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Ex Vivo Culture of Cord Blood CD34$^+$ Cells Expands Progenitor Cell Numbers, Preserves Engraftment Capacity in Nonobese Diabetic/Severe Combined Immunodeficient Mice, and Enhances Retroviral Transduction Efficiency

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ABSTRACT

***Ex vivo* culture of hematopoietic stem/progenitor cells could potentially improve the efficacy of human placental/umbilical cord blood (CB) in clinical hematopoietic stem cell (HSC) transplantation and allow gene transduction using conventional retroviral vectors. Therefore, we first examined the effects of a 7-day period of *ex vivo* culture on the hematopoietic capacity of CB CD34$^+$ cells. Medium for the *ex vivo* cultures contained either serum and six recombinant human hematopoietic growth factors (GFs), including Flt-3 ligand (FL), Kit ligand (KL = stem cell factor), thrombopoietin (Tpo), interleukin 3 (IL-3), granulocyte colony-stimulating factor (G-CSF), and interleukin 6 (IL-6), or a serum-free medium containing only FL, KL, and Tpo. After culture under both *ex vivo* conditions, the total numbers of viable cells, CD34$^+$ cells, colony-forming cells (CFCs), and long-term culture initiating cells (LTC-ICs) were increased. In contrast, the severe combined immunodeficiency (SCID) mouse engrafting potential (SEP) of cultured cells was slightly decreased, as compared with fresh cells. Nevertheless, cultured human CB CD34$^+$ cells were able to generate engraftment, shown to persist for up to 20 weeks after transplantation. We next tested the efficacy of retroviral transduction of cultured cells. Transduced cultured human cells were able to engraft in NOD/SCID mice, as tested 4 weeks after transplantation, and EGFP$^+$CD34$^+$ cells and EGFP$^+$ CFCs were isolated from the chimeras. Thus, although additional improvements in *ex vivo* culture are still needed to expand the numbers and function of human HSCs, the current conditions appear to allow gene transduction into hematopoietic SCID engrafting cells, while at least qualitatively preserving their *in vivo* engraftment potential.**

OVERVIEW SUMMARY

This study investigated improving *ex vivo* culture conditions for retroviral transduction of human hematopoietic stem cells (HSCs). Since successful hematopoietic gene therapy requires the transduced stem cells to engraft *in vivo*, we first evaluated the effects of *ex vivo* culture on the *in vivo* SCID engrafting potential (SEP) of cord blood (CB) CD34$^+$ cells. We found that the numbers of hematopoietic progenitor cells were increased after a 7-day period of *ex vivo* culture, and SEP was only slightly decreased. We then observed that human CB CD34$^+$ cells transduced during *ex vivo* culture in serum-free medium containing Flt3 ligand (FL), Kit ligand (KL = stem cell factor), and thrombopoietin (Tpo), generated EGFP$^+$CD34$^+$ cells and EGFP$^+$ CFCs in NOD/SCID chimeras. Thus, *ex vivo* culture allowed efficient gene transfer into human hematopoietic stem/progenitor cells, while preserving their *in vivo* engraftment potential.

INTRODUCTION

HUMAN UMBILICAL/PLACENTAL CORD BLOOD (CB) is now in active use as an alternative transplant graft source because of its advantages over bone marrow, including immediate availability of cryopreserved CB, lower risk of transmission of viral diseases, and reduced risk of graft-versus-host disease (GVHD) (Kurtzberg *et al.*, 1996; Cairo and Wagner, 1997). An

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additional advantage of CB is the potentially higher susceptibility of CB stem/progenitor cells to retroviral gene transfer (Lu *et al*., 1993); CB may be a favorable source of "early" or more proliferative hematopoietic stem cells (HSCs). Successful gene transduction by retroviral vectors requires integration of the retroviral insert into cellular DNA, which in turn requires cell proliferation (Miller *et al*., 1990). Thus, CB has been cultured *ex vivo* to increase HSC proliferation and thereby enhance retroviral transduction. Promising clinical results of retroviral transduction have already been achieved with target cells from CB (Lu *et al*., 1993; Kohn *et al*., 1995, 1998), and any significant enhancement of transduction efficiency could provide clear clinical benefit for use in selected inherited diseases including thalassemia, sickle cell anemia, Fanconi anemia, severe combined immunodeficiency (SCID) secondary to adenosine diaminase (ADA) deficiency, and a variety of metabolic/storage deficiencies (Kohn *et al*., 1995; Cairo and Wagner, 1997; Lorincz *et al*., 1997).

Ex vivo culture of hematopoietic cells has utilized hematopoietic growth factors (GFs) (Coutinho *et al*., 1990; Brugger *et al*., 1993; Srour *et al*., 1993; Henschler *et al*., 1994; McKenna *et al*., 1995; Petzer *et al*., 1996b; Conneally *et al*., 1997; Prosper *et al*., 1997; Zandstra *et al*., 1997), with or without bone marrow stromal cells (Koller *et al*., 1996; Breems *et al*., 1998). Several groups have reported that *ex vivo* culture results in increased numbers of viable cells, $CD34^+$ cells, and colony-forming cells (CFCs), including long-term culture initiating cells (LTC-ICs) (Moore and Hoskins, 1994; Koller *et al*., 1996; Petzer *et al*., 1996b; Bhatia *et al*., 1997; Conneally *et al*., 1997; Piacibello *et al*., 1997; Zandstra *et al*., 1997; Luens *et al*., 1998). Several combinations of GFs have been investigated, and combinations that include Flt-3 ligand (FL), Kit ligand (KL), and thrombopoietin (Tpo) have provided promising results (Conneally *et al*., 1997; Luens *et al*., 1998). Results of two clinical trials indicate that *ex vivo* cultured $CD34^+$ cells can be safely transplanted after high-dose chemotherapy, although the kinetics of bone marrow (BM) reconstitution have not yet been shown to be enhanced (Brugger *et al*., 1995; Alcorn *et al*., 1996; Emerson, 1996). More recently, the availability of immunodeficient animal hosts for human hematopoiesis has provided an additional method to test human hematopoietic cell populations for the ability to generate extensive multilineage hematopoiesis *in vivo* (Civin *et al*., 1996; Larochelle *et al*., 1996; Bhatia *et al*., 1997; Conneally *et al*., 1997; Hogan *et al*., 1997; Cheng *et al*., 1998; Leung *et al*., 1998; Luens *et al*., 1998; Ramírez *et al*., 1998; Güenechea *et al*., 1999; Piacibello *et al*., 1999).

Therefore, we first performed quantitative comparisons of *ex vivo* cultured versus fresh CB $CD34^+$ cell populations in hematopoietic assays, including their ability to engraft at high levels in the nonobese diabetic/LtSz-*SCID/SCID* (NOD/SCID) hematopoietic chimera model. After finding that human CB $CD34^+$ cell populations cultured *ex vivo* for 7 days retained the ability to engraft in the NOD/SCID chimera model, we evaluated the efficiency of gene transduction of CB $CD34^+$ cells during culture in serum-free medium containing FL, KL, and Tpo. Our results indicate that *ex vivo* serum-free culture of CB $CD34^+$ cells allows efficient gene transduction of *in vitro* colony-forming cells (CFCs) and *in vivo* NOD/SCID engrafting cells.

MATERIALS AND METHODS

Experimental design

Freshly thawed (previously purified and cryopreserved) human CB $CD34^+$ cells were suspended in a given quantity of tissue culture medium containing GFs. Approximately half the volume of this "fresh" control cell suspension was assayed immediately for content of viable cells, $CD34^+$ cells, CFCs, LTC-ICs, and SCID mouse engrafting potential (SEP). The other "experimental" half of these cells was cultured *ex vivo* for 7 days before assessment by the same hematopoietic assays. Although cell counts were determined both before and after the 7-day *ex vivo* culture period, the input of *ex vivo* cultured cells into the hematopoietic assays was based on volume, rather than cell number. In this way, we directly compared the function of a given number of uncultured "fresh" control cells versus the experimental population of day 7 *ex vivo* "cultured" cells generated by that number of input day 0 fresh cells. Finally, we evaluated the efficiency of retroviral gene transduction (as assessed by marker gene expression in *in vitro* and *in vivo* assayed hematopoietic progeny) of cultured cells.

Human cells

We purchased cryopreserved CB $CD34^+$ cells from Poietic Technologies (Gaithersburg, MD) or PureCell, LLC (San Mateo, CA). $CD34^+$ cells were purified by immunomagnetic selection (Miltenyi Biotechnologies, Auburn, CA), then cryopreserved in Iscove's modified Dulbecco's medium (IMDM; HyClone Laboratories, Logan, UT) containing 50% fetal bovine serum (FBS; StemCell Technologies, Vancouver, BC, Canada) and 15% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO). After thawing, $89 \pm 5\%$ of the cells were viable (trypan blue dye exclusion), and $91 \pm 4\%$ of the cells were $CD34^+$ (Trischmann *et al*., 1993).

Human stromal cells were prepared for LTC-IC assays from BM samples collected from the posterior iliac crests of consenting healthy adult volunteers under an Institutional Review Board (IRB)-approved protocol.

Recombinant human cytokines

Purified recombinant human KL, granulocyte colony-stimulating factor (G-CSF), and interleukin 2 (IL-3) were generously donated by Amgen (Thousand Oaks, CA). IL-6, FL, and Tpo were purchased from either R&D Systems (Minneapolis, MN) or PeproTech (Rocky Hill, NJ).

Antibodies and FACS analysis

Phycoerythrin (PE)-conjugated purified mouse anti-human CD34, CD19, and CD33, and PE-conjugated and fluorescein isothiocyanate (FITC)-conjugated isotype control antibodies were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated rat anti-mouse CD45 and mouse-anti human CD3, and PE-conjugated mouse anti-human CD45 were purchased from Sigma. FITC-conjugated mouse anti-human CD13 was purchased from Dako (Carpinteria, CA). FITC-conjugated mouse anti-human CD41a, PE-Cychrome 5 (PE-CY5)-conjugated mouse anti-human CD45, and PE-CY5-conjugated iso-

type control were purchased from PharMingen (San Diego, CA).

A FACSort (Becton Dickinson) flow cytometer equipped with an Argon laser tuned at 488 nm was used for fluorescence-activated cell sorting (FACS) analysis. Green fluorescence from enhanced green fluorescent protein (EGFP) was detected in the FL1 emission channel (Cheng *et al*., 1997, 1998).

Ex vivo *hematopoietic cultures*

CB CD34$^+$ cells were plated at 5–20 $\times$ 10^4 cells/ml in 75-cm^2 flasks (Becton Dickinson) in either condition 1 (IMDM supplemented with 10% FBS and the following six recombinant humans GFs:FL [100 ng/ml], KL [100 ng/ml] Tpo [20 ng/ml], IL-3 [20 ng/ml], IL-6 [20 ng/ml], and G-CSF [20 ng/ml]) or condition 2 (QBSF-60 serum-free medium [Quality Biological, Gaithersburg, MD] containing the following three recombinant human GFs: FL [100 ng/ml], KL [100 ng/ml], and Tpo [20 ng/ml]). These suspension cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C. In experiments 1–6 (Tables 1 and 2), at ~3.5 days, an equal volume of the same culture medium (either condition 1 or 2) was added to cultures. These cell cultures were then incubated for an additional ~3.5 days (for a total of 7 days of *ex vivo* culture). In the gene transduction experiments, the cells were resuspended onto RetroNectin with retroviral supernatant, as indicated for each single experiment in the footnotes to Tables 3–6.

Viable cell counts and CD34$^+$ cell content

Fresh and cultured cells were counted with a Coulter Z1 cell counter (Coulter Diagnostics, Miami, FL). Viability was assessed by trypan blue dye exclusion and microscopy, then confirmed by LDS-751 (Exciton, Dayton, OH) dye exclusion and flow cytometry (Terstappen *et al*., 1988). Immunophenotype was determined by multicolor flow cytometry after immunostaining (Trischmann *et al*., 1993).

Progenitor cell assays

Colony-forming cell-mix (CFC-Mix), colony-forming cell-granulocyte/monocyte (CFC-GM), and burst-forming unit-erythroid (BFU-E) were evaluated by plating 500–1000 fresh CD34$^+$ cells and the equivalent volume of cultured cells (see Experimental Design, above) in 1 ml of MethoCult GF H4434 methylcellulose medium (StemCell Technologies) in triplicate. Total and green fluorescent colonies were counted after 2 weeks, as described (Cheng *et al*., 1998; Götze *et al*., 1998). Individual colonies were subsequently picked from some plates and lysed for polymerase chain reaction (PCR) analysis (Cheng *et al*., 1997).

CFC assays were also performed on cells recovered from the BM of NOD/SCID mice, using previously described methods shown to be selective for the growth of human colonies in a murine cell background (Carow *et al*., 1993; Leung *et al*., 1998; Ramírez *et al*., 1998).

We quantitated the number of LTC-ICs in the fresh and cultured CB CD34$^+$ cell populations by limiting dilution analysis as described (Sutherland *et al*., 1990). For each evaluation, four concentrations of fresh cells and equivalent volumes of the cultured cell (see Experimental Design, above) were seeded in 20 replicates in 96-well plates containing preestablished adherent stromal cell layers and 200 μl of MyeloCult H5100 (StemCell Technologies) supplemented with 10^{-6} *M* freshly dissolved hydrocortisone. After 5 weeks of culture with medium refeeding as described above, adherent and nonadherent cells were harvested and pooled, then plated in 24-well plates for CFC assays.

Transduction of fresh and cultured cells

Amphotropic retroviral supernatants of the EGFP-encoding MGIN retroviral vector (derived from the murine stem cell virus [MSCV]) were produced in a human 293T packaging cell line (Cheng *et al*., 1997). We performed gene transduction either by centrifugation ("spinoculation") or by incubation on fibronectin fragment CH-296 (RetroNectin; PanVera, Madison, WI)-coated surfaces.

Analysis of retroviral transduction by PCR

The efficiency of gene transfer into human progenitor cells was measured by PCR analysis of CFC colonies derived from CD34$^+$ cells. Nested PCR for the EGFP gene was carried out on individual colonies picked from methylcellulose cultures. PCR amplification of a human endogenous DNA sequence was used as a control, as described (Ramírez *et al*., 1998).

Transplantation of human cells for measurement of SEP

Mice were bred and maintained under pathogen-free conditions, as approved by the Animal Care Committee of the Johns Hopkins Medical Institutions (Ramírez *et al*., 1998). Fresh or equivalent volumes of *ex vivo* cultured human cells (see Experimental Design, above) were transplanted by tail vein injection into sublethally irradiated (300 cGy, using a ^{137}Cs γ-irradiator) 6- to 8-week-old NOS/SCID mice. Mice were sacrificed 2–20 weeks after transplantation of human cells, as described for each experiment. Single-cell suspensions were prepared from the spleens, and BM cells were flushed from the removed femurs and tibias (Ramírez *et al*., 1998). Cells were counted and viability determined (89 $\pm$ 3%). Because these four bones have been shown to contain ~25% of the total BM of a mouse (Boggs, 1984), the total number of BM cells per mouse was estimated by multiplying the number of cells obtained by 4.

Human cells in the BMs and spleens of the human–mouse chimeras were enumerated and cell lineages determined by three-color flow cytometry of cells immunostained with murine monoclonal antibodies, as described previously (Leung *et al*., 1998; Ramírez *et al*., 1998). SEP was defined as the (average) number of human CD45$^+$ cells recovered from the BM of an experimental group of NOD/SCID mice per human CD34$^+$ cell initially transplanted (Leung *et al*., 1998).

Statistical analysis

We used a multiple linear regression model to compare the engraftment potential of the cultured versus fresh cells in experiments 1–5 (Leung *et al*., 1998). Briefly, the dose–response relationship between the number of human hematopoietic cells transplanted and the number of human CD45$^+$ cells in the BM of the chimera was determined by plotting the two variables

and then calculating the Pearson correlation coefficient. SEP was defined as the estimated slope of the resulting linear regression line, which represented the average number of human $CD45^+$ cells detected divided by the number of human $CD34^+$ cells transplanted.

We used single-hit Poisson statistics to analyze LTC-IC frequency and number in fresh or cultured cell populations (Sutherland *et al*., 1990).

RESULTS

The numbers of total cells and CD34+ cells are increased after ex vivo *culture*

Fresh cells were 89 ± 5% viable and cultured cells were 82 ± 8% viable. As shown in Table 1, the number of viable cells increased by more than 40-fold after ex vivo culture in either condition 1 (serum-containing medium plus six GFs: experiments 1–4) or condition 2 (serum-free medium plus three GFs: experiments 5 and 6). The frequency of $CD34^+$ cells fell from a mean of 91% in the fresh cells to 18% in the cultured cells (Fig. 1). Nevertheless, the total numbers of $CD34^+$ cells increased by an average of 9- to 10-fold after *ex vivo* culture (Table 1). In two experiments using condition 1, the cultured cells were also immunostained for the expression of several leukocyte differentiation antigens (Trischmann *et al*., 1993). $CD13^+$ (myelomonocytic) cells comprised 70–80% of the cells, and $CD41a^+$ (megakaryocytic) cells comprised 5–10% of the cells. No $CD19^+$ (B lymphoid) or $CD3^+$ (T lymphoid) cells were detected (data not shown).

The numbers of CFCs are increased after ex vivo *culture*

On average in experiments 1–5, 1000 fresh CB $CD34^+$ cells generated 22 CFC-Mix, 52 CFC-GM, and 71 BFU-E colonies. In comparison, 1000 *ex vivo* cultured cells generated one CFC-Mix, seven CFC-GM, and seven BFU-E colonies. Because of the increase in total cell numbers during the *ex vivo* culture, the total numbers of CFCs in the entire cell population after 7 days in culture condition 1 increased by twofold for CFC-Mix, sixfold for CFC-GM, and fourfold for BFU-E (Table 1). These results were similar (and not statistically different) for culture condition 2.

Numbers of LTC-ICs after ex vivo *culture*

LTC-IC assays were performed in only three experiments (experiments 2, 3, and 5). The frequency of LTC-ICs was on

TABLE 1. EFFECTS OF 7-DAY *ex Vivo* CULTURE OF CORD BLOOD $CD34^+$ CELLS ON HEMATOPOIETIC CAPACITY: EXPERIMENTS 1–6[a]

Culture condition[b]	*Fold increase in viable cells*	*Fold increase in $CD34^+$ cells*	*Fold increase in CFCs*			*Fold increase in LTC-IC*[c]	*Fold increase in SEP*
			CFC-Mix	*CFC-GM*	*BFU-E*		
1. IMDM + FBS + FKT36G (experiments 1–4)	43.2 ± 12.5	9.7 ± 8.0	1.5 ± 0.3	6.2 ± 0.6	4.1 ± 2.5	2.2 ± 0.3	0.3 ± 0.2
2. QBSF-60 + FKT (experiments 5 and 6)	44.4 ± 18.5	8.8 ± 2.6	5.6 ± 3.9	6.1 ± 1.7	5.1 ± 2.0	1.3	0.9 ± 0.6

[a]Values represent the mean ± standard error increase in the total numbers of viable cells, $CD34^+$ cells, CFCs, LTC-ICs, and SEP of 7 day-cultured CB $CD34^+$ cells relative to fresh cells. Because large numbers of cells were needed for experiments 1–5, cells were pooled from three or four donor CB samples for each of these experiments; this also minimizes effects due to variability among donors in stem/progenitor cell number and function (Leung *et al*., 1998). In experiments 6–10, cells from only one or two CB donors were pooled, due to the smaller size of the experiments. In experiments 1–6, mice received intraperitoneal injections of the following growth factors three times per week posttransplantation: IL-3, GM-CSF, G-CSF, and KL (10 μg of each/dose) combined in a single injection for each mouse, in order to maximize levels of human cells in the engrafted human–mouse chimeras (Leung *et al*., 1998). In experiments 1–5, three doses of fresh and cultured cells were transplanted. This design enabled us to obtain easily detectable levels of human cell engraftment and dose–response curves for analysis using a multiple linear regression model (Leung *et al*., 1998). In experiment 6, a single dose of fresh versus the equivalent volume of cultured cells was transplanted. In experiment 6, the probability of a significant difference in human $CD45^+$ cell levels in the human–mouse chimeras was determined by Wilcoxon scores (rank sums) test.

[b]Experiments 1–4 were conducted in culture condition 1 (IMDM + 10% FCS + FL, KL, Tpo, IL-3, IL-6, and G-CSF). Experiments 5 and 6 were conducted in culture condition 2 (QBSF-60 + FL, KL, and Tpo). $CD34^+$ cells were plated at the following concentrations: experiment 1, 1×10^5; experiment 2, 1.4×10^5; experiment 3, 1.6×10^5; experiment 4, 9×10^4; experiments 5–8, 5×10^4.

[c]LTC-IC assays were performed in only two experiments using culture condition 1, and in one experiment using culture condition 2, as described in Materials and Methods. For preparation of allogeneic stromal cells for the LTC-IC assays, MNCs were isolated by density-gradient centrifugation (Ficoll-Hypaque; Pharmacia Biotech, Piscataway, NJ) and suspended in myeloid long-term culture medium (H5100; StemCell Technologies) supplemented with 10^{-6} *M* water-soluble hydrocortisone (Sigma). Cells were plated onto 75-cm^2 tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C in a humidified 5% CO_2 atmosphere with weekly culture refeeding (Gartner and Kaplan, 1980). When confluent stromal cell layers were present (3–4 weeks after initiation of the cultures), stromal cells were harvested and cryopreserved in DMSO-containing medium. Ten days before the initiation of an LTC-IC assay, stromal cells were thawed and subcultured in 96-well tissue culture plates (Becton Dickinson) at 100–150 cells/μl in the same stromal cell culture medium. Subcultured stromal cell layers were irradiated (1500 cGy, using a ^{137}Cs γ irradiator) 2–6 days prior to initiation of LTC-IC assays.

average 1 per 1000 fresh CB $CD34^+$ cells. After culture in condition 1, the frequency of LTC-ICs decreased on average to 0.05 per 1000 cultured cells. After culture in condition 2, the LTC-IC frequency decreased to 0.02 per 1000 cultured cells. The average total numbers of LTC-ICs in the cell populations after 7 days of *ex vivo* culture increased by twofold in condition 1 and remained essentially constant in condition 2 (Table 1).

Effect of ex vivo *culture on SEP*

A total of 173 sublethally irradiated NOD/SCID mice was transplanted with fresh or cultured human cells in experiments 1–6. As we and others have previously reported (Leung *et al.*, 1998; van der Loo *et al.*, 1998), a dose–response relationship between the numbers of $CD34^+$ cells transplanted and the numbers of human $CD45^+$ cells recovered from the BMs and spleens of the hematopoietic chimeras was observed overall (Figs. 2 and 3). Human cells were easily detected in all mice transplanted with fresh or cultured cells by flow cytometry. Up to 98×10^6 human $CD45^+$ cells were identified in the BMs of chimeras transplanted with fresh cells, and up to 39×10^6 human $CD45^+$ cells were identified in the BMs of the chimeras transplanted with cultured cells (Fig. 2). In experiments 1–4 (condition 1), and in experiment 5 (condition 2), the SEP was lower for cultured cells than for the corresponding fresh cells (Fig. 2, Tables 1 and 2). In experiments 3–5, we randomized mice to be sacrificed 2–3 or 7–20 weeks after transplantation; we operationally defined these lengths of follow-up as "short term" and "long term," respectively. In all cases, the cultured cells had lower SEP than the corresponding fresh cells (the range of the ratio of the SEP of cultured versus fresh cells was 0.1–0.7), even though the difference was not always significant (Table 2, Fig. 2). Experiment 6 was a smaller experiment performed with a single dose of fresh versus cultured cells, which prevented linear regression analysis. In this experiment, the average number of $CD45^+$ cells in the BMs of the NOD/SCID mice transplanted with fresh cells was 10.6 (± 9) $\times 10^6$, while the average number in the BMs of the animals transplanted with cultured cells was 18.2 (± 12.5) $\times 10^6$. This 1.7-fold increase in engraftment of the cultured cells versus fresh cells was not significant by Wilcoxon scores test ($p = 0.4$). Finally, for all these results taken together (both culture conditions, analyzed at both time points), 1 fresh $CD34^+$ cell was able to generate 38 $CD45^+$ cells in the BMs of the chimeras, while 1 cultured $CD34^+$ cell was able to generate 14 $CD45^+$ cells. Overall, the weighted average SEP ratio of cultured to fresh cells was 0.4; i.e., fresh cells had a 2.8-fold higher SEP ($p = 0.0002$) (Table 2). Figure 3 shows the combined regression fits for all 173 mice analyzed in our study.

All animals transplanted with fresh or cultured cells had human $CD13^+$, $CD19^+$, and $CD34^+$ cells in their BMs by flow cytometry (data not shown). As has been found previously in these human–mouse chimeras (Leung *et al.*, 1998; Ramírez *et al.*, 1998), no $CD3^+$ (T lymphoid) cells were detected. In experiments 2, 4, 5, and 6, the chimera BM cells were plated for CFC assays. Human myeloid and erythroid colonies were generated from all the chimeras transplanted with either fresh or cultured cells, assessed at short-time and long-term time points (data not shown). There was an overall dose–response relationship between the number of colonies recovered and the

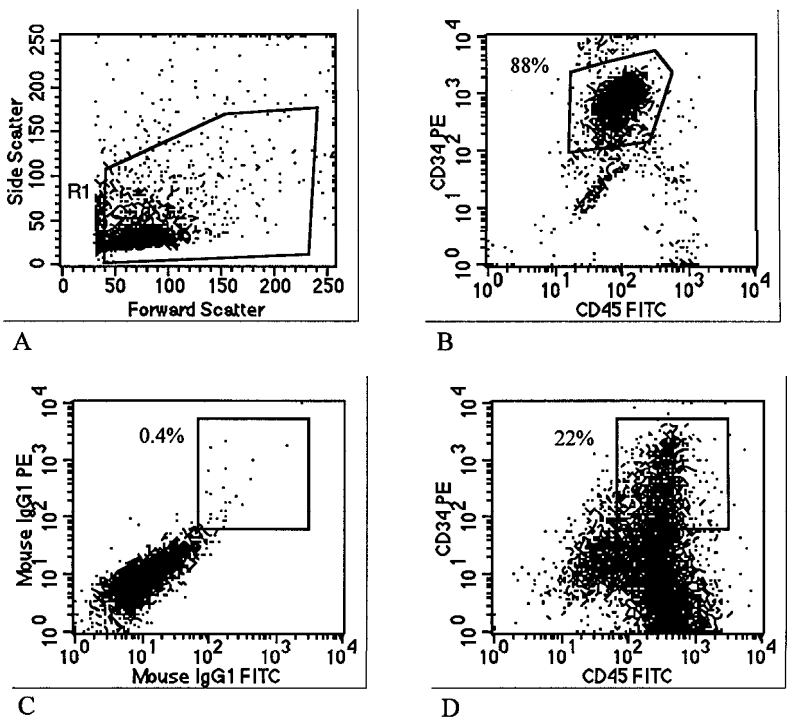

FIG. 1. $CD34^+$ cell frequency in fresh versus *ex vivo* cultured cells. Aliquots of fresh versus cultured cells were immunostained and analyzed by flow cytometry: (**A** and **B**) Fresh $CD34^+$ cells; (**C** and **D**) 7 day-cultured cells. Percentages of $CD34^+$ cells are shown. These results are from experiment 4 (Table 1) and are representative of all the experiments.

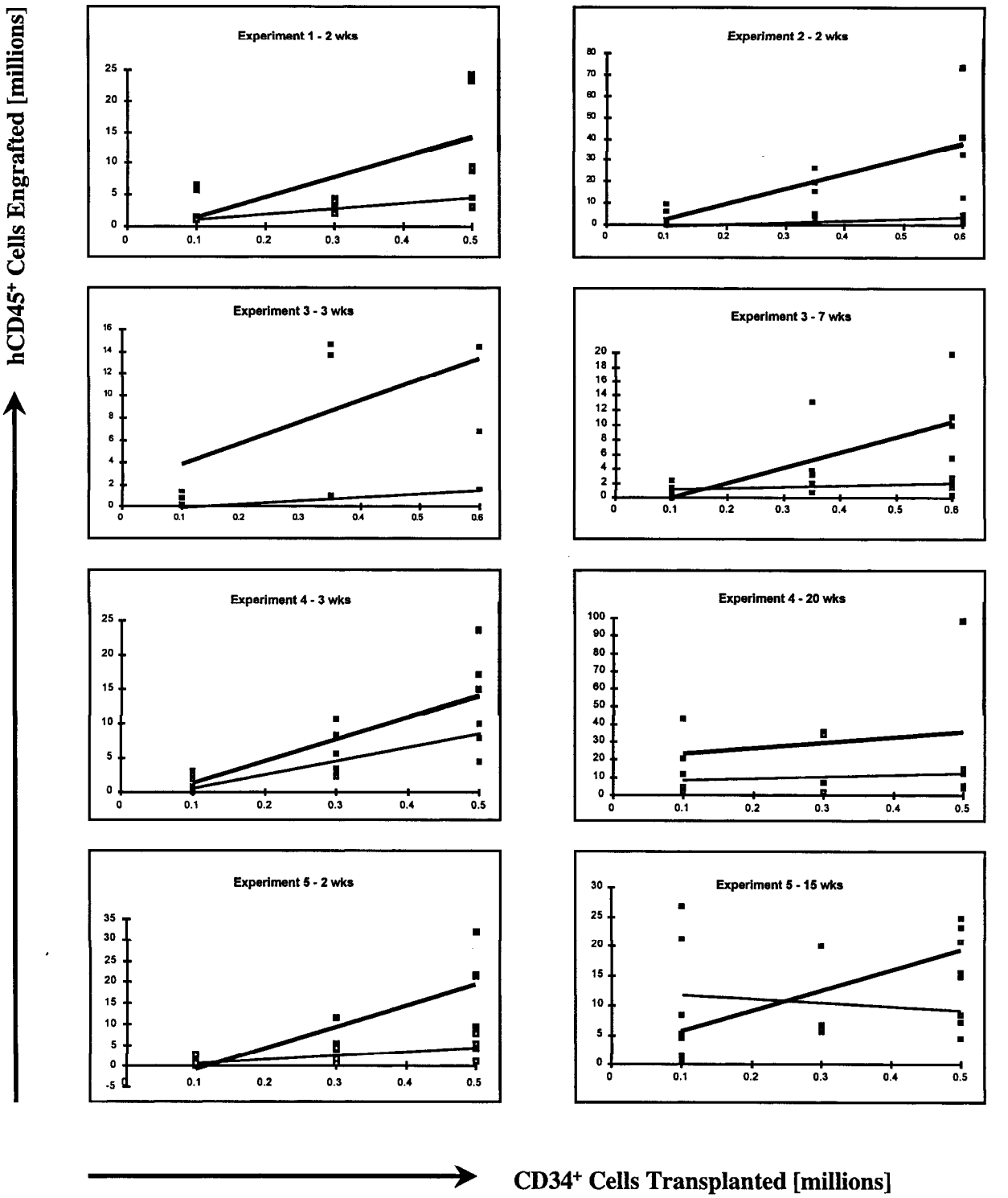

FIG. 2. SEP of fresh versus cultured cells. Three cell doses of fresh or cultured cells were transplanted into sublethally irradiated NOD/SCID mice in experiments 1–5 (Table 1). Individual experiments are indicated, along with the time point (weeks after transplant) at which the NOD/SCID chimeras were assayed for human cell content. The dose of $CD34^+$ cells transplanted is plotted on the *x* axis, and the number of human $CD45^+$ ($hCD45^+$) cells engrafted is plotted on the *y* axis. Data from each chimera transplanted with fresh cells (closed squares) or cultured cells (open squares) are shown. Each line represents the multiple linear regression fit for the data from the transplanted chimeras (fresh cells, thick line; cultured cells, thin line).

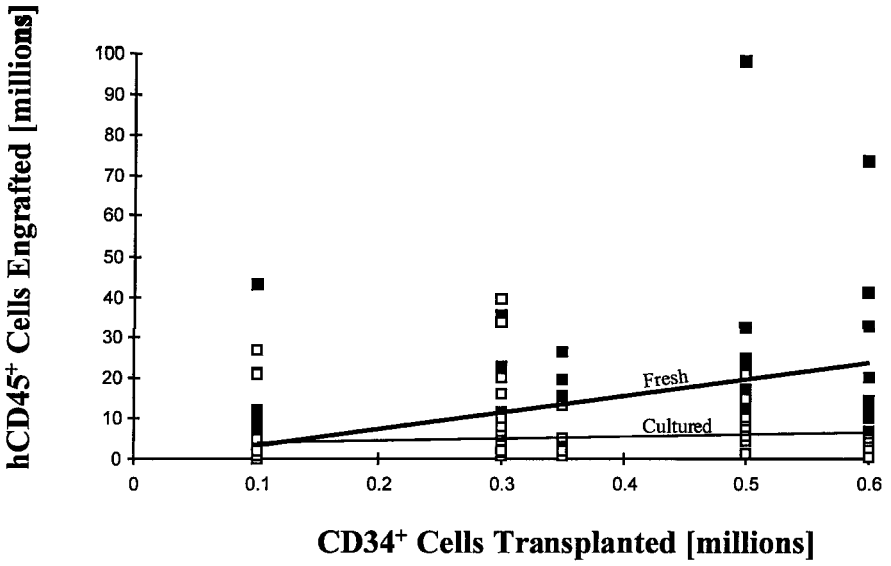

FIG. 3. SEP of fresh versus cultured cells. This multiple linear regression analysis includes data from all six experiments (n = 173; Table 1). The SEP of fresh cells was 37.9, and the SEP of cultured cells was 13.7. The difference between the two values was significant (p = 0.0002). Symbols and lines are as in Fig. 2.

TABLE 2. *Ex Vivo*-CULTURED CORD BLOOD CD34+ CELL POPULATION ENGRAFTMENT *in Vivo*, AND EFFECT ON SCID ENGRAFTMENT POTENTIAL: EXPERIMENTS 1–6

Culture condition[a]	*Experiment*	*Assay time point (weeks after transplant)*[b]	*SEP*			p *Value*	*No. of mice*
			Fresh	*Cultured*	*Cultured/fresh*[c]		
1. IMDM + FCS + FKT36G	1	2	27.5	10.3	0.4	0.06	23
	2	2	61.4	5.2	0.1	0.004	24
	3	3	23.2	2.6	0.1	0.04	12
		7	16.5	5.4	0.3	0.04	23
	4	3	26.9	16.7	0.6	0.4	23
		20	72.9	33.1	0.4	0.3	15
2. QBSF-60 + FKT	5	2	35.5	9.1	0.3	0.01	25
		15	39.4	26.9	0.7	0.006	21
	6	10	10.6	18.2	1.7	0.4	7
	1–6	2–20	37.9	13.7	0.4	0.0002	173

[a]Same abbreviations as in Table 1. The last row combines all data obtained under both culture conditions, and at both short- and long-term time points.

[b]NOD/SCID mice were sacrificed after 2–20 weeks, as indicated in text and in Fig. 2.

[c]Ratio of SEPs. We also analyzed the results of the experiments grouped by culture condition, time point of assay ("short term" or "long term"), and a combination of the two. In the experiments utilizing culture condition 1, the SEP of fresh cells was 38.2, the SEP of cultured cells was 10.1, and the weighted average SEP ratio (cultured : fresh) was 0.3 (p = 0.005, n = 0120 mice). In the experiments utilizing culture condition 2, the SEP of fresh cells was 37.0, the SEP of cultured cells was 22.9, and the weighted average SEP ratio (cultured : fresh) was 0.6 (p = 0.0002, n = 53 mice). In the experiments utilizing culture condition 1 analyzed at 2–3 weeks after transplant, the SEP of fresh cells was 38.7, the SEP of cultured cells was 8.7, and the weighted average SEP ratio (cultured : fresh) was 0.2 (p = 0.0001, n = 82 mice). In the experiments utilizing culture condition 1 analyzed at 7–20 weeks, the SEP of fresh cells was 37.1, the SEP of cultured cells was 13.2, and the weighted average SEP ratio (cultured : fresh) was 0.3 (p = 0.04, p = 38 mice). In the experiments utilizing culture condition 2 analyzed at 7–20 weeks, the SEP of fresh cells was 38.6, the SEP of cultured cells was 33.5, and the weighted average SEP ratio (cultured : fresh) was 0.9 (p = 0.01, p = 28 mice). For all the experiments in which the SEP was determined 2–3 weeks after transplant (including both culture conditions), the SEP of fresh cells was 38.1, the SEP of cultured cells was 8.8, and the weighted average SEP ratio (cultured : fresh) was 0.2 (p = 0.0001, p = 107 mice). For all the experiments in which the SEP was determined at 7–20 weeks after transplant (including both culture conditions), the SEP of fresh cells was 37.6, the SEP of cultured cells was 21.7, and the weighted average SEP ratio (cultured : fresh) was 0.6 (p = 0.3, p = 28 mice).

$CD34^+$ cell dose transplanted (data not shown). The data obtained from the spleens of the animals paralleled those obtained from the BM, although the levels of engraftment were lower (data not shown).

Ex vivo *culture enhances retroviral gene transduction of* in vitro *progenitor cells*

In experiment 7, we transduced either fresh CB $CD34^+$ cells or cells "precultured" (cultured *ex vivo* prior to transduction) in condition 2 for 2 days (the duration of preculture used in several published retroviral transduction protocols) (Larochelle *et al*., 1996; Cheng *et al*., 1997, 1998). In the cell population that had been transduced after 2 days of preculture, 12% of the $CD34^+$ cells and 19% of the $CD34^-$ cells were $EGFP^+$, by flow cytometry on day 7 (Table 3). In contrast, in the cell population that had been transduced without preculture, only 0.5% of the $CD34^+$ cells and 1.8% of the $CD34^-$ cells were $EGFP^+$. About 28% of CFCs transduced on day 2 expressed EGFP by fluorescence microscopy, while only ~8% of the CFC from cells transduced on day 0 were $EGFP^+$ (Table 3). PCR analyses of plucked colonies confirmed these results (Table 3).

In experiment 8, we compared gene transduction efficiency after three different periods of preculture (condition 2) and using three transduction strategies. Three to 24% of $CD34^+$ cells, $CD45^+$ cells, or $CD13/33^+$ cells expressed EGFP by flow cytometry. Each of these strategies resulted in efficient transduction, with up to 60% of $EGFP^+$ CFCs (Table 4).

In experiment 9, we explored the effects of multiple additions of retroviral supernatant to *ex vivo* cultures of CB $CD34^+$ cells incubated on RetroNectin. After *ex vivo* culture under these conditions for 7 days, with daily addition of retroviral supernatant from days 2 to 6, 52% of $CD34^+$ cells and 52% of CFCs were $EGFP^+$ (Table 5).

In experiment 10, we compared 4 days (total duration) of culture with addition of retroviral supernatant on days 2 and 3,

TABLE 3. ENHANCEMENT OF RETROVIRAL TRANSDUCTION EFFICIENCY IN 2-DAY CULTURE OF $CD34^+$ CELLS IN SERUM-FREE MEDIUM CONTAINING THREE GROWTH FACTORS, AS ASSESSED BY *in Vitro* ASSAYS: EXPERIMENT 7

Days in culture[a]	*Transduction*	*EGFP*$^+$					
		CD34$^+$[b]	*CD34*$^-$[b]	*CFC-Mix*[c]	*CFC-GM*[c]	*BFU-E*[c]	*CFC*[d]
0 + 7	Mock spinoculation	0	0	0 / 2 ± 1	0 / 28 ± 4	0 / 41 ± 4	0 / 10
	Spinoculation	0.5 ± 0.1	1.8 ± 0.2	0 / 2 ± 1	3 ± 2 / 34 ± 2	3 ± 1 / 37 ± 13	0 / 10
2 + 5	Mock spinoculation	0	0	0 / 2 ± 1	0 / 51 ± 12	0 / 55 ± 2	0 / 10
	Spinoculation	12 ± 0.3	19 ± 0.2	2 ± 2 / 5 ± 2	22 ± 6 / 54 ± 4	13 ± 6 / 74 ± 22	3 / 10

[a]In order to study the effects of *ex vivo* culture on gene transduction efficiency, we transduced fresh versus cultured (condition 2) CB $CD34^+$ cells with the EGFP-encoding MGIN retroviral vector, which was derived from the murine stem cell virus (MSCV) (Cheng *et al*., 1997). The cells were transduced with the MGIN retrovirus by spinoculation for 4 hr. Briefly, in both experiments 7 and 8, freshly thawed retroviral supernatants from the packaging cell line were mixed 1:1 (v/v) in a 15-ml polypropylene tube (Becton Dickinson) with an aliquot of target cells in QBSF-60 containing FL, KL, and Tpo (GFs at the same concentrations as used for *ex vivo* cultures) and 8 mg/ml polybrene (Sigma). This transduction suspension was centrifuged at 1800 × *g* at 32–35°C for 4 hr. After this 4-hr "spinoculation," cells were washed once and immediately aliquoted for *in vitro* assays. In addition, in experiments 8–10, we followed the manufacturer recommendations for retrovirus-mediated gene transfer on RetroNectin. In brief, aliquots of freshly thawed retroviral supernatants from the packaging cell line were preincubated on RetroNectin-coated 6-well plates (Becton Dickinson) for 1 hr at room temperature. The cultured cell suspension and the remaining viral supernatant were then added to the plate. The transduction mixture was incubated for 2–3 days in a 5% CO_2 humidified atmosphere at 37°C. An aliquot was then plated for CFC assay, and the remaining cells were cultured under condition 2 for an additional 7 days (cells transduced on day 0), or 5 days (cells transduced on day 2) prior to assessment by flow cytometry. *0 + 7* indicates that $CD34^+$ cells were transduced on day 0 and cultured for an additional 7 days. *2 + 5* indicates that cells were transduced on day 2 and cultured for an additional 5 days.

[b]Percentage of $CD34^+$ and $CD34^-$ cells that were $EGFP^+$ by flow cytometry.

[c]Number of $EGFP^+$ (numerator) and total (denominator) CFCs were scored by fluorescence microscopy. Results are expressed as means ± standard error (five replicates).

[d]Ten CFCs were randomly plucked from the plates and tested for the presence of the EGFP transgene by PCR. Type of CFC was not noted. All EGFP PCR primers were within the EGFP cDNA: EGFP1 sense primer: 5′-GGAGAGGGTGAAGGTGATGC-3′; EGFP1 antisense primer: 5′-CCATGTGTAATCCCAGCAGC-3′; EGFP2 sense primer: 5′-CAAGAGTGCCATGCCC-GAAGG-3′; EGFP2 antisense primer: 5′-CATGTGGTCTCTCTTTTCGTTGGG-3′. The conditions for nested EGFP PCR were 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, for 10 cycles with the EGFP1 primers, using the RoboCycler 40 (Stratagene, La Jolla, CA). A 3-μl reaction mixture (from a total of 50 μl) was then used for the nested EGFP PCR with EGFP2 primers, under the same conditions but for 35 cycles. A fragment of 380 bp was expected and produced from control EGFP plasmid DNA.

TABLE 4. RETROVIRAL TRANSDUCTION OF CULTURED CELLS, USING THE SPINOCULATION AND/OR RETRONECTIN METHODS: EXPERIMENT 8

Days in culture[b]	*Method of transduction*[c]	*CD34+*[d]	*EGFP+*[a]			
			CD45+	*CD34+*	*CD13/33+*	*CFC*
2 + 3	M	92	0	0	0	0
	S	92	12.6	10.3	11.2	8.5 ± 1
	S + RN	92	18.8	17.2	17.4	ND
	RN	91	24.0	23.0	24.0	ND
	BSA	93	0	0	0	0
5 + 3	M	51	0	0	0	0
	S	51	11.7	5.9	8.2	6.5 ± 1
	S + RN	48	17.3	12.0	13.6	19.7 ± 6
	RN	50	18.5	15.0	15.9	27.0 ± 6
	BSA	47	0	0	0	0
7 + 3	M	44	0	0	0	0
	S	40	3.5	2.7	3.2	17.9 ± 3
	S + RN	28	15.4	11.4	10.8	61.6 ± 7
	RN	32	13.8	13.5	12.6	52.0 ± 9
	BSA	32	0	0	0	0

[a]The percentage of EGFP+CD45+, CD34+, and CD13/33+ cells was assessed by flow cytometry. Percentage of EGFP+ CFCs and total CFCs was determined by fluorescence microscopy; EGFP+ CFC-Mix, CFC-GM, and BFU-E were observed, but the data are not broken down by type of CFC.

[b]CB CD34+ cells were cultured under culture condition 2 for 2, 5, or 7 days, transduced, and cultured for an additional 3 days.

[c]M, mock spinoculation; S, spinoculation; RN, incubation on RetroNectin; BSA, incubation on bovine serum albumin.

[d]Percentage of total CD34+ cells per total cells.

a transduction strategy used in several previous studies (Cheng *et al*., 1998; van Hennik *et al*., 1998), versus 7 days (total duration) of culture with a third addition of retroviral supernatant on day 5. We did not observe a significant difference in the percentages of cultured CD45+ or CD34+ cells that were EGFP+ in the populations of cells cultured for 7 versus 4 days. However, the percentages of EGFP+ cells and CFCs recovered from the animals 4 weeks posttransplantation were somewhat increased in the mice transplanted with cells that had been cultured for 7 days (Table 6), as compared with 4 days. Both erythroid and myeloid (EGFP+ CFCs were recovered from mice transplanted with both 4 and 7 day-cultured cells (data not shown).

We purified human CD34+ cells from the BMs of two highly engrafted mice transplanted with 7 day-cultured cells; evaluation and purification of CD34+ cells from the BMs of the

TABLE 5. INCREASE IN EFFICIENCY OF TRANSDUCTION ON RETRONECTIN AFTER DAILY ADDITION OF VIRAL SUPERNATANT TO *EX VIVO* CULTURES OF CORD BLOOD CD34+ CELLS: EXPERIMENT 9

Days in culture[b]	*Method of transductions*[c]	*CD34+*[a]	*EGFP+*[a]				
			CD45+	*CD34+*	*CFC-Mix*	*CFC-GM*	*BFU-E*
0	0	97	0	0	0 3 ± 1	0 27 ± 6	0 29 ± 5
3	1	89	9	9	ND	ND	ND
4	2	76	35	35	ND	ND	ND
5	3	72	42	40	ND	ND	ND
6	4	64	49	51	ND	ND	ND
7	5[d]	54	54	52	8 ± 1 15 ± 1	73 ± 1 98 ± 6	48 ± 3 136 ± 7
8	5[d]	58	56	56	ND	ND	ND

[a]See Table 3 footnotes.

[b]CB CD34+ cells were cultured under culture condition 2 for 2 days, plated on RetroNectin, and cultured for an additional 6 days.

[c]Number of viral supernatant additions: each day from day 2 to day 6, cells were centrifuged and resuspended in fresh QBSF-60 + FKT and fresh viral supernatant (1:1).

[d]An aliquot of cells was left in culture from day 7 to day 8, without further additions of viral supernatant, to allow full expression of the EGFP transcript.

TABLE 6. COMPARISON OF THE EFFECT OF 4- VERSUS 7-DAY CULTURE ON THE EFFICIENCY OF TRANSDUCTION OF $CD34^+$ CELLS: EXPERIMENT 10

			$EGFP^{+a}$							
Days in culture[b]	*Number of transductions*[b]	$CD34^{+a}$	$CD45^+$	$CD34^+$	*CFC-Mix*	*CFC-GM*	*BFU-E*	$CD45^+$ *from chimera BM*	$CD34^+$ *from chimera BM*	*CFC from chimera BM*
4	2	99	38	35	45 ± 20	46 ± 10	18 ± 5	13 ± 2	ND	2 ± 1
7	3	39	44	36	48 ± 15	53 ± 13	28 ± 7	23 ± 2	19 ± 3	31 ± 8

[a]See Table 3 footnotes. In this experiment, we also transplanted cultured, transduced cells for measurement of $EGFP^+$ SCID engrafting cells. On average, 1 (±1) × 10^6 human $CD45^+$ cells were recovered from the BMs of chimeras transplanted with day 4 cultured cells, while 4.8 (±6.7) × 10^6 human cells were recovered from the chimeras transplanted with day 7 cultured cells. This difference was not significant, considering the high variation in the levels of engraftment among the chimeras in this experiment.

[b]CB $CD34^+$ cells were cultured for 2 days, plated on RetroNectin and either cultured for an additional 2 days, with two additions of viral supernatant, or cultured for an additional 5 days with three additions of viral supernatant and replacement of the RetroNectin layer on day 5. In this experiment, we used retroviral supernatant collected in QBSF-60, to avoid the introduction of small amounts of FCS under condition 2. In preliminary studies, we had observed that comparable titers of viral supernatant are obtained when the packaging cell line is cultured overnight in QBSF-60, as compared with DMEM containing 10% FCS; viral supernatants obtained in either serum-free medium or DMEM + 10% FCS were equally efficient in transducing CB $CD34^+$ cells and CFCs (data not shown).

chimeras transplanted with day 4 cultured cells were not possible because of the low levels of engraftment in this group. We obtained ~10^6 viable $CD34^+$ cells from the BM of the two chimeras (pooled). Of these purified $CD34^+$ cells, 28% were $EGFP^+$ by flow cytometry (Fig. 4). After plating in CFC assays, 17% of the colonies generated by these $CD34^+$ cells purified from the BM of the chimeras were $EGFP^+$.

DISCUSSION

CB is currently in clinical use as an alternative source of HSCs for hematopoietic transplantation (Cairo and Wagner, 1997). Reports have demonstrated that it is possibe to genetically engineer CB HSCs (Lu *et al*., 1993; Kohn *et al*., 1995, 1998), but improved gene transduction protocols appear to be necessary to obtain therapeutic effects (Emerson, 1996; Kohn *et al*., 1998). In the experiments discussed herein, we explored the effects of *ex vivo* suspension culture of CB $CD34^+$ cells on the ability of the cultured cell population to engraft *in vivo* and to express a retrovirally transduced gene.

Our first set of *ex vivo* cultures was performed with medium supplemented with 10% FBS plus six GFs (FL, KL, Tpo, IL-3, IL-6, and G-CSF). Previously, it had been shown that culture of human CB $CD34^+CD38^-$ cells with these GFs resulted in increases in numbers of total cells, progenitor cells, and LTC-ICs (Petzer *et al*., 1996a,b). The same group also reported that cultured human CB $CD34^+CD38^-$ cells engrafted in NOD/SCID mice. They found that the frequency of SCID engrafting cells, by limiting dilution analysis, was approximately twofold higher in cultured cells then in fresh cells. However, these cells appeared to have reduced proliferative potential, since the numbers of progeny recovered from the BMs of the chimeras were somewhat decreased (Conneally *et al*., 1997). Similarly, an independent report showed that the SCID repopulating capacity of CB $CD34^+CD38^-$ cells was slightly en-

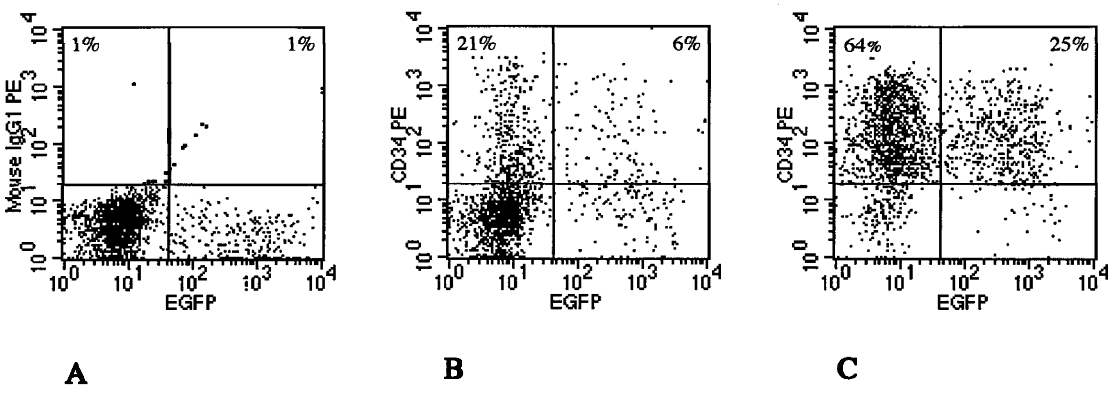

FIG. 4. Flow cytometric analysis of $CD34^+$ cells detected and isolated from the BMs of two mice transplanted with 7 day-cultured CB $CD34^+$ cells (from experiment 10, Table 6). (**A**) Isotype control; (**B**) cell population prior to the purification; (**C**) enriched $CD34^+$ cells after purification. The percentages of cells in selected quadrants are indicated.

hanced (fourfold) by *ex vivo* culture for 4 days in serum-free medium supplemented with the same GF cocktail, but was completely lost after 9 days of culture (Bhatia *et al*., 1997), in spite of increases in CFC numbers. It was reported that when $CD34^+$ cells were cultured in serum-free medium in the presence of KL, FL, and either IL-3 or Epo/IL-6, the SCID repopulating capacity decreased by 2-fold after 1 day of culture and by 25-fold by day 3 (Rebel *et al*., 1999). The results of our *in vitro* assays were consistent with these studies, in that culture of CB $CD34^+$ cells, in both the culture conditions tested, resulted in increases in numbers of total cells, $CD34^+$ cells, CFCs, and LTC-ICs. In our experiments, the SCID repopulating capacity (calculated as SEP)(Leung *et al*., 1998) of cultured cells was 2.8-fold decreased, as compared with fresh cells. We had hypothesized that increased numbers of colony-forming progenitor cells (which generate *in vitro* colonies enumerated 2 weeks after plating) might correlate with higher levels of *in vivo* engraftment 2–3 weeks after transplant, but this was not observed. SEP was somewhat lower for cultured than for fresh cells, even at these time points early after transplantation. Overall in our experiments (averaged), 1 fresh CB $CD34^+$ cell yielded 38 $CD45^+$ cells in the BMs of the chimeras, while 1 initially cultured CB $CD34^+$ cell yielded only 14 $CD45^+$ cells. Thus, we did not obtain evidence for the hypothesis that *ex vivo* culture increases *in vivo* engrafting capacity. Nevertheless, these results confirm and extend the conclusion that a cultured CB $CD34^+$ cell population is capable of *in vivo* engraftment, with the capacity to generate high levels of multilineage hematopoiesis at both short-term and long-term time points after transplantation in the NOD/SCID chimera model. It is possible that slightly different culture conditions may further improve the SEP of the cultured cell population. For instance, several groups have shown that maximal CFC, LTC-IC, and SEP expansion occurs when subpopulations of $CD34^+$ cells are plated at low cell concentrations (Emerson, 1996; Conneally *et al*., 1997), with continuous resupply of fresh medium and GFs (Emerson, 1996; Piacibello *et al*., 1999), and with extended periods of culture (Piacibello *et al*., 1999). These factors may be responsible for the variability in *ex vivo* "expansion" observed among different studies.

Several groups have reported that inclusion of IL-3 in *ex vivo* culture medium results in diminished stem/progenitor cell function (Yonemura *et al*., 1996; Dao *et al*., 1997; Zandstra *et al*., 1997). Others found that *ex vivo* culture in FL plus Tpo, with or without KL, but without "late-acting" growth factors was sufficient to increase progenitor cell numbers (Piacibello *et al*., 1997). Furthermore, it was shown (Luens *et al*., 1998) that culture of PKH26-labeled BM $CD34^+Thy1^+Lin^-$ cells in FL, KL, plus Tpo for 6 days resulted in persistent $CD34^+$ cells, all of which had divided (i.e., had the phenotype $CD34^{high}PKH26^{low}$); nevertheless, the cultured, purified $CD34^+$ cells retained the capacity to engraft in immunodeficient mice. Therefore, we next investigated the effects of *ex vivo* culture in the presence of only these three GFs (FL, KL, and Tpo). To avoid unknown effects of variable serum proteins and to better enable future clinical application, we utilized a serum-free medium, QBSF-60. The effects of *ex vivo* culture in these simpler conditions were similar to those observed after culture in medium containing FBS plus six GFs: the cultured cells generated high levels of short-term and long-term human engraftment, although the SEP was slightly decreased as compared with fresh cells. In averaging the experiments conducted in culture condition 2, the ratio of SEP for cultured versus fresh cells was 0.9. It should be noted that the decrease in SEP of cultured versus fresh cells varied among experiments and did not always reach significance.

Taken together with the current literature, the above results suggested that culture of $CD34^+$ cells in serum-free medium containing FL, KL, plus Tpo might result in enhanced retroviral gene transduction of *in vivo* SCID engrafting cells. We decided to test this hypothesis by transducing cultured versus fresh $CD34^+$ cells with the MSCV-based MGIN vector carrying the EGFP reporter gene. Previous observations showed that transduction with MSCV-based vectors yields a higher level of transgene expression in hematopoietic progeny, as compared with cells transduced using the conventional Moloney murine leukemia virus (Mo-MuLV)(Cheng *et al*., 1998; van Hennik *et al*., 1998). Since there are several different methods of retroviral transduction, we began by testing different transduction approaches. We evaluated these approaches by *in vitro* assays of transduction efficiency (experiments 7–9). We then selected a single approach that worked well and tested this approach by both *in vivo* and *in vitro* assays (experiment 10). We first explored the effects of 2 days of *ex vivo* "preculture" on gene transduction; similar durations of preculture have been utilized widely to enhance retroviral transduction efficiency (Larochelle *et al*., 1996; Cheng *et al*., 1997, 1998; Dao *et al*., 1997; Conneally *et al*., 1998; Schilz *et al*., 1998; van Hennik *et al*., 1998). As expected, the level of EGFP expression in hematopoietic progenitor cells was increased severalfold by a 2-day *ex vivo* preculture (Table 3). It had been suggested that immature $CD34^+38^{low}$ cells may require at least 5 days of preculture in the presence of GF exposure before they divide (Conneally *et al*., 1997, 1998). Therefore, we next explored the effects of different lengths of preculture (in condition 2) on gene transduction efficiency by either spinoculation, transduction in the presence of RetroNectin, or a combination of these two transduction methods. Transduction by spinoculation (alone) resulted in EGFP expression in up to 30% of CFCs. This result is consistent with previously published reports using murine retroviral vectors (Nolta *et al*., 1996; Cheng *et al*., 1997, 1998; Conneally *et al*., 1998). When transduction was performed on RetroNectin, up to 60% of CFCs were $EGFP^+$. It is possible that the enhancement provided by the use of RetroNectin in this single experiment may be an overestimate, especially since toxicity due to the presence of Polybrene may have negatively affected transduction by spinoculation (Conneally *et al*., 1998). In addition, the lengths of exposure of cells to the viral supernatant were different among the groups. Further experiments and an optimized spinoculation protocol (i.e., using protamine sulfate [Conneally *et al*., 1998] instead of Polybrene) will be required to determine which procedure is more efficient in transducing HSCs. Nevertheless, both these experiments indicate that cells in this serum-free medium with three GFs can be readily transduced with a retroviral vector. Since only a fraction of the HSCs may divide during transduction on any single day and since the half-life of retrovirus is <1 day (Kotani *et al*., 1994), we tested whether daily addition of fresh virus in the presence of RetroNectin might result in increasing levels of transduction. Intriguingly, up to 50% of the human cells in NOD/SCID mice carried the retroviral marker after transplantation of cells that

had undergone multiple transductions by spinoculation (Schilz *et al*., 1998). Experiment 9 (Table 5) shows a limited increase in the percentage of cells that expressed EGFP after multiple additions of viral supernatant. The percentage of cells that expressed EGFP increased slightly, from ~40% (two transductions) to 56% (five transductions). RetroNectin was not replaced throughout the duration of this experiment, so it is possible that saturation of RetroNectin binding sites after the early additions of viral supernatant may have prevented greater increases in transduction efficiency.

Since Luens *et al*. (1998) had reported that $CD34^+$ cells, repurified after 6 days of culture in the above three GFs, had all divided and were capable of engraftment in the Hu/SCID model, we decided to test whether the retrovirally transduced cells in our cultures could engaft *in vivo* in NOD/SCID mice (experiment 10). As shown in Table 6, cells cultured and transduced for either 4 or 7 days were capable of engraftment in NOD/SCID mice, as assessed after 4 weeks. $EGFP^+$ CFCs were recovered from the chimeras, and $EGFP^+CD34^+$ cells purified from the chimeras were able to generate $EGFP^+$ hematopoietic colonies.

In summary, we have first confirmed that *ex vivo* cultured CB $CD34^+$ cells can generate multilineage engraftment in NOD/SCID mice. Whether modifications to these culture conditions will result in actual expansion of SEP will be the subject of further investigations. For example, future research may indicate that IL-6 or a fusion protein of the IL-6α chain joined to an IL-6 receptor fragment may increase the survival/proliferation of SCID repopulating cells (Rappold *et al*., 1998). We have also shown that serum-free culture with FL, KL, and Tpo enhances the efficiency of gene transfer into $CD34^+$ progenitor cells, by *in vitro* and *in vivo* assays. In the experiments presented herein, cells precultured in serum-free medium with three GFs were efficiently transduced by one or more transductions with MSCV and RetroNectin. The cultured, transduced cells generated $EGFP^+$ human cells in NOD/SCID transplant recipients. Transduction may be further enhanced by improved retroviral vectors and packaging cell lines, e.g., MSCV vectors pseudotyped with the gibbon ape leukemia virus envelope protein (Kiem *et al*., 1997; Schilz *et al*., 1998; van Hennik *et al*., 1998), or Mo-MuLV pseudotyped with the vesicular stomatitis virus G protein (Rebel *et al*., 1999). We hypothesize that such strategies, when fully optimized with respect to vector, titer, and transduction conditions, will support adequate levels of gene transduction to provide therapeutic benefit in clinical settings. Testing for persistence of transducted cells for a longer times (>2–3 months) should be done in chimera models prior to such clinical studies.

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REFERENCES

ALCORN, M., HOLYOAKE, T., RICHMOND, L., PEARSON, C., FARRELL, E., KYLE, B., DUNLOP, D., FITZSIMMONS, E., STWARD, W., PRAGNELL, I., and FRANKLIN, I. (1996). CD34-positive cells isolated from cryopreserved peripheral blood cells can be expanded ex vivo and used for transplantation with little or no toxicity. J. Clin. Oncol. **14,** 1839–1847.

BHATIA, M., BONNET, D., KAPP, U., WANG, J.C.Y., MURDOCH, B., and DICK, J.E. (1997). Quantitative analysis reveals expansion of human repopulating cells after short-term ex vivo culture. J. Exp. Med. **186,** 619–624.

BOGGS, D.R. (1984). The total marrow mass of the mouse: A simplified method of measurement. Am. J. Hematol. **16,** 277–286.

BREEMS, D.A., BLOKLAND, E.A.W., SIEBEL, K.E., MAYEN, A.E.M., ENGELS, L.J.A., and PLOEMACHER, R.E. (1998). Stroma-contact prevents loss of hematopoietic stem cell quality during ex vivo expansion of $CD34^+$ mobilized peripheral blood stem cells. Blood **91,** 111–117.

BRUGGER, W., MICKLIN, W., HEIMFELD, S., BERENSON, R.J., MERTELSMANN, R., and KANZ, L. (1993). Ex vivo expansion of enriched peripheral blood $CD34^+$ cells by stem cell factor, interleukin-1, IL-6, IL-3, interferon-gamma, and erythropoietin. Blood **81,** 2579–2584.

BRUGGER, W., HEIMFELD, S., BERENSON, R.J., MERTELSMANN, R., and KANZ, L. (1995). Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. N. Engl. J. Med. **333,** 283–287.

CAIRO, M.S., and WAGNER, J.E. (1997). Placental and/or umbilical cord blood: An alternative source of hematopoietic stem cells for transplantation. Blood **90,** 4665–4678.

CAROW, C.E., HARRINGTON, M.A., and BROXMEYER, H.E. (1993). Detection of human myeloid progenitor cells in a murine background. Exp. Hematol. **21,** 66–69.

CHENG, L., DU, C., MURRAY, D., TONG, X., ZHANG, Y.A., CHEN, B.P., and HAWLEY, R.G. (1997). A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells. Gene Ther. **4,** 1013–1022.

CHENG, L., DU, C., LAVAU, C., CHEN, S., TONG, J., CHEN, B.P., SCOLLAY, R., HAWLEY, R.G., and HILL, B. (1998). Sustained gene expression in retrovirally transduced, engrafting human hematopoietic stem cells and their lympho-myeloid progeny. Blood **92,** 83–92.

CIVIN, C.I., ALMEIDA-PORADA, G., LEE, M.J., OLWEUS, J., TERSTAPPEN, L.W.M.M., and ZANJANI, E.D. (1996). Sustained, retransplantable, multilineage engraftment of highly purified adult bone marrow stem cells in vivo. Blood **88,** 4102–4109.

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. U.S.A. **94,** 9836–9841.

CONNEALLY, E., EAVES, C.J., and HUMPHRIES, R.K. (1998). Efficient retroviral-mediated gene transfer to human cord blood stem cells with in vivo repopulating potential. Blood **91,** 3487–3493.

COUTINHO, L.H., WILL, A., and RADFORD, J. (1990). Effects of recombinant human granulocyte colony-stimulating factor (CSF), human granulocyte macrophage-CSF, and gibbon interleukin-3, on hematopoiesis in human long-term BM culture. Blood **74,** 2118–2129.

DAO, M.A., HANNUM, C.H., KOHN, D.B., and NOLTA, J.A. (1997). FLT3 ligand preserves the ability of human CD34+ progenitors to sustain long-term hematopoiesis in immune-deficient mice after ex-vivo retroviral-mediated transduction. Blood **89,** 446–456.

EMERSON, S.G. (1996). Ex vivo expansion of hematopoietic precursors, and stem cells: The next generation of cellular therapeutics. Blood **87,** 3082–3088.

GARTNER, S., and KAPLAN, H.S. (1980). Long-term culture of human bone marrow cells. Proc. Natl. Acad. Sci. U.S.A. **77,** 4756–4759.

GÖTZE, K.S., RAMIREZ, M., TABOR, K., SMALL, D., MATTHEWS, W., and CIVIN, C.I. (1998). Flt3high and Flt3low progenitor cells isolated from human bone marrow are functionally distinct. Blood **91,** 1–13.

GÜENECHEA, G., SEGOVIA, J.C., ALBELLA, B., RAMIREZ, M., REGIDOR, C., FERNANDEZ, M.N., and BUEREN, J.A. (1999). Delayed engraftment of nonobese diabetic/severe combined immunodeficient mice transplanted with *ex-vivo*-expanded human CD34+ cord blood cells. Blood **93,** 1097–1105.

HENSCHLER, R., BRUGGER, W., LUFT, T., FREY, T., MERTELSMANN, R., and KANZ, L. (1994). Maintenance of transplantation potential in ex vivo expanded CD34+-selected human peripheral blood progenitor cells. Blood **84,** 2898–2903.

HOGAN, C.J., SHPALL, E.J., McNULTY, O., McNIECE, I., DICK, J.E., SHULTZ, L.D., and KELLER, G. (1997). Engraftment and development of human CD34+-enriched cells from umbilical cord blood in NOD/LtSz-scid/scid mice. Blood **90,** 85–96.

KIEM, H.P., HEYWARD, S., WINKLER, A., POTTER, J., ALLEN, J.M., MILLER, A.D., and ANDREWS, R.G. (1997). Gene transfer into marrow repopulating cells: Comparison between amphotropic and gibbon ape leukemia virus pseudotyped retroviral vectors in a competitive repopulation assay in baboons. Blood **90,** 4638–4645.

KOHN, D.B., WEINBERG, K.I., NOLTA, J.A., HEISS, L.N., LENARSKY, C., CROOKS, G.M., HANLEY, M.E., ANNETT, G., BROOKS, J.S., EL-KHOUREIY, A., LAWRENCE, K., WELLS, S., MOEN, R.C., BASTIAN, J., WILLIAMS-HERMAN, D.E., ELDER, M., WARA, D., BOWEN, T., HERSHFIELD, M.S., MULLEN, C.A., BLAESE, R.M., and PARKMAN, R. (1995). Engraftment of gene-modified umbilical cord blood cells in neonates with adenosine deaminase deficiency. Nature Med. **1,** 1017–1023.

KOHN, D.B., HERSHFIELD, M.S., CARBONARO, D., SHIGEOKA, A., BROOKS, J., SMOGORZEWSKA, E.M., BARSKY, L.W., CHAN, R., BUROTTO, F., ANNETT, G., NOLTA, J.A., CROOKS, G., KAPOOR, N., ELDER, M., WARA, D., BOWEN, T., MADSEN, E., SNYDER, F.F., BASTIAN, J., MUUL, L., BLAESE, R.M., WEINBERG, K., and PARKMAN, R. (1998). T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood CD34+ cells in ADA-deficient SCID neonates. Nature Med. **4,** 775–780.

KOLLER, M.R., MANCHEL, I., BROTT, D.A., and PALSSON, B.O. (1996). Donor-to-donor variability in the expansion potential of human bone marrow cells is reduced by accessory cells but not by soluble growth factors. Exp. Hematol. **24,** 1484–1493.

KOTANI, H., NEWTON, P.B., ZHANG, S., CHIANG, Y.L., OTTO, E., WEAVER, L., BLAESE, R.M., ANDERSON, W.F., and McGARRITY, G.J. (1994). Improved methods of retroviral vector transduction and production for gene therapy. Hum. Gene Ther. **5,** 19–28.

KURTZBERG, J., LAUGHLIN, M., GRAHAM, M.L., SMITH, C., OLSON, J.F., HALPERIN, E.C., CIOCCI, J., CARRIER, C., STEVENS, C.E., and RUBINSTEIN, P. (1996). Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. N. Engl. J. Med. **335,** 157–166.

LAROCHELLE, A., VORMOOR, J., HANENBERG, H., WANG, J.C.Y., BHATIA, M., LAPIDOT, T., MORITZ, T., MURDOCH, B., XIAO, X.L., KATO, I., WILLIAMS, D.A., and DICK, J.E. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy. Nature Med. **2,** 1329–1337.

LEUNG, W., RAMIREZ, M., NOVELLI, E.M., and CIVIN, C.I. (1998). In vivo engraftment potential of clinical hematopoietic grafts. J. Invest. Med. **46,** 303–311.

LORINCZ, M., HERZENBERG, L.A., DIWU, Z., BARRANGER, J.A., and KERR, W.G. (1997). Detection and isolation of gene-corrected cells in Gaucher disease via a fluorescence-activated cell sorter assay for lysosomal glucocerebrosidase activity. Blood **89,** 3412–3420.

LU, L., XIAO, M., CLAPP, D.W., LI, Z.-H., and BROXMEYER, H.E. (1993). High efficiency retroviral mediated gene transduction into single isolated immature and replatable CD34+ hematopoietic stem/progenitor cells from human umbilical cord blood. J. Exp. Med. **178,** 2089–2096.

LUENS, K.M., TRAVIS, M.A., CHEN, B.P., HILL, P.L., SCOLLAY, R., and MURRAY, L.J. (1998). Thrombopoietin, kit ligand and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin− cells with preserved ability to engraft SCID-hu bone. Blood **91,** 1206–1215.

McKENNA, H.J., DE VRIES, P., BRASEL, P., LYMAN, S.D., and WILLIAMS, D.E. (1995). Effect of Flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. Blood **86,** 3413–3420.

MILLER, D.G., ADAM, M.A., GARCIA, J.V., 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.

MOORE, M.A.S., and HOSKINS, I. (1994). Ex vivo expansion of cord blood-derived stem cells and progenitors. Blood Cells **20,** 468–479.

NOLTA, J.A., DAO, M.A., WELLS, S., SMOGORZEWSKA, E.M., and KOHN, D.B. (1996). Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. Proc. Natl. Acad. Sci. U.S.A. **93,** 2414–2419.

PETZER, A.L., HOGGE, D.E., LANSDORP, P.M., REID, D.S., and EAVES, C.J. (1996a). Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating-cells) in vitro and their expansion in defined medium. Proc. Natl. Acad. Sci. U.S.A. **93,** 1470–1474.

PETZER, A.L., ZADSTRA, P.W., PIRET, J.M., and EAVES, C.J. (1996b). Differential cytokine effects on primitive (CD34+CD38−) human hematopoietic cells: Novel responses to Flt3-ligand and thrombopoietin. J. Exp. Med. **183,** 2551–2558.

PIACIBELLO, W., SANAVIO, F., GARETTO, L., SEVERINO, A., BERGANDI, D., FERRARIO, J., FAGIOLI, F., BERGER, M., and AGLIETTA, M. (1997). Extensive amplification and self-renewal of human primitive hematopoietic stem cell from cord blood. Blood **89,** 2644–2653.

PIACIBELLO, W., SANAVIO, F., SEVERINO, A., DANÉ, A., GAMMAITONI, L., FAGIOLI, F., PERISSINOTTO, E., CAVALLONI, G., KOLLET, O., LAPIDOT, T., and AGLIETTA, M. (1999). Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34+ cord blood cells after ex vivo expansion: Evidence for the amplification and self-renewal of repopulating stem cells. Blood **93,** 3736–3749.

PROSPER, F., VANOVERBEKE, K., STRONCEK, D., and VERFAILLIE, C.M. (1997). Primitive long-term culture initiating cells (LTC-ICs) in granulocyte colony-stimulating factor mobilized peripheral blood progenitor cells have similar potential for ex vivo expansion as primitive LTC-ICs in steady state bone marrow. Blood **89,** 3991–3997.

RAMÍREZ, M., ROTTMAN, G.A., SHULTZ, L.D., and CIVIN, C.I. (1998). Mature human hematopoietic cells in donor bone marrow complicate interpretation of stem/progenitor cell assays in xenogeneic hematopoietic chimeras. Exp. Hematol. **26,** 332–344.

RAPPOLD, I., WATT, S.M., ROSE-JOHN, S., and PLOEMACHER, R.E. (1998). GP130 signalling synergizes with FL and Tpo for the long-term expansion of human hematopoietic progenitors. Blood **92,** 724a.

REBEL, V.I., TANAKA, M., LEE, J.-S., HARTNETT, S., PULSIPHER, M., NATHAN, D.G., MULLIGAN, R.C., and SIEFF, C.A. (1999). One-day ex vivo culture allows effective gene transfer into human nonobese diabetic/severe combined immune-deficient repopulating cells using high-titer vesicular stomatitis virus G protein pseudotyped retrovirus. Blood **93,** 2217–2224.

SCHILZ, A.J., BROUNS, G., KNÖß, H., OTTMANN, O.G., HOELZER, D., FAUSER, A.A., THRASHER, A.J., and GREZ, M. (1998). High efficiency gene transfer to human hematopoietic SCID-repopulating cells under serum-free conditions. Blood **92,** 3153–3171.

SROUR, E.F., BRANDT, J.E., BRIDDELL, R.A., GRIGSBY, S., LEEMHUIS, T., and HOFFMAN, R. (1993). Long-term generation and expression of human primitive hematopoietic progenitor cells in vitro. Blood **81,** 661–669.

SUTHERLAND, H.J., LANSDORP, P.M., HENKELMAN, D.H., EAVES, A.C., and EAVES, C.J. (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc. Natl. Acad. Sci. U.S.A. **87,** 3584–3588.

TERSTAPPEN, L.W.M.M., SHAH, V.O., CONRAD, M.P., RECKTENWALD, D., and LOKEN, M.R. (1988). Discriminating between damaged and intact cells in fixed flow cytometric samples. Cytometry **9,** 477–484.

TRISCHMANN, T.M., SCHEPERS, K.G., and CIVIN, C.I. (1993). Measurement of $CD34^+$ cells in bone marrow by flow cytometry. J. Hematother **2,** 305–313.

VAN DER LOO, J.C.M., HANENBERG, H., COOPER, R.J., LUO, F.-Y., LAZARIDIS, E.N., and WILLIAMS, D.A. (1998). Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood cells. Blood **92,** 2556–2570.

VAN HENNIK, P.B., VERSTEGEN, M.M.A., BIERHUIZEN, M.F.A., LIMON, A., WOGNUM, A., CANCELAS, J.A., BARQUINERO, J., PLOEMACHER, R.E., and WAGEMAKER, G. (1998). Highly efficient transduction of the green fluorescent protein gene in human umbilical cord blood stem cells capable of cobblestone formation in long-term cultures and multilineage engraftment of immunodeficient mice. Blood **92,** 4013–4022.

YONEMURA, Y., KU, H., HIRAYAMA, F., SOUZA, L.M., and OGAWA, M. (1996). Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. Proc. Natl. Acad. Sci. U.S.A. **93,** 4040–4044.

ZANDSTRA, P.W., CONNEALLY, E., PETZER, A.L., PIRET, J.M., and EAVES, C.J. (1997). Cytokine manipulation of primitive human hematopoietic cell self-renewal. Proc. Natl. Acad. Sci. U.S.A. **94,** 4698–4703.

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