engrafted CD45⁺ human hematopoietic cells, $13 \pm 2\%$ were CD19⁺ cells belonging to the B cell lineage and $26 \pm 4\%$ were CD33⁺ myeloid cells (Fig. 3B). In contrast to the negative results obtained with adult H-2^b Rag2^{-/-} γ_c^- recipients, transplantation of CD34⁺ human hematopoietic progenitor cells into sublethally irradiated H-2^b Rag2^{-/-} γ_c^- newborns leads to *de novo* development of T cells, B cells, natural killer cells, myeloid cells, and plasmacytoid dendritic cells, formation of structured primary and secondary lymphoid organs, and production of functional immune responses (Gimeno *et al.*, 2004; Traggiai *et al.*, 2004).

Human Hematopoietic Repopulating Cell Assay Protocol

1. Isolate mononuclear cells from human cord blood as described in the previous section. Enrich for cells expressing CD34 with a CD34 MicroBead kit (cat. no. 130-046-703; Miltenyi Biotec) utilizing superparamagnetic beads conjugated to a monoclonal mouse anti-human CD34 antibody and a VarioMACS separator. Follow the manufacturer's recommendations and obtain $\sim 1 \times 10^6$ CD34⁺ cells/ml with $>95\%$ purity (if necessary, repeat the enrichment with a second MACS cell separation column). Retain the CD34⁻Lin⁺ flow-through cells for coadministration as accessory cells.

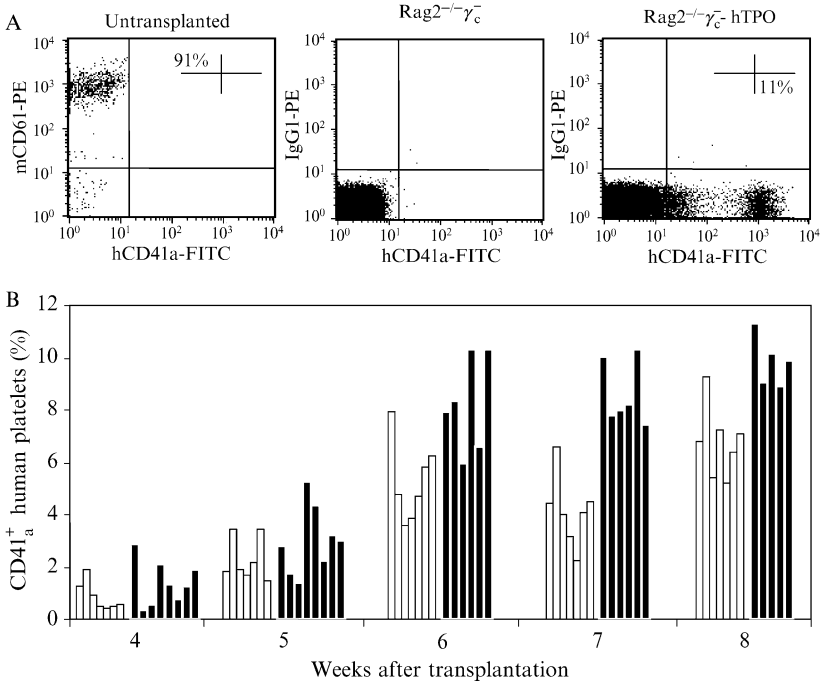


FIG. 2. Human platelet production in the peripheral blood of $Rag2^{-/-}\gamma_c^{-}$ -hTPO mice transplanted with candidate human HSCs. (A) Sublethally irradiated (350 cGy) $Rag2^{-/-}\gamma_c^{-}$ -hTPO and $Rag2^{-/-}\gamma_c^{-}$ mice were transplanted with 5×10^5 human $CD34^+$ cord blood cells. Human platelets were detected in the peripheral blood of all $Rag2^{-/-}\gamma_c^{-}$ -hTPO mice but not $Rag2^{-/-}\gamma_c^{-}$ mice, determined by staining with an anti-human CD41a monoclonal antibody and gating on low forward and side scatter (platelet population gate). Shown are representative examples. Flow cytometry data were acquired with a FACSCalibur instrument and analyzed with CellQuest software (BD Biosciences Immunocytometry Systems). (B) Summary of the analysis of human $CD41a^+$ platelets within the platelet population in the peripheral blood of individual $Rag2^{-/-}\gamma_c^{-}$ -hTPO mice 4 to 8 weeks after transplantation with 5×10^5 human $CD34^+$ cells plus (solid columns) or minus (open columns) 1×10^6 $CD34^+$ Lin^+ accessory cells.

2. All animal procedures are carried out in accordance with Institutional Animal Care and Use Committee guidelines. $H-2^b$ $Rag2^{-/-}\gamma_c^{-}$ ((C57BL/6J \times C57BL/10SgSnAi)-[KO] γ_c^{-} -[KO] $Rag2$, cat. no. 004111; Taconic, Hudson, NY) and NOD/SCID (NOD.CB17-*Prkdc*^{scid}, cat. no. 001303; Jackson Laboratory, Bar Harbor, ME) immunodeficient mice are housed in sterile microisolator cages on laminar flow racks to minimize the chance of adventitious infections. Two to 6 h before transplantation, the mice are exposed to a single sublethal dose of total body γ irradiation from a ^{137}Cs source (350 cGy for $H-2^b$ $Rag2^{-/-}\gamma_c^{-}$ mice; 250 cGy for NOD/SCID mice).

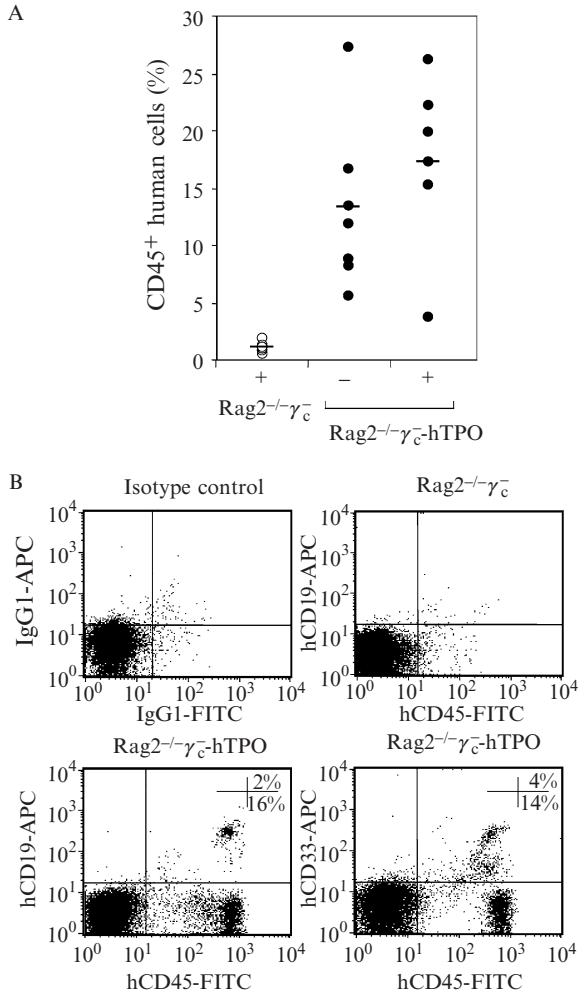


FIG. 3. Multilineage human hematopoietic engraftment in the peripheral blood of $Rag2^{-/-}\gamma_c^{-}$ -hTPO mice transplanted with candidate human HSCs. (A) Sublethally irradiated (350 cGy) mice were transplanted with 5×10^5 human $CD34^+$ cord blood cells plus (+) or minus (-) 1×10^6 $CD34^-\text{Lin}^+$ accessory cells. Shown is a summary of the percentages of $CD45^+$ human cell engraftment in the peripheral blood of transplanted $Rag2^{-/-}\gamma_c^{-}$ (open circles) and $Rag2^{-/-}\gamma_c^{-}$ -hTPO (solid circles) mice 6 weeks after transplantation. Each circle represents data for an individual mouse and the horizontal lines indicate the mean levels of human cells. (B) Flow cytometric analyses showing percentages of human $CD45^+CD19^+$ B cells and $CD45^+CD33^+$ myeloid cells in the peripheral blood of a representative $Rag2^{-/-}\gamma_c^{-}$ -hTPO mouse 6 weeks posttransplantation. Flow cytometry data were acquired with a FACSCalibur instrument and analyzed with CellQuest software (BD Biosciences Immunocytometry Systems).

Baytril (active ingredient, enrofloxacin; Bayer HealthCare, Animal Health Division, Shawnee, KS) is added to the drinking water (2 ml/250 ml) immediately after irradiation and treatment is continued for 3 weeks as an additional prophylactic measure to prevent possible deaths due to adventitious infections.

3. Prepare aliquots of $\sim 5 \times 10^5$ CD34⁺ cells with or without 1×10^6 CD34⁻Lin⁺ cells (as accessory cells) in 200 μ l of PBS and transplant into sublethally irradiated 8- to 10-week-old immunodeficient mice via intravenous tail vein injection, using a 27-gauge needle.

4. Detection of human platelets in mouse peripheral blood: Mouse bleeding (from the retroorbital venous sinus) is performed after inhalation anesthesia with isoflurane and administration to the eye of one drop of a local anesthetic (tetracaine ophthalmic solution, 0.5% solution; Phoenix Scientific, St. Joseph, MO). At weekly intervals posttransplantation, collect peripheral blood from the retroorbital venous sinus, using microhematocrit capillary tubes (cat. no. 22-362-566; Fisher Scientific, Pittsburgh, PA), and place ~ 100 μ l of blood into microcollection tubes containing potassium ethylenediaminetetraacetic acid (EDTA, cat. no. 41.1395.105; Sarstedt, Nümbrecht, Germany). Centrifuge at 1500g for 5 min at 22° and resuspend the pellet in 500 μ l of PBS containing 2% FBS. Stain 50- μ l aliquots of the cell/platelet suspension for 30 min at 22° with the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD41a (cat. no. 555466; BD Biosciences Pharmingen) or FITC-conjugated mouse IgG₁ isotype control (cat. no. 349041; BD Biosciences Pharmingen), and anti-mouse CD61-PE (cat. no. 553347; BD Biosciences Pharmingen) or PE-conjugated hamster IgG₁ isotype control (cat. no. 553972; BD Biosciences Pharmingen). Centrifuge at 750g for 5 min at 22°. Decant the supernatant and drain. Wash in 2 ml of PBS containing 2% FBS plus 0.1% NaN₃. Centrifuge at 750g for 5 min at 22°. Decant the supernatant and drain. Resuspend in 500 μ l of PBS containing 2% FBS plus 0.1% NaN₃. Platelets are analyzed on a flow cytometer equipped for excitation wavelengths of 488 and 633 nm by gating for low SSC and forward scatter signals (Perez *et al.*, 2001).

5. Detection of human hematopoietic cells in mouse bone marrow: Mice are killed under inhalation anesthesia with isoflurane by cervical dislocation at or greater than 6 weeks posttransplantation. Single-cell bone marrow suspensions are prepared by flushing the femurs and tibias with PBS containing 2% FBS, using a 21-gauge needle. Erythrocytes are removed by hypotonic lysis in 0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA, pH 7.2-7.4 (Eaker *et al.*, 2004; Ramezani *et al.*, 2000). Prepare aliquots of $\sim 1 \times 10^5$ cells/100 μ l in PBS containing 2% FBS and stain as described in the previous section with the following monoclonal antibodies

(all from BD Biosciences Pharmingen): anti-human CD45-FITC (cat. no. 555482), FITC-conjugated mouse IgG₁ isotype control (cat. no. 349041), anti-human CD19-APC (cat. no. 555415), anti-human CD33-APC (cat. no. 551378), and APC-conjugated mouse IgG₁ isotype control (cat. no. 555751). As an additional negative control, stain the bone marrow from an untransplanted mouse. Incubate at 4° for 20 min. Add 1 ml of PBS containing 2% FBS plus 0.1% NaN₃. Centrifuge at 375g for 10 min at 4°. Decant the supernatant and drain. Resuspend in 500 μ l of PBS containing 2% FBS plus 0.1% NaN₃ and analyze by flow cytometry.

Long-Term Culture of Candidate HSCs and Progenitors

Overview

Although a variety of culture conditions support some self-renewal of human hematopoietic progenitors, long-term maintenance of HSCs *in vitro*

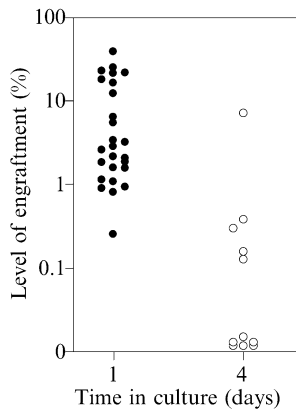


FIG. 4. Loss of human hematopoietic repopulating potential during short-term *in vitro* culture of CD34⁺ cord blood cells. The potential of human CD34⁺ cord blood cells to engraft in the bone marrow of NOD/SCID mice was compared for cells cultured *in vitro* for 1 or 4 days in X-VIVO-15 serum-free medium supplemented with 10% BIT 9500 serum substitute, 100 μ M 2-mercaptoethanol, SCF (100 ng/ml), TPO (20 ng/ml), and Flt3 ligand (100 ng/ml). The cells (1.5×10^6) were harvested, mixed with 1×10^6 CD34⁻Lin⁺ accessory cells, and transplanted into sublethally irradiated (250 cGy) NOD/SCID mice. Twelve weeks after transplantation, the mice were killed and bone marrow cells were collected for flow cytometric analysis. Human cells in the mouse bone marrow were detected after staining with anti-human CD45-PE-Cy5 (cat. no. 555484; BD Biosciences Pharmingen) monoclonal antibody. Transplantation of mice with CD34⁺ cord blood cells after 1 day of *in vitro* culture resulted in ~5% (0.5–50%) human hematopoietic cell engraftment (solid circles). The ability to repopulate NOD/SCID mouse bone marrow was significantly reduced (0–8%; mean, 0.2%) when the CD34⁺ cord blood cells were cultured *in vitro* for 4 days (open circles).

remains a major challenge (Sauvageau *et al.*, 2004). As illustrated in Fig. 4, a significant decrease in human hematopoietic repopulating activity in NOD/SCID mice was observed after *in vitro* culture of CD34⁺ cord blood cells for 4 days in serum-free medium supplemented with a combination of SCF, TPO, and Flt3 ligand (Laroche *et al.*, 1996; Petzer *et al.*, 1996). However, modest expansion of SRCs (2- to 6-fold net increases) has been reported after short-term culture in serum-free medium in more complex cocktails of hematopoietic growth factors (Bhatia *et al.*, 1997a; Conneally *et al.*, 1997; Gammaitoni *et al.*, 2003). Evidence for a limited degree of *in vitro* expansion of candidate human HSCs has also been obtained under other culture conditions (Ando *et al.*, 2006; Chute *et al.*, 2005; Madlambayan *et al.*, 2005; Piacibello *et al.*, 1999; Ueda *et al.*, 2000).

We have explored another approach based on the observation that human embryonic stem cells circumvent cellular senescence by expressing the catalytic subunit of telomerase reverse transcriptase (hTERT), a specialized ribonucleoprotein complex that is responsible for adding telomeric DNA (repetitive TTAGGG sequences) to the ends of chromosomes to prevent shortening during replication (Smogorzewska and de Lange, 2004; Thomson *et al.*, 1998). Candidate human HSCs express relatively high levels of hTERT (Yui *et al.*, 1998), and telomere length analysis of human HSC subpopulations indicates that cells with the longest telomeres have the greatest proliferative potential (Bartolovic *et al.*, 2005; Van Ziffle *et al.*, 2003). Conversely, patients with aplastic anemia have short telomeres and mutations in telomerase have been identified as the cause of hematopoietic failure (Vulliamy *et al.*, 2001; Yamaguchi *et al.*, 2005). Besides progressive telomere shortening, human cells undergo senescence in response to various types of stress (Campisi, 2005). Regardless of the senescence-initiating stimuli, the signaling pathways triggered converge to varying extents on the p53 and retinoblastoma (Rb) tumor suppressors. Therefore, we employed HIV-1-based SIN lentiviral vectors to introduce the hTERT gene and the human papillomavirus type 16 (HPV16) E6 and E7 genes (Okamoto *et al.*, 2002), which accelerate the degradation of p53 and Rb, respectively (Munger *et al.*, 2004), into human CD34⁺ cord blood cells. The transduced CD34⁺ cells were then maintained under serum-free conditions in the presence of SCF, TPO, and Flt3 ligand, with or without IL-3 (Akimov *et al.*, 2005). Although this strategy did not result in the immortalization of human HSCs, several SCF-dependent cell lines resembling human myeloid/mast cell progenitors were established in this manner, two of which express low levels of the HSC surface antigen CD133. It is important to point out, however, that the cell lines contain chromosomal aberrations (Table I). Abnormal karyotypes notwithstanding, the progenitor cell lines were not leukemogenic when injected into sublethally

TABLE I
CHARACTERISTICS OF hTERT- PLUS HPV16 E6/E7-IMMORTALIZED HUMAN CORD
BLOOD-DERIVED HEMATOPOIETIC PROGENITOR CELL LINES

Cell line	ET1a	ET2
Cell surface phenotype ^a	CD133 ^{lo} CD235a ^{lo} CD71 ⁺ CD203c ⁺ CD33 ⁺ CD13 ⁺	CD133 ^{lo} CD235a ^{lo} CD71 ⁺ CD203c ⁺ CD33 ⁺ CD13 ⁺
Growth factor responsiveness ^b	SCF dependent	SCF dependent
Karyotype ^c	46,XY,der(22)t(17;22)[9]	45,XY,der(14)t(9;14), der(19)t(19;22),-22[8]

^a In addition to the CD133 cell surface antigen, candidate human HSCs have been suggested to express CD33 and CD13 (Taussig *et al.*, 2005).

^b The ET1a and ET2 human hematopoietic progenitor cell lines require SCF for survival and proliferation but grow optimally in the presence of SCF, TPO, Flt3 ligand, and IL-3. On the basis of growth factor responsiveness, the cell lines are presumed to express CD117 (c-Kit receptor) and CD123 (the low-affinity binding subunit of the IL-3 receptor).

^c See Akimov *et al.* (2005) for details.

irradiated NOD/SCID mice (Akimov *et al.*, 2005). These findings establish the feasibility of bypassing senescence in human hematopoietic progenitors through genetic engineering, providing proof-of-principle for approaches that might eventually lead to the establishment of permanent human HSC lines. Accordingly, our future efforts will focus on extending these results by assessing the combinatorial effects of novel hematopoietic growth factors such as Notch ligands, Hedgehog proteins, Wnt molecules, bone morphogenic proteins, HOXB4 homeoprotein, and angiopoietin-like proteins (Sauvageau *et al.*, 2004; Zhang *et al.*, 2006a).

Protocol for Derivation of Human Hematopoietic Progenitor Cell Lines

1. The HIV-1-based SIN3-MU3-hTERT-IRES-GFP-W-S and SIN3-MU3-E6E7-IRES-YFP-W-S lentiviral vectors used to immortalize human hematopoietic progenitors have been described previously (Akimov *et al.*, 2005). SIN3-MU3-hTERT-IRES-GFP-W-S contains the hTERT cDNA upstream of an encephalomyocarditis virus internal ribosome entry site (IRES)-green fluorescent protein (GFP) gene cassette and SIN3-MU3-E6E7-IRES-YFP-W-S contains the HPV16 E6/E7-coding region upstream of an IRES-yellow fluorescent protein (YFP) gene cassette. In both cases, transgene transcription is driven by an internal murine stem cell virus (MSCV) long terminal repeat (LTR) promoter (Hawley *et al.*, 1994; Ramezani *et al.*, 2000, 2003).

2. Culture human embryonic kidney 293T cells in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (50 IU/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$) at 37° in a humidified atmosphere containing 5% CO₂. Plate the 293T cells (4×10^6) into 10-cm tissue culture dishes containing 7 ml of complete medium the day before transfection. Mix 15 μg of the transfer vector plasmid (SINF-MU3-hTERT-IRES-GFP-W-S or SINF-MU3-E6E7-IRES-YFP-W-S), 10 μg of the packaging plasmid pCMV Δ R8.91 (Zufferey *et al.*, 1997), and 5 μg of the VSV-G glycoprotein envelope plasmid pMD.G (Naldini *et al.*, 1996). Bring the volume up to 450 μl with sterile water. Add 50 μl of 2.5 M CaCl₂ and mix. Add the DNA–CaCl₂ solution dropwise to 500 μl of 2 \times N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)-buffered saline [0.283 M NaCl, 0.023 M HEPES (cat. no. H0887; Sigma-Aldrich), 1.5 mM Na₂HPO₄, pH 7.05] in a 15-ml conical tube. Use a 5-ml pipette to bubble the 2 \times HEPES-buffered saline while adding the DNA–CaCl₂ solution. Vortex immediately for 5 s and incubate for 20 min at 22°. Add the precipitate dropwise over the cells and mix gently. Incubate the cells overnight (16 h) at 37°. The next day, remove medium from the plate, rinse the cells with 5 ml of PBS, and add 7 ml of fresh medium. Collect the vector-containing medium after another 48 h, centrifuge at 2000g for 10 min to remove cellular debris, and filter through a 0.45- μm pore-size filter (Nalgene; Nalge Nunc International, Rochester, NY). Ultracentrifuge vector supernatants in 70-ml bottles (Beckman Coulter, Fullerton, CA) at 45,000g for 90 min at 4°. Resuspend pellets in 500 μl of medium by gentle vortexing for 2 h at 4°. Spin down the debris at 2000g for 5 min and store the concentrated vector particles at –80°. Titer vector stocks on human fibrosarcoma HT1080 cells and assay for the presence of replication-competent virus as previously described (Ramezani and Hawley, 2002b, 2003).

3. Coat 24-well non-tissue culture-treated plates (Lux suspension dish, cat. no. ICNLX171099; Fisher Scientific) with recombinant fibronectin fragment (RetroNectin, 2 $\mu\text{g}/\text{cm}^2$, cat. no. TAK_T100A; Takara Mirus Bio, Madison, WI). Culture CD34⁺ cells isolated as described in the previous section at a density of 1×10^6 cells/ml for 24 h in X-VIVO-15 serum-free medium (cat. no. BW04-418Q; Fisher Scientific) supplemented with 10% BIT 9500 serum substitute (bovine serum albumin, insulin, and human transferrin) [cat. no. 09500; StemCell Technologies), 100 μM 2-mercaptoethanol, SCF (100 ng/ml), TPO (20 ng/ml), and Flt3 ligand (100 ng/ml), with or without IL-3 (20 ng/ml)] (all cytokines from PeproTech, Rocky Hill, NJ) at 37° in a humidified atmosphere containing 5% CO₂. Transduce the cells with lentiviral vector particles (2×10^6 transducing units/ml; multiplicity of infection, 2) in the presence of protamine sulfate (4 $\mu\text{g}/\text{ml}$; Sigma-Aldrich)

(Ramezani and Hawley, 2002b, 2003; Ramezani *et al.*, 2003). Change the medium after 24 h and continue culturing the cells.

4. Harvest the hematopoietic progenitor cells after an additional 48–72 h of culture (cell dissociation buffer, cat. no. 13151–014; Invitrogen), wash, and resuspend in PBS containing 2% FBS. Under the conditions employed, the majority of cells retain the CD34⁺ phenotype (Ramezani *et al.*, 2000). Isolate GFP⁺YFP⁺ cells to >95% purity by fluorescence-activated cell sorting (Akimov *et al.*, 2005; Cheng *et al.*, 1997; Dorrell *et al.*, 2000; Hawley *et al.*, 2004). The YFP and GFP signals are separated with a 525-nm shortpass dichroic filter and collected with a 550/30 nm BP filter and a 510/20 nm BP filter, respectively (cat. no. XCY-500; Omega Optical, Brattleboro, VT).

5. Maintain the hematopoietic progenitor cells in continuous culture in X-VIVO-15 serum-free medium supplemented with 10% BIT 9500 serum substitute, 100 μ M 2-mercaptoethanol, SCF (100 ng/ml), TPO (20 ng/ml), and Flt3 ligand (100 ng/ml), with or without IL-3 (20 ng/ml) at 37° in a humidified atmosphere containing 5% CO₂.

Acknowledgments

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[8] Hemangioblasts and Their Progeny

By URSULA M. GEHLING

Abstract

In the developing embryo, the hemangioblast, a mesodermal precursor, gives rise to hematopoietic and endothelial cells. Recent work has shown that during postnatal life, a subset of hematopoietic progenitor cells also displays this dual differentiation capacity and can function as endothelial progenitor cells that contribute to neovascularization. Thus, this subset might be useful for therapy of various hematopoietic and vascular diseases. Here, we describe a two-step culture system that results in the generation of endothelial and hematopoietic cells from adult progenitor cells with hemangioblastic potential. We have developed growth conditions that allow retroviral gene marking of the adult hemangioblast. This culture system is amenable for single-cell analyses at distinct stages of endothelial and hematopoietic differentiation from mobilized CD133⁺ progenitor cells.

Introduction

Identification of adult progenitor cells with endothelial differentiation potential has proved difficult because hematopoietic stem and progenitor cells and mature endothelial cells share expression of a number of different markers, such as CD34, Tie-2, and the vascular endothelial growth factor receptor-2 (VEGFR-2; reviewed by Rafii and Lyden, 2003). The human stem cell antigen CD133 (previously termed AC133) is expressed on a subset of hematopoietic CD34-positive (+) stem and progenitor cells but not on mature endothelial cells (Yin *et al.*, 1997). In addition, it has been