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Article

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Avoidance of stimulation improves engraftment of cultured and retrovirally transduced hematopoietic cells in primates

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Recent reports suggest that cells in active cell cycle have an engraftment defect compared with quiescent cells. We used nonhuman primates to investigate this finding, which has direct implications for clinical transplantation and gene therapy applications. Transfer of rhesus CD34$^+$ cells to culture in stem cell factor (SCF) on the CH-296 fibronectin fragment (FN) after 4 days of culture in stimulatory cytokines maintained cell viability but decreased cycling. Using retroviral marking with two different gene transfer vectors, we compared the engraftment potential of cytokine-stimulated cells versus those transferred to nonstimulatory conditions (SCF on FN alone) before reinfusion. In vivo competitive repopulation studies showed that the level of marking originating from the cells continued in culture for 2 days with SCF on FN following a 4-day stimulatory transduction was significantly higher than the level of marking coming from cells transduced for 4 days and reinfused without the 2-day culture under nonstimulatory conditions. We observed stable in vivo overall gene marking levels of up to 29%. This approach may allow more efficient engraftment of transduced or ex vivo expanded cells by avoiding active cell cycling at the time of reinfusion.

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Introduction

Recent studies have suggested that actively cycling primitive hematopoietic cells have an engraftment defect compared with quiescent cells in the G0 phase of the cell cycle (1). In the murine model, there is some evidence that this defect may be reversible, if cells are allowed to come back out of active cycle before transplantation (2). The mechanism for this phenomenon is unknown, but it most likely involves modulation of the expression or the activation state of adhesion molecules that are involved in homing to and lodging in the marrow microenvironment. These findings could greatly impact the ability to manipulate hematopoietic cells ex vivo for applications including stem cell expansion and gene therapy. Hematopoietic cells that are successfully transduced with standard retroviral vectors will by necessity have cycled in vitro, and therefore an engraftment defect related to cycling could contribute to disappointing in vivo levels of transduced cells. Thus far, there is little convincing evidence that human repopulating progenitor cells or stem cells can be expanded ex vivo, but cytokines used to stimulate cells to proliferate could also prevent optimal engraftment.

Improving the in vivo levels of genetically modified progeny of repopulating hematopoietic stem cells (HSCs) has been a very important issue for clinical applications of stem cell gene therapy. In murine models, reproducible retroviral gene transfer to a high percentage of long-term repopulating HSCs has been achieved, with 10–50% or more cells from all hematopoietic lineages containing vector long-term (3). However, these levels have not yet been approached in human clinical trials. In human trials, in vivo marking levels have been below those necessary for clinical utility, even in the setting of full myeloablation, except when transduced cells and their progeny have a strong in vivo selective advantage, such as in X-linked severe combined immunodeficiency (4). We have focused on the nonhuman primate transplantation model as a much better predictor of human stem cell behavior and gene transfer efficiency than murine models or in vitro progenitor assays (5). Rhesus macaques appear to have stem cell kinetics, hematopoietic demand, cytokine responsiveness, and retroviral receptor levels that are similar to those of humans. The inclusion of either autologous marrow stroma or the CH-296 fragment of fibronectin (RetroNectin, abbreviated throughout this article as FN), along with a combination of cytokines that are active on primitive hematopoietic cells, has recently been shown to greatly improve results in nonhuman primates, with long-term levels of 5–10% or greater (5–7).

We hypothesized that avoidance of active cell cycling at the time of reinfusion of retrovirally transduced HSCs could improve engraftment and thus in vivo marking levels by reducing competition against both remaining endogenous HSCs and nontransduced

HSCs in the graft. To test this hypothesis, culture conditions that maintained viability of primitive cells without further stimulating cell cycle progression were required. In vitro and murine studies have shown that the CH-296 fragment of fibronectin (RetroNectin) prevents apoptosis without inducing active cell cycling (8–10). Stem cell factor used as a single agent has also been shown to promote survival without resulting in proliferation or differentiation (11). We investigated the effect of transferring primitive hematopoietic cells from rhesus macaques to these conditions after transduction and culture in the presence of the stimulatory cytokines necessary for efficient gene transfer (5, 6, 12).

Methods

Collection of peripheral blood stem cells from rhesus macaques. Young rhesus macaques (*Macaca mulatta*) were housed and handled in accordance with guidelines set by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. The animals received recombinant human (rh) stem cell factor (SCF) (200 mg/kg; Amgen Inc., Thousand Oaks, California, USA) and rhG-CSF (10 mg/kg; Amgen Inc.) as daily subcutaneous injections for 3 days, and twice daily on day 4. Mobilized peripheral blood (PB) cells were collected by apheresis on day 5 as described (13). Mononuclear cells (MCs) were isolated using density gradient centrifugation over lymphocyte separation media (Organon Teknika Corp., Durham, North Carolina, USA), and enrichment of $CD34^+$ cells was performed using an anti-CD34 monoclonal antibody (12.8) that is biotinylated and thus able to be used for immunoselection using streptavidin-coated microbeads as directed (14). The degree of progenitor enrichment was calculated from CFU assays performed before and after column purification.

Culture conditions and cell cycle analysis. CD34-enriched cells were cultured in DMEM (Mediatech Inc., Herndon, Virginia, USA) supplemented with 10% FBS (Atlanta Biologicals Inc., Norcross, Georgia, USA), 4 mM L-glutamine, 50 mg/ml penicillin, and 50 mg/ml streptomycin at 37°C in 5% CO_2. Cells were maintained in the presence of 100 ng/ml rhSCF, 100 ng/ml rhFlt-3 ligand (FLT; Immunex Corp., Seattle, Washington, USA), and 100 ng/ml rh megakaryocyte growth and development factor (rhMGDF; Amgen Inc.) in flasks previously coated with RetroNectin (Takara Shuzo Co., Shiga, Japan) per the manufacturer's instructions.

For cell cycle analysis of the S, G2, and M phases (S/G2/M) versus the G0 and G1 phases, fractions were fixed in 80% ice-cold ethanol, washed with PBS (BioSource International, Rockville, Maryland, USA), and resuspended in PBS supplemented with 50 mg/ml propidium iodide (PI; Sigma Chemical Co., St. Louis, Missouri, USA), 1 mM EDTA (Quality Biological Inc., Gaithersburg, Maryland, USA), and 0.1% Triton X-100 (ICN Biomedicals Inc., Aurora, Ohio, USA). This mixture was incubated for 15 minutes at 37°C. PI-stained cells were analyzed for DNA content using a Coulter Epics XL Flow Cytometry System (Beckman Coulter Inc., Miami, Florida, USA). For cell cycle analysis of G1 phase versus G0 versus S/G2/M, fractions were fixed in 80% ice-cold ethanol, washed with PBS, resuspended in PBS supplemented with 20 μl of Ki-67 antibody MIB-1–FITC (Beckman Coulter Inc., Fullerton, California, USA), 950 mg/ml PI, 1 mM EDTA, and 0.1% Triton X-100, and then incubated for 15 minutes at 37°C. Ki-67 and PI double-stained cells were analyzed for DNA content using a Coulter Epics XL Flow Cytometry System. Analysis of apoptosis was performed using the Annexin V assay from CLONTECH Laboratories Inc. (Palo Alto, California, USA) per the manufacturer's instructions.

Vectors and transduction procedures. G1Na and LNL6 are Moloney virus–derived retroviral vectors that carry an identical bacterial neomycin phosphotransferase (*neo*) gene (15). A 16-bp polylinker insertion upstream of the *neo* gene allows quantitative assessment of marking from the two vectors within one PCR reaction (5, 16). The biologic titers of the G1Na and LNL6 supernatants used were both 7×10^6 biologically active vector particles per ml, as assayed by transfer of G418 resistance to HeLa cells. Retroviral supernatant was harvested from subconfluent producer cells cultured in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 50 mg/ml penicillin, and 50 mg/ml streptomycin at 37°C in 5% CO_2. Fresh vector supernatant was passed through a 0.45-mm filter (Millipore Corp., Bedford, Massachusetts, USA) to remove cellular debris before transduction. An amphotropic clone (a kind gift of Brian Sorrentino, St. Jude Children's Research Hospital, Memphis, Tennessee, USA) producing the murine stem cell virus–based vector MgirL22Y, which expresses the enhanced green fluorescent protein gene (eGFP), was also used, and generated supernatants containing approximately 5×10^5 biologically active particles per ml, as assayed by flow cytometry of transduced HeLa cells (17).

CD34-enriched cells from each animal were divided into two equal fractions; each was cultured at a starting concentration of 200,000 cells/ml in fresh vector supernatant in the presence of SCF, MGDF, and FLT, at the concentrations noted above, on FN support (SCF/MGDF/FLT/FN) (5). Every 24 hours, nonadherent cells were collected, spun down, resuspended in fresh vector supernatant and cytokines, and then returned to the same FN-coated flask. The starting cell concentration was 1×10^5 cells/ml. At the end of 96 hours, one fraction was cryopreserved and the other was washed and continued in culture for two additional days in the same FN-coated flask in the presence of SCF alone, then cryopreserved. After each animal received two treatments of 500 cGy total body irradiation, both fractions of transduced cells were thawed and reinfused via a central venous catheter. Twenty-

four hours later, the animals were started on intravenous G-CSF at 5 mg/ml/kg daily until the white blood cell count reached 6,000/ml. Standard supportive care was administered (18).

Analysis of posttransplantation samples. PB and bone marrow samples were collected at the time of recovery of leukocyte counts to greater than 2,000 ml and 1, 2, 3, 4, 5, 6, 8, 10, and 12 months after transplantation. MCs were isolated by density gradient centrifugation over lymphocyte separation media. Granulocytes of greater than 95% purity were obtained as previously described (7). PB CD34-enriched cells collected before and after transduction, and bone marrow samples obtained after transplantation were analyzed by CFU assays using MethoCult M4230 methylcellulose media (StemCell Technologies Inc., Vancouver, British Columbia, Canada) supplemented with 5 U/ml rh erythropoietin (Amgen Inc.), 10 ng/ml rhIL-3, 10 ng/ml rhGM-CSF (Sandoz Pharmaceuticals Corp., East Hanover, New Jersey, USA), and 100 ng/ml rhSCF at 37°C in 5% CO_2. At day 14, colonies of more than 50 cells were scored. At least 20 individual CFUs were plucked from the plates at each time point for PCR analysis.

PCR analysis. Genomic DNA was extracted using the QIAamp Blood Kit (QIAGEN Inc., Chatsworth, California, USA). PCR analysis for *neo* and β-*actin* sequences was performed using primers and conditions described previously, with the following modifications to yield linear amplification in the range of the test samples: template DNA was reduced to 100 ng for the outer reaction, and the cycle number was reduced to18 for the inner reaction (16). In every PCR analysis, negative controls included DNA from normal rhesus PB samples extracted concurrently with the test samples, and a reagent control. Serial dilutions of G1Na DNA (containing two integrated copies per cell) into normal rhesus PB DNA were used as positive controls. Two hundred nanograms of genomic DNA from PB or marrow samples was added to a master mix containing dNTPs, buffer, and Taq polymerase from the GeneAmp kit from Perkin-Elmer (Cetus Corp., Norwalk, Connecticut, USA), and then divided equally into tubes containing either the primer pair for the control β-*actin* gene or for the *neo* gene. All *neo* and β-*actin* PCR reactions were run under conditions optimized to yield linear results in the range of the intensity of the in vivo samples. The PCR products were separated by PAGE. The expected band sizes were 483 bp for G1Na *neo*, 467 bp for LNL6 *neo*, and 232 bp for β-*actin*. Band intensity was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA). The *neo* band intensity was normalized for amplifiable DNA content based on the β-*actin* signal, and the overall contribution of each vector to in vivo marking was calculated by plotting the signal intensity of each band on a standard curve derived from controls with a known number of integrated copies amplified concurrently. Mixtures of different ratios of LNL6 and G1Na DNA in a background of rhesus PB DNA were also amplified simultaneously to document the ability of the assay to detect both vectors simultaneously and quantitatively.

Southern blot analysis. Ten micrograms of genomic DNA was digested with KpnI (Boehringer Mannheim Biochemicals Inc., Indianapolis, Indiana, USA), which cuts once within each viral LTR and thus can distinguish integrated retroviral G1Na and LNL6 DNA. The DNA fragments were transferred to a nylon membrane and hybridized with a radiolabeled *neo* gene–specific probe generated by PCR using the following primers: forward primer 5′-TCC ATC ATG GCT GAT GCA ATG CGG C-3′ and reverse primer 5′-GAT AGA AGG CGA TGC GCT GCG AAT CG-3′.

Statistical analysis. Analysis of significance using the two-tailed Student's *t* test and regression analysis was carried out using Excel software (Microsoft Corp., Seattle, Washington, USA).

Results

In vitro analysis of rhesus CD34⁺ PBSC proliferation and cell cycle characteristics. To investigate the impact of various cytokine conditions on active proliferation versus simple maintenance of cell viability during in vitro culture, SCF/G-CSF–mobilized, CD34-enriched rhesus PB cells were cultured in the presence of SCF/MGDF/FLT on flasks coated with FN for 4 days. The cell culture was then divided, and cells were either continued in culture on FN with the same stimulatory cytokines (SCF/MGDF/FLT), with SCF alone, or without exogenous cytokines (Figure 1a). For the first 4 days, in the presence of SCF/MGDF/FLT/FN, the cells had a doubling time of approximately 44 hours, with acceleration towards the end of the 4-day period. By day 4, the cell number had increased an average of 2.2-fold, and by day 6, the viable cell number had increased 7.1-fold. The cells that remained in SCF/MGDF/FLT/FN continued exponential growth until reaching a peak on day 10, followed by a slowing of proliferation and increased numbers of nonviable cells, with evidence for apoptosis (Figure 1d). Cells transferred to SCF/FN stopped proliferating but maintained viability through days 8–10. Cells removed completely from cytokines and maintained in flasks with FN rapidly lost viability and underwent apoptosis (Figure 1, a and d). Rhesus CD34⁺ PB cells appear to require some cytokine support to prevent apoptosis and loss of viability, even in the presence of FN.

In the same experiments, the percentage of cells in active cell cycle over time was determined using PI staining and flow cytometry (Figure 1b). As expected, a very small percentage of CD34⁺ PBSCs were in active cycle (S/G2/M phases) at the time of culture initiation (13, 19). Over the first 96 hours in SCF/MGDF/FLT/FN, the fraction of cells in S/G2/M increased to a mean of over 25%. This was the peak fraction of cells in active cycle: even with continued time in SCF/MGDF/FLT/FN the percentage of cells in active cycle began to decrease by days 6–8. A significantly lower percentage of cells transferred to SCF/FN after 4 days of stimulatory culture

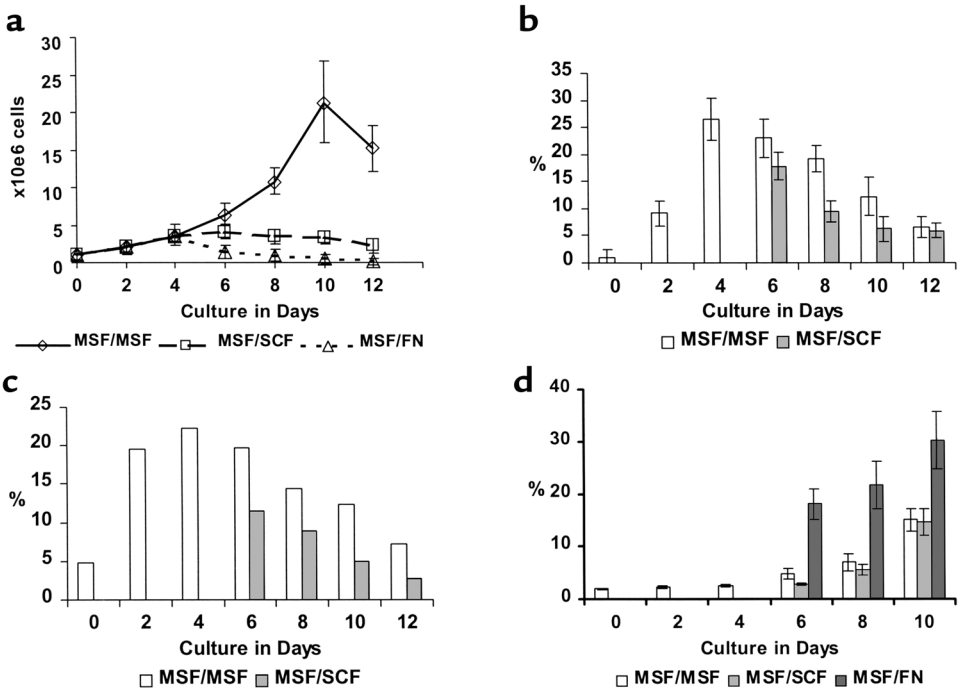

Figure 1

(**a**) In vitro cell growth of PB CD34$^+$ cells. The CD34$^+$ cells were cultured in the presence of MGDF/SCF/FLT/FN for 4 days. On day 4 the cells were split into three equal fractions. One was continued in culture in the presence of SCF/MGDF/FLT/FN (MSF/MSF). The second was transferred to SCF/FN (MSF/SCF). The third was maintained without cytokines on FN (MSF/FN). Shown are the mean ± SD of viable cell numbers obtained in independent experiments using cells from three different animals. (**b**) Cell cycle analysis. PB CD34$^+$ cells were stained with PI and analyzed for DNA content. The percentage of cells in S/G2/M phases of the cell cycle are shown. A significantly lower percentage of cells in active cycle with MSF/SCF treatment than with MSF/MSF at days 6, 8, and 10 ($P < 0.05$ for each time point). (**c**) Cell cycle analysis of PB CD34$^+$ cells cultured and transduced for 4 days in the presence of MGDF/SCF/FLT/FN with a retroviral vector expressing the eGFP gene, and then either continued in MGDF/SCF/FLT/FN without further transduction (MSF/MSF) or transferred to SCF/FN (MSF/SCF). The percentage of GFP-positive cells in the S/G2/M phases of the cell cycle are shown. (**d**) Apoptosis analysis. The percentage of apoptotic cells is shown for cells cultured with SCF/MGDF/FLT/FN for 4 days, and then either continued in the three cytokines (MSF/MSF), transferred to SCF/FN (MSF/SCF), or transferred to FN alone (MSF/FN). Transfer to FN alone (MSF/FN) resulted in a significant increase in apoptosis compared with both MSF/MSF and MSF/SCF ($P < 0.005$ for both comparisons).

were in S/G2/M, although there were still actively cycling cells present ($P < 0.05$). The fraction of cells in S/G2/M after complete removal of cytokines fell even more sharply, but as noted above, the total number of viable cells also greatly decreased. Actively cycling cells that were still detectable on days 6–8 in the presence of SCF alone or no cytokines may have initiated cell cycle progression at the end of the initial 4 days of culture in the presence of SCF/MGDF/FLT/FN.

We also distinguished G0-phase cells from G1-phase cells using concurrent PI and Ki-67 antibody staining. At culture initiation, 90.2% of the cells were in G0 phase and 3.9% were in G1 phase. After 96 hours in SCF/MGDF/FLT/FN, the fraction of G0 cells fell to 34.0% and G1 cells increased to 35.5%. After transfer to SCF/FN alone for an additional 48 hours, the G0 cells increased to 40.2% and the G1 cells were at 36.6%. The transfer to SCF/FN increased the fraction of G0 cells, and since the cell numbers remained constant in SCF/FN, the absolute number of G0 cells increased as well.

To confirm that cells stimulated to cycle during the first 4 days of culture could be brought back out of cell cycle by culture in SCF/FN, we performed transduction studies using a retroviral vector (MgirL22Y) expressing GFP (20). During the initial 4 days of culture in SCF/MGDF/FLT/FN, vector supernatant was added daily. On day 4, transduced cells were washed and split into two treatments: continued culture in SCF/MGDF/FLT/FN and culture in SCF/FN. Cells that were successfully transduced, and therefore GFP-positive, must have traversed M phase during the initial 4-day culture and transduction period. Cells were simultaneously analyzed for cell cycle fraction and GFP positivity. Within the GFP-positive gate, there was a decrease in the fraction of S/G2/M cells after transfer to SCF/FN that was similar to the decrease in the overall cultures (compare Figure 1b with Figure 1c).

Experimental design of the in vivo repopulation assay. Overall, the in vitro studies suggested that 4 days of culture and transduction in the presence of SCF/MGDF/FLT/FN followed by 2 days of additional culture in the presence of SCF/FN supports cell viability, but decreases the percentage of actively cycling cells. We next tested whether the transfer of rhesus PBSCs to these nonstimulatory culture conditions

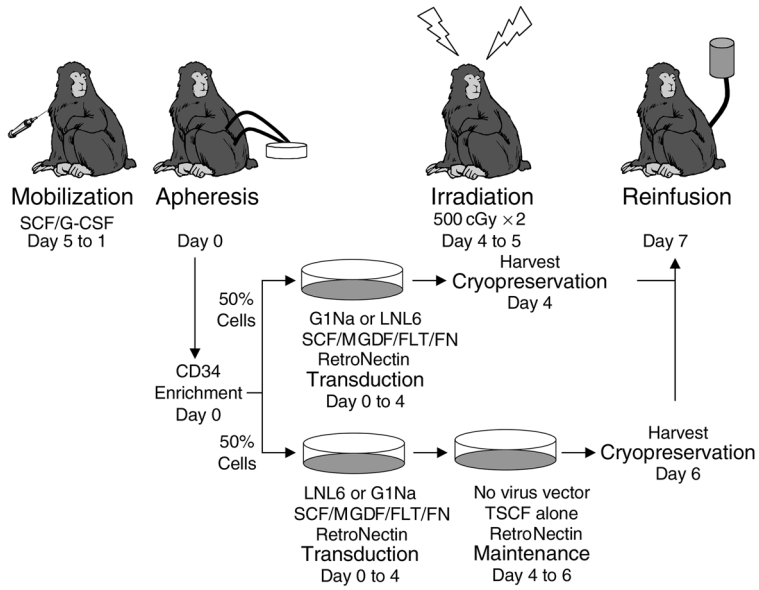

Figure 2
Experimental design. In each animal, if G1Na was used to transduce one fraction, LNL6 was used to transduce the other fraction. For each subsequent animal, the vector used to transduce each fraction was alternated.

could result in enhanced engraftment of transduced progenitor and stem cells in vivo, as compared directly to cells transduced under the same conditions but without culture for an additional time period under the nonstimulatory conditions. The experimental design is depicted in Figure 2. Three rhesus macaques had SCF/G-CSF–mobilized PB cells collected, CD34-enriched, split into two equal fractions, and transduced with one of two retroviral marking vectors for 4 days in the presence of SCF/MGDF/FLT/FN. One fraction was cryopreserved at the end of transduction and the second fraction was continued in culture, but without vector, for an additional 2 days in the presence of SCF/FN, then cryopreserved. Both fractions were thawed and reinfused simultaneously.

The two vectors used, LNL6 and G1Na, have identical titers on HeLa cells and have been found to be equivalent for in vivo marking of primitive repopulating cells in several prior studies (5, 12, 16). Table 1 summarizes the PBSC collection and progenitor enrichment, in vitro transduction efficiency, and engraftment kinetics for each animal. There were no significant differences in the efficiency of transduction of committed progenitors (CFUs) between the two fractions, suggesting that the LNL6 and G1Na vectors had equivalent titers for transduction of hematopoietic cells. All animals engrafted rapidly.

Analysis of engraftment with transduced cells. After transplantation, semiquantitative PCR analysis of PB samples allowed assessment of the relative contribution of each fraction to in vivo marking. Figure 3 shows representative gels from each animal. A summary of marking levels in PBMCs and PB granulocytes over time, assuming one vector integration per cell, is depicted in Figure 4. Total marking levels (adding together marking from both transduced fractions) in two of the three animals were in clinically relevant ranges long-term, at 6.2–9.5% in PBMCs and 6.0–10.0% in PB granulocytes in animal 96E019; and 14.9–22.5% in PBMCs and 14.4–20.4% in PB granulocytes in animal RC706. In all animals, the contribution to in vivo marking from the cells cultured for two additional days in SCF/FN without further transduction was significantly higher (3.4- to 4.2-fold higher for animal 96E019, 2.4- to 5.0-fold higher for animal RC706, and 1.6- to 2.3-fold higher for animal 96E025) than from the cells cryopreserved immediately following the 4-day transduction in stimulatory cytokines ($P < 0.01$ in all animals).

The levels and ratio of marking originating from the two transduced fractions were confirmed by genomic Southern blotting of PB samples from selected time points in the two animals with overall high-level marking (Figure 3b). Analysis of band intensity indicated that marking from cells cultured for two additional days in SCF/FN, without further transduction, was

Table 1
Summary of CD34 enrichment, infusion, transduction efficiency, and engraftment

Animal	CD34 enrichment (fold)[A]	Transduction	Total CFUs $\times 10^5$	No. cells infused/kg $\times 10^6$	Transduction efficiency (%)[B]	Engraftment (days)[C]	Follow-up (months)
96E025	87.8	4 days	5.2	6.8	55.0	9	>12
		4 + 2 days	5.9	7.2	61.9		
96E019	127.5	4 days	8.1	9.8	55.0	11	>12
		4 + 2 days	8.8	10.3	61.9		
RC706	112.0	4 days	9.4	8.3	61.9	8	>12
		4 + 2 days	11.0	8.6	65.0		
RQ2280	88.3	6 days	9.4	9.9	65.0%	9	2
		4 + 2 days	8.8	8.8	61.9%		

[A]Degree of progenitor cell enrichment by CD34 selection was calculated by enumerating total CFUs before and after column purification. [B]Transduction efficiency evaluated by the percentage of β-*actin*–positive colonies that were also *neo*-positive at the end of transduction, as determined by PCR of individual colonies. [C]Days to reach an absolute neutrophil count greater than 500/μl.

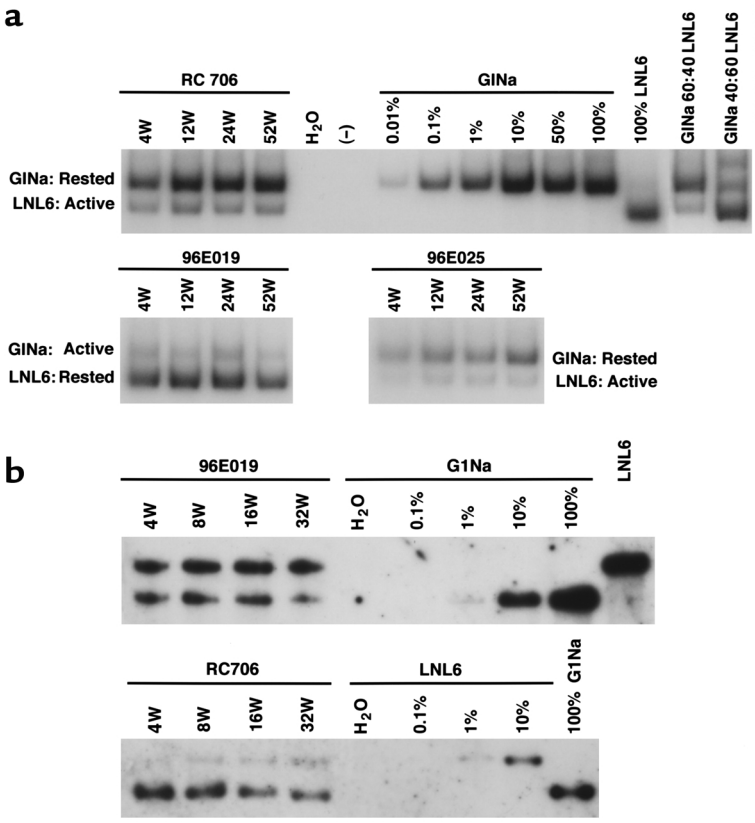

Figure 3
Analysis of engraftment with transduced cells in vivo. (**a**) PCR analysis of *neo* sequences in PBMCs. 96E025 and RC706 received cells transduced with LNL6 for 4 days in MGDF/SCF/FLT/FN (active) and cells transduced with G1Na for 4 days in MGDF/SCF/FLT/FN that were then transferred to SCF/FN for two additional days without further exposure to vector (rested). 96E019 cells received the reverse vector treatment: G1Na for 4 days in MGDF/SCF/FLT/FN (active) and LNL6 for 4 days then transferred to SCF/FN for two additional days without further exposure to vector (rested). DNA from PBMCs at the indicated number of weeks after transplantation (W) was analyzed by PCR. Serial dilutions of G1Na vector–containing DNA with a known number of integrated copies were made using normal rhesus PB DNA at the indicated percentages; known-copy-number LNL6 DNA and mixtures of LNL6 and G1Na controls are shown. Dash (–) indicates concurrently extracted control rhesus PB DNA; H_2O: reagent control. (**b**) Southern blot analysis of genomic marking levels in RC706 and 96E019, using DNA samples from PBMCs at the indicated number of weeks after transplantation, and positive standards made by diluting known-copy-number cell-line DNA into normal rhesus DNA. Samples were digested with KpnI, an enzyme that cuts within the LTRs in both LNL6 and G1Na. Due to residual *env* sequences in LNL6 that are not present in G1Na, the expected fragment size is 3.0 kb in LNL6 and 2.3 kb in G1Na.

higher than the marking from the cells cryopreserved immediately after the 4 days of transduction in stimulatory cytokines, confirming the PCR analysis. Marking was 2.3-fold higher in animal 96E019 and 5.2-fold higher in animal RC706.

Individual CFUs grown from posttransplantation marrow samples were also analyzed by PCR for vector sequences. The overall marking levels were similar to PB marking, confirming the likelihood of one vector copy per cell. The pattern was the same as in the PB granulocytes and PBMCs, with a higher contribution of marked CFUs originating from the fraction of cells cultured for 2 days in SCF/FN after transduction in all animals (data not shown).

To exclude the possibility that the improved marking in the treatment consisting of 4 days in SCF/MGDF/FLT/FN followed by 2 days in SCF/FN was not simply due to a more prolonged period in culture (allowing integration of proviral particles after removal from the stimulatory cytokines), a final competitive repopulation assay was performed. Animal RQ2280 had SCF/G-CSF–mobilized, CD34-enriched cells collected and split into two fractions: for transduction either for 6 days in the presence of SCF/MGDF/FLT/FN, with daily addition of vector supernatant, or for 4 days in SCF/MGDF/FLT/FN, followed by washing and culture without vector in the presence of SCF/FN for 2 days (Table 1). Both fractions were collected and reinfused simultaneously. As shown in Figure 5, engraftment with marked cells at 20 weeks after transplantation is 3.4-fold higher from the "4 + 2 fraction" than from the 6-day transduction fraction.

Discussion

Since the discovery and purification of the first hematopoietic cytokines almost two decades ago, scientists and clinicians have pursued ex vivo culture techniques for stem cell expansion and gene therapy applications (21). The ability to expand hematopoietic stem and progenitor cells could potentially widen the availability and improve the efficacy of cord-blood transplantation, speed recovery from cytopenias after autologous or allogeneic transplantation, allow complex graft manipulation procedures to purge tumor cells, and ensure engraftment in mismatched or nonablative allogeneic transplantation (22). Current methods for HSC genetic modification require ex vivo stimulation with cytokines in order to stimulate mitosis and thus allow passage of retroviral vectors to the cell nucleus (23). Because primitive stem cells in both marrow and (especially) mobilized PB are in the quiescent G0 phase of the cell cycle, susceptibility to retroviral transduction or ex vivo expansion requires culture conditions that induce cells to enter active cell cycle (13, 19, 24, 25). However, several studies have documented a disturbing loss of in vivo repopulating stem cell activity after culture in stimulatory cytokines for 4 days or more (21, 26–28). In the nonhuman primate model, a direct comparison using gene marking to track ex vivo–manipulated PBSCs found complete loss of in vivo engraft-

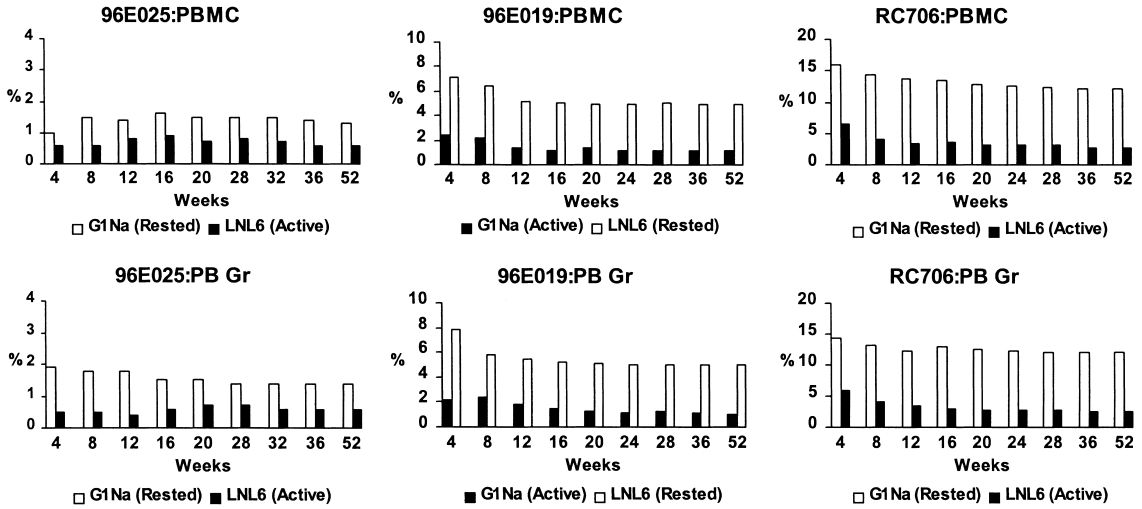

Figure 4
Summary of in vivo marking levels. DNA marking in PBMCs and PB granulocytes (PB Gr) calculated from PCR analysis, assuming one vector copy per cell, for 96E025, 96E019, and RC706. Animals received cells that were transduced for 4 days in MGDF/SCF/FLT/FN (active), and cells transduced for 4 days and cultured for an additional 2 days in SCF/FN without further vector exposure (rested). The percentage of marked cells was calculated from individual values of *neo*/β-*actin* band intensities plotted on the regression curve generated from the G1Na controls. Active: marking level from the vector used to transduce cells cultured for 4 days in MGDF/SCF/FLT/FN; Rested: marking from the vector used to transduce cells cultured in SCF/MGDF/FLT/FN followed by an additional 2 days without vector in SCF/FN.

ment of cells cultured for 10 days as compared with those cultured for 4 days (7, 29).

Loss of functional stem cell activity during ex vivo culture could result from several factors. The most obvious is irreversible terminal differentiation. Using membrane dyes to track cell divisions of murine marrow stem cells during ex vivo culture, it has been shown that repopulating activity was preferentially retained in cells that had not divided in culture (30). Under different cytokine conditions, cells in human cord blood that had divided could still engraft NOD/SCID mice (31). One study showed that with more divisions in culture, there was increased apoptosis of cells with a primitive phenotype (32). Several groups began to study the relationship between cell cycle status and in vivo stem cell function. Quesenberry and coworkers reported decreased engraftment of cells induced into cell cycle, both in vivo with 5-fluorouracil and in vitro with cytokines, compared with steady-state marrow. They postulated that this defect could result from reversible changes in engraftment and homing, as opposed to irreversible loss of stem cells or diminished self-renewal capacity (33–35). Srour and coworkers have performed several studies using the NOD/SCID model, and reported that engrafting ability in marrow and in mobilized blood was primarily in G0-phase cells as opposed to G1 cells, although cord blood cells seemed to have the ability to engraft in either G0 or G1 cells, similar to results from other investigators (1, 31, 36).

Initial attempts to demonstrate reversibility of this engraftment "defect" failed, but used ex vivo exposure to agents such as TGF-β, a cytokine that inhibits cell cycle progression but also induces apoptosis of primitive cells (37–39). The most convincing demonstration of reversibility to date took advantage of the observation that murine stem cells go in and out of active cell cycle synchronously for the first 36 hours of cytokine exposure ex vivo. Investigators reported a cyclic loss and then gain in engraftment activity at different time points in culture (2).

Nonhuman primates and other large animal models have been shown to have great predictive value for understanding human hematopoiesis and gene transfer, due to similar hematopoietic demand, response to cytokines, expression of cell surface receptors, and localization of hematopoietic activity (40). It may be especially important to use primate models when investigating mechanisms of stem cell engraftment, because

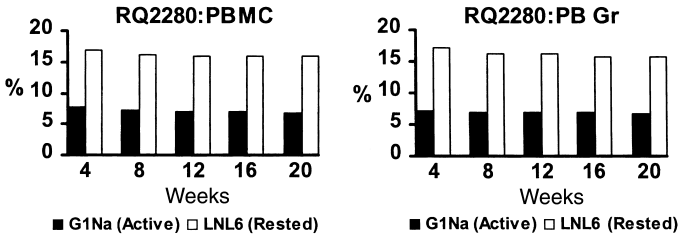

Figure 5
In vivo analysis of 6-day stimulatory culture versus 4-day stimulatory culture followed by 2 days in SCF/FN. PCR analysis of DNA from an animal receiving cells cultured for 6 days in the presence of the G1Na vector and SCF/MGDF/FLT/FN, versus cells cultured for 4 days in the presence of LNL6 vector and SCF/MGDF/FLT/FN followed by culture without vector for two additional days in the presence of SCF and FN.

murine/human xenograft models may be limited by differences between murine and human adhesion receptors and inefficient homing. We have identified a simple culture manipulation that improves the engraftment ability of primate hematopoietic cells stimulated to cycle ex vivo. The design of the competitive repopulation experiment allowed direct comparison of the engrafting ability of primitive cells that were transduced for 4 days in stimulatory cytokines versus an identical cell population stimulated and transduced for 4 days that was then shifted to culture in SCF/FN for an additional 2 days without further transduction. Despite the lack of further exposure to vector, long-term engraftment in all animals was significantly higher from vector-containing cells originating from the fraction that was continued in culture for two additional days with SCF/FN, suggesting that transduced primitive cells regained engrafting ability. Direct comparison of cells transduced for 6 days under stimulatory conditions with cells transduced for 4 days followed by 2 days without further transduction in SCF/FN also demonstrated a marked advantage for cells cultured without stimulatory cytokines before reinfusion. This suggests that the improvement was not simply due to a more prolonged ex vivo culture period that allowed integration of vector.

There are several mechanisms that may be responsible for these findings. It is most likely that transfer of the cells to SCF/FN maintained viability of primitive cells, but allowed exit from the active cell cycle and an associated improvement in engraftment ability. Previous murine experiments have shown that SCF alone maintains HSCs in culture without inducing proliferation (11, 41). Culture on FN retained the repopulating ability of murine HSCs, and was crucial even in the presence of cytokines for the maintenance of human cord-blood engraftment in immunodeficient mice (8, 9). Adhesion of primitive cells to stroma or fibronectin has also been shown to inhibit active cycling of primitive cells, but this block could be overcome by the addition of stimulatory cytokine combinations (42, 43). In our study, cell cycle analysis demonstrated that culture in SCF/FN did decrease the fraction of cells in S/G2/M, although to a less marked degree than could completely account for the in vivo engraftment results. However, the analysis was performed on the entire population of $CD34^+$ cells, due to lack of adequate reagents to further discriminate primitive rhesus macaque hematopoietic populations. The cell cycle effect may have been more marked in the most primitive subset of $CD34^+$ responsible for engraftment. A second potential explanation for the findings is that culture in SCF/FN actually expanded the number of transduced primitive cells, but there is little existing data suggesting that these conditions would induce primitive cells to proliferate, and the cell cycle analysis does not support this explanation.

Presumably, the engraftment defect associated with active proliferation of primitive cells results from changes in the expression or function of cell surface molecules. These molecules function in the complex processes of homing or passive trapping of cells in the marrow, passage through the marrow endothelium, lodging in the appropriate microenvironmental niches, and initiation of self-renewal and replenishment of hematopoietic lineages (44). Thus far there is no clear understanding of the exact cell surface molecules necessary or sufficient for these processes, but various models implicate integrins such as VLA-4 and VLA-5, chemokine receptors such as CXCR4, selectins, leukosialins, and an ever increasing number of other adhesion receptors found on primitive hematopoietic cells. Changes in the expression and function of cell surface adhesion receptors upon cell cycle progression or cytokine stimulation are beginning to be investigated (45). Cytokine stimulation was shown to increase both the expression and the inside-out signaling function of VLA-4, an integrin that can bind both fibronectin and VCAM-1 components of the bone marrow microenvironment (46). It is possible that increased expression of adhesion molecules induced by cycling could result in initial homing to an inappropriate site, such as the spleen or lungs, and therefore loss of in vivo engraftment function (47). Further insights will have to await a better understanding of the critical pathways for each step in engraftment, and studies are ongoing using the rhesus model.

Regardless of the mechanism, our findings have a number of implications. Irreversible loss or failure to expand HSCs ex vivo should not be assumed without an attempt to rescue engraftment potential by culture in SCF/FN, or under other conditions that have been found to decrease cycling without loss of viability. Clinical and experimental ex vivo expansion protocols should be developed to investigate these hypotheses further, using retroviral marking to directly track progeny of transduced, and thus cycling, cells. At present, most clinical investigations of ex vivo expansion involve cord blood expansion for allogeneic transplantation. Murine xenograft studies indicate that the engraftment defect may be less pronounced with cord blood than with adult HSCs, but given the potential clinical implications, study of the issue should incorporate gene marking of progeny of "expanded" cord blood cells following reinfusion.

There are also several implications for gene therapy. The ability of lentiviral vectors to transduce noncycling cells during a brief ex vivo exposure to vector may increase efficiency of gene transfer to primitive cells, but also enhance engraftment ability, and thus increase competition against endogenous stem cells or nontransduced stem cells in the graft (48). But even using standard retroviral vectors, we achieved encouraging levels of gene transfer, with stable marking in multiple lineages from the fraction of cells "rested" before reinfusion being as high as 15–29%, at this point more than one year after transplantation. Improving the ability of transduced cells to compete against endogenous stem cells is most important in nonablative situations, and we will now proceed to investigate the utility of this approach in such a setting, with potential applications in gene therapy of nonmalignant diseases.

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1. Gothot, A., van der Loo, J.C.M., Clapp, D.W., and Srour, E.F. 1998. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood $CD34^+$ cells in non-obese diabetic/severe combined immune-deficient mice. *Blood.* **92**:2641–2649.
2. Habibian, H.K., et al. 1998. The fluctuating phenotype of the lymphohematopoietic stem cell with cell cycle transit. *J. Exp. Med.* **188**:393–398.
3. Dunbar, C.E. 1996. Gene transfer to hematopoietic stem cells: implications for gene therapy of human disease. *Annu. Rev. Med.* **47**:11–20.
4. Cavazzana-Calvo, M., et al. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science.* **288**:669–672.
5. Wu, T., et al. 2000. Prolonged high-level detection of retrovirally marked hematopoietic cells in non-human primates after transduction of CD34+ progenitors using clinically feasible methods. *Mol. Ther.* **1**:285–293.
6. Kiem, H.P., et al. 1998. Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. *Blood.* **92**:1878–1886.
7. Tisdale, J.F., et al. 1998. Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. *Blood.* **92**:1131–1141.
8. Dao, M.A., Hashino, K., Kato, I., and Nolta, J.A. 1998. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. *Blood.* **92**:4612–4621.
9. Yokota, T., et al. 1998. Growth-supporting activities of fibronectin on hematopoietic stem/progenitor cells in vitro and in vivo: structural requirements for fibronectin activities of CS1 and cell-binding domains. *Blood.* **91**:3263–3272.
10. Moritz, T., et al. 1996. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. *Blood.* **88**:855–862.
11. Li, C.L., and Johnson, G.R. 1994. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood.* **84**:408–414.
12. Huhn, R.D., et al. 1999. Retroviral marking and transplantation of rhesus hematopoietic cells by nonmyeloablative conditioning. *Hum. Gene Ther.* **10**:1783–1790.
13. Donahue, R.E., et al. 1996. Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. *Blood.* **87**:1644–1653.
14. Berenson, R.J., Andrews, R.G., Bensinger, W.I., Kalamasz, D., and Knitter, G. 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* **81**:951–955.
15. Cassel, A., Cottler-Fox, M., Doren, S., and Dunbar, C.E. 1993. Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. *Exp. Hematol.* **21**:585–591.
16. Dunbar, C.E., et al. 1995. Retrovirally marked CD34-enriched peripheral blood and bone marrow cells contribute to long-term engraftment after autologous transplantation. *Blood.* **85**:3048–3057.
17. Donahue, R.E., et al. 2000. High levels of lymphoid expression of enhanced green fluorescent protein in nonhuman primates transplanted with cytokine-mobilized peripheral blood CD34(+) cells. *Blood.* **95**:445–452.
18. Nienhuis, A.W., et al. 1987. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) shortens the period of neutropenia after autologous bone marrow transplantation in a primate model. *J. Clin. Invest.* **80**:573–577.
19. Uchida, N., et al. 1997. The unexpected G0/G1 cell cycle status of mobilized hematopoietic stem cells from peripheral blood. *Blood.* **89**:465–472.
20. Persons, D.A., et al. 1998. Use of the green fluorescent protein as a marker to identify and track genetically modified hematopoietic cells. *Nat. Med.* **4**:1201–1205.
21. Srour, E.F., Abonour, R., Cornetta, K., and Traycoff, C.M. 1999. Ex vivo expansion of hematopoietic stem and progenitor cells: are we there yet? *J. Hematother.* **8**:93–102.
22. Emerson, S.G. 1996. Ex vivo expansion of hematopoietic precursors, progenitors, and stem cells: the next generation of cellular therapeutics. *Blood.* **87**:3082–3088.
23. Miller, D.G., Adam, M.A., and Miller, A.D. 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol. Cell. Biol.* **10**:4239–4242.
24. Uchida, N., et al. 1998. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA.* **95**:11939–11944.
25. Jordan, C.T., Yamasaki, G., and Minamoto, D. 1996. High-resolution cell cycle analysis of defined phenotypic subsets within primitive human hematopoietic cell populations. *Exp. Hematol.* **24**:1347–1355.
26. Williams, D.A. 1993. Ex-vivo expansion of hematopoietic stem and progenitor cells: robbing Peter to pay Paul? *Blood.* **81**:3169–3172.
27. Dorrell, C., Gan, O.I., Pereira, D.S., Hawlet, R.G., and Dick, J.E. 2000. Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood.* **95**:102–110.
28. Holyoake, T.L., et al. 1997. CD34 positive PBPC expanded ex vivo may not provide durable engraftment following myeloablative chemoradiotherapy regimens. *Bone Marrow Transplant.* **19**:1095–1101.
29. Conneally, E., Cashman, J., Petzer, A., and Eaves, C. 1997. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc. Natl. Acad. Sci. USA.* **94**:9836–9841.
30. Traycoff, C.M., Cornetta, K., Yoder, M.C., Davidson, A., and Srour, E.F. 1996. Ex vivo expansion of murine hematopoietic progenitor cells generates classes of expanded cells processing different levels of bone marrow repopulating potential. *Exp. Hematol.* **24**:299–306.
31. Glimm, H., and Eaves, C.J. 1999. Direct evidence for multiple self-renewal divisions of human in vivo repopulating hematopoietic cells in short-term culture. *Blood.* **94**:2161–2168.
32. Traycoff, C.M., et al. 1998. Proliferation-induced decline of primitive hematopoietic progenitor cell activity is coupled with an increase in apoptosis of ex vivo expanded CD34+ cells. *Exp. Hematol.* **26**:53–62.
33. Stewart, F.M., Crittenden, R.B., Lowry, P.A., Pearson-White, S., and Quesenberry, P.J. 1993. Long-term engraftment of normal and post-5-fluorouracil murine marrow into normal nonmyeloablated mice. *Blood.* **81**:2566–2571.
34. Ramshaw, H.S., et al. 1995. Engraftment of bone marrow cells into normal unprepared hosts: effects of 5-fluorouracil and cell cycle status. *Blood.* **86**:924–929.
35. Peters, S.O., Kittler, E.L.W., Ramshaw, H.S., and Quesenberry, P.J. 1995. Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. *Exp. Hematol.* **23**:461–466.
36. Wilpshaar, J., et al. 2000. Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34(+) cells in NOD/SCID mice. *Blood.* **96**:2100–2107.
37. Wiesmann, A., et al. 2000. Modulation of hematopoietic stem/progenitor cell engraftment by transforming growth factor-β. *Exp. Hematol.* **28**:128–139.
38. Glimm, H., Oh, I.H., and Eaves, C.J. 2000. Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their $S/G_2/M$ transit and do not reenter G_0. *Blood.* **96**:4185–4193.
39. Jacobsen, F.W., Stokke, T., and Jacobsen, S.E.W. 1995. Transforming growth factor-β potently inhibits the viability-promoting activity of stem cell factor and other cytokines and induces apoptosis of primitive murine hematopoietic progenitor cells. *Blood.* **86**:2957–2966.
40. Donahue, R.E., and Dunbar, C.E. 2001. An update on the use of nonhuman primate models for preclinical testing of gene therapy approaches targeting hematopoietic cells. *Hum. Gene Ther.* **12**:607–617.
41. Broudy, V.C. 1997. Stem cell factor and hematopoiesis. *Blood.* **90**:1345–1364.
42. Hurley, R.W., McCarthy, J.B., and Verfaillie, C.M. 1995. Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J. Clin. Invest.* **96**:511–519.
43. Jiang, Y., Prosper, F., and Verfaillie, C.M. 2000. Opposing effects of engagement of integrins and stimulation of cytokine receptors on cell cycle progression of normal human hematopoietic progenitors. *Blood.* **95**:846–854.
44. Quesenberry, P.J., and Becker, P.S. 1998. Stem cell homing: rolling, crawling, and nesting. *Proc. Natl. Acad. Sci. USA.* **95**:15155–15157.
45. Becker, P.S., et al. 1999. Adhesion receptor expression by hematopoietic cell lines and murine progenitors: modulation by cytokines and cell cycle status. *Exp. Hematol.* **27**:533–541.
46. Levesque, J.P., Leavesley, D.I., Niutta, S., Vadas, M., and Simmons, P.J. 1995. Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *J. Exp. Med.* **181**:1805–1815.
47. Szilvassy, S.J., Bass, M.J., Van Zant, G., and Grimes, B. 1999. Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood.* **93**:1557–1566.
48. Case, S.S., et al. 1999. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc. Natl. Acad. Sci. USA.* **96**:2988–2993.