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Stable Transduction with Lentiviral Vectors and Amplification of Immature Hematopoietic Progenitors from Cord Blood of Preterm Human Fetuses

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ABSTRACT

Umbilical cord blood (CB) from the early gestational human fetus is recognized as a rich source of hematopoietic stem cells. To examine the value of fetal CB for gene therapy of inborn immunohematopoietic disorders, we tested the feasibility of genetic modification of CD34⁺ cells from CB at weeks 24 to 34 of pregnancy, using lentiviral vector-mediated transfer of the green fluorescent protein (GFP) gene. The transduction rate of CD34⁺ cells was 42 ± 9%, resulting in GFP expression in 23 ± 4% of colonies derived from colony-forming units (CFUs) and 11 ± 1% from primitive long-term culture-initiating cells (LTC-ICs). Cell cycle analysis demonstrated transduction and GFP expression in cells in the G₀ phase, which contains immature hematopoietic progenitors. Transduced fetal CD34⁺ cells could be expanded 1000-fold in long-term cultures supplemented with megakaryocyte growth and development factor along with Flt-3 ligand. At week 10, expression of GFP was observed in 40.5 ± 11.7% of CFU-derived colonies. While prestimulation of CD34⁺ cells with cytokines prior to transduction increased the efficiency of GFP transfer 2- to 3-fold, long-term maintenance of GFP-expressing CFUs occurred only in the absence of prestimulation. The GFP gene was found integrated into the genomic DNA of 35% of LTC-IC-derived colonies initiated at week 10, but GFP expression was not detectable, suggesting downregulation of transgene activity during the extended culture period. These results indicate that human fetal CB progenitors are amenable to genetic modification by lentiviral vectors and may serve as a target for gene therapy of hematopoietic disorders by prenatal autologous transplantation.

OVERVIEW SUMMARY

Although hematopoietic stem cells in preterm umbilical CB are considered as targets for gene therapy of congenital diseases *in utero*, gene transfer to these cells has not been attempted. We used HIV-derived lentiviral vectors for GFP gene transfer to human CD34⁺ CB progenitors from weeks 24–34 of pregnancy. Transduction efficiency was 42 ± 9%, resulting in GFP expression in 23 ± 4 and 11 ± 1% of CFU- and primitive LTC-IC-derived colonies, respectively. When transduced fetal CD34⁺ cells were expanded about 1000-fold in cytokine-supplemented cultures for 10 weeks, expression of GFP was maintained in 40.5 ± 11.7% of CFUs and the GFP gene was found integrated into the DNA of

35% of LTC-ICs. GFP transfer efficiency was increased by cytokine prestimulation preceding transduction of CD34⁺ cells, but long-term maintenance of transgene expression was superior in the absence of prestimulation. These results provide evidence that preterm CB can be targeted for gene transfer by lentiviral vectors.

INTRODUCTION

GENETIC MODIFICATION of hematopoietic stem cells is a promising approach for the treatment of inherited disorders of the hematopoietic system, provided the relevant gene can be stably expressed in cells with the capacity to self-renew

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and differentiated to the affected hematopoietic lineages. Lentiviral vectors, based on the human immunodeficiency virus type 1 (HIV-1), have been developed and shown to be efficient for the delivery and expression of genes in nondividing cells (Naldini *et al.*, 1996; Zufferey *et al.*, 1997). For transduction of quiescent hematopoietic progenitors, these vectors may offer a significant advantage over murine leukemia virus-derived vectors, since they do not require mitotic cell divisions for stable integration into the host genome (Roe *et al.*, 1993; Uchida *et al.*, 1998). The value of lentiviral vectors for gene delivery to bone marrow-repopulating stem cells was demonstrated by sustained transgene expression in recipient nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice transplanted with transduced human CD34⁺ progenitors (Miyoshi *et al.*, 1999).

Umbilical cord blood (CB) is an established source of hematopoietic stem cells for allogeneic transplantation (Gluckman *et al.*, 1997; Rubinstein *et al.*, 1998). Moreover, autologous CB has been used for gene therapy in children with adenosine deaminase deficiency (Kohn *et al.*, 1995, 1998). With advances in prenatal diagnosis, congenital defects can now be identified in the first trimester of pregnancy, calling for the earliest possible therapeutic intervention to prevent the manifestation of disease (Holzgreve, 1997). This could be achieved by stem cell transplantation *in utero* before the development of any tissue damage (Flake and Zanjani, 1999; Schneider and Coutelle, 1999). Allogeneic transplantation has been successful in fetuses with congenital immunodeficiency syndromes profiting from a selective survival advantage of genetically corrected lymphoid progenitors (Touraine *et al.*, 1989, 1992; Flake *et al.*, 1996; Wengler *et al.*, 1996). However, engraftment has not been possible in other hereditary disorders, such as hemoglobinopathies or storage diseases, likely because of immune intolerance and competition by the host bone marrow environment (Flake and Zanjani, 1999). The limitations and risks associated with immunological barriers in acceptance of allogeneic grafts might be circumvented by gene therapy with autologous genetically corrected CB cells transplanted *in utero* (Zanjani and Anderson, 1999).

As a result of the transition of the hematopoietic site from fetal liver to bone marrow during hematologic ontogeny (Tavassoli, 1991), stem cells are present in the fetal circulation during the second and third trimester of pregnancy. The content of hematopoietic progenitors in CB is higher during fetal life than at birth (Clapp *et al.*, 1989; Jones *et al.*, 1994; Thialganathan *et al.*, 1994; Migliaccio *et al.*, 1996; Shields and Andrews, 1998; Surbek *et al.*, 1998) and, furthermore, preterm CB is rich in immature progenitors, defined phenotypically as CD34⁺CD38⁻ cells and functionally as long-term culture-initiating cells (LTC-ICs) (Wyrsh *et al.*, 1999). However, the total number of available fetal cells is low, limited by the volume of CB that can be retrieved by fetal blood sampling. If the number of genetically corrected transplanted cells is insufficient, they may be unable to compete with and eventually replace the defective host hematopoiesis. Experience in transplantation of stem cells from bone marrow or "mobilized" peripheral blood has shown that escalation of the stem cell dose can facilitate and accelerate engraftment (Sykes *et al.*, 1997; Aversa *et al.*, 1998; Reisner and Martelli, 1999). To obtain adequate numbers of cells, expansion of pluripotent hematopoietic progenitors *in vitro* might

prove clinically useful, and suitable culture conditions have been particularly well described for CB cells (Broxmeyer *et al.*, 1992; Moore and Hoskins, 1994; Piacibello *et al.*, 1997). We have shown also that fetal CB progenitors can be extensively expanded *ex vivo* (Wyrsh *et al.*, 1999).

The goal of this work was to establish conditions for gene delivery to immature fetal CB progenitors, using lentiviral vectors for transfer of the green fluorescent protein (GFP) gene. We show that transduction of human CB progenitors from the second and early third trimester of pregnancy is as efficient as that of term CB, and that extensive amplification of transduced fetal CB cells with sustained transgene expression is possible. These findings may prove useful for developing protocols for autologous gene therapy of genetic diseases amenable to prenatal stem cell transplantation.

MATERIALS AND METHODS

Study population

Samples from term healthy newborns ($n = 4$; weeks ≥ 35) were collected after uneventful vaginal births or cesarean sections. Fetal CB included samples from the second ($n = 3$; weeks 24–28) and early third ($n = 6$; weeks 31–34) trimester of pregnancy, collected after abortions or preterm labor. Exclusion criteria included clinically overt chorioamnionitis and preeclampsia. There was no evidence of hematopoietic abnormalities in the fetuses. Informed consent was obtained from the mothers prior to delivery, and the investigations were approved by the Ethics Committee of University Hospital Basel (Basel, Switzerland).

Cord blood cells

CB collection was performed according to Swisscord Commission standard procedure. Shortly after delivery, the umbilical cord was double clamped and dissected. CB was harvested aseptically by umbilical vein puncture, and up to 10 ml was collected in heparin-containing tubes. The delay between collection and sample processing did not exceed 12 hr. CB mononuclear cells were isolated by centrifugation on Histopaque ($d < 1.077$ g/ml; Sigma, St. Louis, MO), and cryopreserved in liquid nitrogen in Iscove's modified Dulbecco's medium (IMDM) containing 20% fetal calf serum (FCS; both from GIBCO, Gaithersburg, MD) and 10% dimethyl sulfoxide (DMSO; Sigma). On thawing, cells were left overnight in IMDM–25% FCS containing DNase (100 IU/ml; Sigma) at 4°C. CD34⁺ cells were isolated with superparamagnetic MACS (magnetic cell sorting) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer instructions. The purity of the CD34⁺ cell population ranged from 85 to 95%.

Growth factors

The following recombinant human growth factors were used: PEGylated megakaryocyte growth and development factor (MGDF; Amgen, Thousand Oaks, CA), Flt-3 ligand (FL; Immunex, Seattle, WA), stem cell factor (SCF; Amgen), interleukin 3 (IL-3), IL-6, granulocyte-macrophage colony-stimu-

lating factor (GM-CSF; all from Novartis, Basel, Switzerland), granulocyte colony-stimulating factor (G-CSF; Rhône-Poulenc, Antony, France), and erythropoietin (Epo; Boehringer Mannheim, Mannheim, Germany). Growth factors were used at a concentration of 100 ng/ml, except for MGDF at 50 ng/ml, IL-3 and IL-6 at 20 ng/ml, and Epo at 36 U/ml.

Transduction of CD34⁺ progenitors with lentiviral vectors

Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviral vectors were produced by cotransfection of human 293T cells with a packaging construct PCWT, a plasmid (pMD.G) encoding protein G of VSV, and a transfer vector containing the enhanced green fluorescent protein (GFP) gene under the control of the phosphoglyceratekinase (PGK) promoter, as described previously (Costello *et al.*, 2000). Control vector, lacking the VSV-G envelope, was generated by omitting the pMD.G plasmid from transfections (Δenv). Viral supernatants were concentrated by ultracentrifugation and titers were determined on HeLa cells. Vector stocks with titers of $2-5 \times 10^7$ IU/ml were used. Vector supernatants were tested for the presence of replication-competent HIV, as described (Costello *et al.*, 2000). No replication-competent virus was detected. To induce cell cycling prior to transduction, purified CD34⁺ cells were prestimulated for 3 days with IL-3, IL-6, SCF, and FL. For transduction, 10^5 prestimulated or nonprestimulated CD34⁺ cells were resuspended in 50 μ l of IMDM with protamine sulfate (5 μ g/ml; Sigma) in V-bottom tubes coated with fibronectin fragment CH296 (Retronectin, 20 μ g/cm²; TaKaRa, Shiga, Japan) or bovine serum albumin (BSA; Behring, Marburg, Germany). Lentivirus-containing supernatant was added (multiplicity of infection [MOI] of 15) and cells were centrifuged at 3000 rpm for 3 hr (spinoculation). Cells were then resuspended in 1 ml of IMDM containing 20% FCS, and incubated for 24 hr at 37°C. In samples prestimulated with IL-3, IL-6, SCF, and FL the same growth factors were present during transduction. Cells were washed twice with IMDM supplemented with 2% FCS. Transduction was repeated by spinoculation with a fresh aliquot of lentiviral supernatant, and incubation for another 24 hr. For cell cycle analysis (see below), transduction was carried out for 48 hr under the conditions described above, in the presence of IL-3, IL-6, SCF, and FL, using nonprestimulated CD34⁺ cells.

Cell culture assays

After the 48-hr transduction period, cells were used to initiate the following cultures.

Colony-forming unit assay. Aliquots of transduced cells from term and fetal CB samples were plated into 1% methylcellulose cultures supplemented with Epo, SCF, IL-3, G-CSF, and GM-CSF (Wodnar-Filipowicz *et al.*, 1992). Hematopoietic colonies were counted after 14 days of culture and GFP-expressing colonies were identified with a fluorescence microscope (Zeiss, Thornwood, NY).

Long-term culture-initiating cell assay. Determination of LTC-ICs was performed as described (Sutherland *et al.*, 1990). The murine fibroblast cell line M210B4 (American Type Cul-

ture Collection, Manassas, VA) was used as a feeder layer after irradiation with 80 Gy and readherence in 96-well plates overnight. Aliquots of transduced cells were seeded in quadruplicate wells over the feeders in 200 μ l of IMDM containing 12.5% horse serum, 12.5% FCS, 0.016 mM folic acid, 0.16 mM *i*-inositol (all from GIBCO), and 10^{-6} M hydrocortisone (Sigma). Cultures were maintained at 33°C for 5 weeks with weekly half-medium changes. At the end of the culture period, nonadherent cells were combined with the corresponding trypsinized adherent cells, washed, and assayed in secondary methylcellulose cultures for CFUs and GFP expression.

Amplification of transduced progenitors

Long-term liquid cultures were carried out as described (Piacibello *et al.*, 1997). After the 48-hr transduction period, 5000–20,000 transduced cells were seeded into 1 ml of LT medium (IMDM containing 10% FCS, iron-saturated human transferrin [380 mg/ml], 1% BSA), and supplemented with MGDF and FL. Half-medium changes were performed weekly. At weeks 1, 3, 6, and 10, harvested cells were counted and analyzed by flow cytometry (fluorescence-activated cell sorting, FACS) for GFP and CD34 expression, and aliquots were plated into secondary methylcellulose cultures to determine CFUs. In addition, at week 10, cells were seeded over M210B4 feeder layers, and LTC-ICs and GFP expression were determined.

FACS analysis of GFP expression in CD34⁺ cells

After the 48-hr transduction period, cells were maintained for 3 days in LT medium containing IL-3, IL-6, SCF, and FL. FACS analysis was performed on day 3 after transduction, thus avoiding GFP expression background due to pseudotransduction. Staining was for 30 min with anti-CD34-phycoerythrin (PE) (HPCA-2; Becton Dickinson, San Jose, CA) or the corresponding isotype control antibodies at concentrations recommended by the manufacturer, in FACS buffer containing phosphate-buffered saline (PBS), 0.5% BSA, and 0.02% sodium azide. Dead cells were stained with propidium iodide (PI) for 10 min at 4°C. Cells were then washed, fixed for 10 minutes with 2% paraformaldehyde (Sigma), and resuspended in FACS buffer. FACS analysis for CD34⁺ cells and the corresponding GFP expression was performed on a FACS Calibur (Becton Dickinson) acquiring no less than 10,000 events. Gates were set according to forward and side scatter, and PI-positive cells were excluded. Analysis was performed with CellQuest software (Becton Dickinson).

Cell cycle analysis

Analysis was performed as described (Jordan *et al.*, 1996) with minor modifications. Freshly isolated or transduced cells were resuspended in 1 ml of FACS buffer containing 0.4% paraformaldehyde. After 30 min at 4°C, 1 ml of FACS buffer with 0.2% Triton X-100 (Sigma) was added, and cells were permeabilized overnight at 4°C, washed twice in FACS buffer, and stained with anti-Ki67-PE (Dako, Glostrup, Denmark) or an isotype control antibody (IgG1-PE) for 60 min at 4°C. After washing, cells were resuspended in FACS buffer containing 7-aminoactinomycin D (7-AAD, 5 μ g/ml; Sigma) and incubated for 3 hr on ice. Whenever possible, 20,000 events were acquired

on a FACSCalibur, using channels FL-1 for GFP, FL-2 for Ki67-PE, and FL-3 for 7-AAD. Analysis was performed with CellQuest software.

Detection of the GFP gene

Single CFUs and LTC-IC-derived colonies were plucked from culture dishes with a micropipette tip and incubated for 1 hr at room temperature in 1 ml PBS. Cells were pelleted and lysed for 1 hr at 56°C in 20 μ l of cell lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, gelatin [0.1 mg/ml], 0.45% Nonidet P-40, and 0.45% Tween 20) supplemented with 3 μ l of proteinase K (10 mg/ml; Sigma). Samples were heated at 95°C for 10 min at 2 μ l of the lysate was used for polymerase chain reaction (PCR) amplification of a 304-bp fragment of the GFP gene, using the sense primer 5'-GCCA-CAAGTTCAGCGTGCC-3', the antisense primer 3'-AGCT-CGATGCGGTTACCCAG-5', and AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ). The PCR was carried out with 40 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. A 438-bp fragment of human β -actin was amplified to confirm the presence of DNA and a control reaction containing water was performed in each experiment. PCR products were separated on 1.2% agarose gels stained with ethidium bromide, and bands were visualized with a Gel Doc 2000 (Bio-Rad, Hercules, CA).

Statistical analysis

The transduction efficiency of fetal and term CB cells was compared by means of the Student unpaired *t* test, using StatView software (Abacus, Berkeley, CA).

RESULTS

Transduction efficiency of CD34⁺ fetal CB cells by lentiviral vectors

Our first goal was to optimize conditions for GFP gene transfer by lentiviral vectors to CD34⁺ progenitors isolated from fe-

tal CB. We tested various gene transfer protocols and investigated the need for cytokine-induced cell cycling. Preliminary experiments revealed that the highest efficiency of GFP gene transfer, as analyzed by FACS on day 3 after transduction, was obtained when cells were exposed to the virus twice at 24-hr intervals and transduced by the spinoculation method in the presence of protamine sulfate; the use of a fibronectin fragment brought no improvement (data not shown). Prestimulation with IL-3, IL-6, SCF, and FL for 3 days prior to exposure to the lentiviral vector significantly increased the transduction efficiency (Table 1, Fig. 1). Using this combination of growth factors, the frequency of GFP-expressing fetal CD34⁺ cells was 42 \pm 9% compared with 16 \pm 2% of cells transduced without cytokine pretreatment, and, accordingly, the total number of GFP⁺CD34⁺ cells was about 4-fold higher. The transduction efficiency of fetal cells was on average higher than that of neonatal CB cells from term deliveries, both with and without cytokine prestimulation (Table 1). These results demonstrate that a transgene can be introduced and expressed in fetal CB using lentiviral vectors.

We analyzed the cell cycle status of fetal CB progenitors, and the efficiency of transduction with lentiviral vectors relative to the cell cycle phases. The distribution of CD34⁺ cells in the subcompartments of the cell cycle was defined by FACS analysis of DNA by 7-AAD staining plotted against expression of the nuclear antigen Ki67 expressed by cycling but not dormant G₀ cells (Jordan *et al.*, 1996). Freshly isolated fetal CD34⁺ cells were almost exclusively in G₀/G₁ phases: 71.5 \pm 3.4% of G₀-phase cells and 22.4 \pm 3.9% of G₁-phase cells were found in CB cells from weeks 24 to 31 of pregnancy, similar to the proportions seen with term CB cells (Fig. 2A). When CD34⁺ cells were exposed to the lentiviral supernatant on two consecutive days in the presence of IL-3, IL-6, SCF, and FL, the vast majority of G₀ cells progressed to G₁ and S/G₂/M phases, while a small cell population remained quiescent (Fig. 2B). The number of cells in S/G₂/M phases was reproducibly higher in fetal than in term CB, indicating that fetal cells responded more readily to cytokine stimulation. The higher proportion of cycling cells may account for the better transduction efficiency in fetal

TABLE 1. EFFICIENCY OF TRANSDUCTION OF FETAL AND TERM CB CD34⁺ PROGENITOR CELLS, AND OF CFU- AND LTC-IC-DERIVED COLONIES

	Pre stimulation ^f	GFP ⁺ CD34 ⁺ cells ^a		GFP ⁺ CFUs ^b		PCR ⁺ / GFP ⁻ CFUs ^e	GFP ⁺ LTC-ICs ^b		PCR ⁺ /GFP ⁻ LTC-ICs ^e
		%	Total ^d	%	Total ^d		%	Total ^d	
Fetal CB ^f	+	42 \pm 9*	49,772 \pm 168	23 \pm 4	4,061 \pm 282	0/10	11 \pm 1	226 \pm 65	1/10
	-	16 \pm 2**	11,232 \pm 56	10 \pm 1	1,184 \pm 235	0/9	9 \pm 4	120 \pm 54	2/9
Term CB	+	24 \pm 3*	32,032 \pm 200	23 \pm 1	4,039 \pm 479	0/9	11 \pm 4	185 \pm 45	0/9
	-	5 \pm 2**	4,792 \pm 112	9 \pm 4	1,010 \pm 65	0/10	16 \pm 5	138 \pm 32	4/10

^aThe frequency of GFP⁺CD34⁺ cells was analyzed by FACS 3 days after the 48-hr transduction period. Significance of the difference between fetal and term CB samples: **p* < 0.04; ***p* < 0.01.

^bCFU and LTC-IC cultures were initiated immediately after the 48-hr transduction period. GFP⁺ colonies were detected by fluorescence microscopy.

^cCB CD34⁺ cells were transduced either after prestimulation with IL-3, IL-6, SCF, and FL for 3 days (+) or without prestimulation (-).

^dCalculated per 1 \times 10⁵ CD34⁺ cells subjected to prestimulation, if any, and transduction.

^ePCR for the detection of the GFP gene was performed with DNA isolated from single CFU- or LTC-IC-derived colonies scored GFP⁻ by fluorescence microscopy.

^fFetal CB cells were from weeks 26, 31, 32, and 34 of gestation.

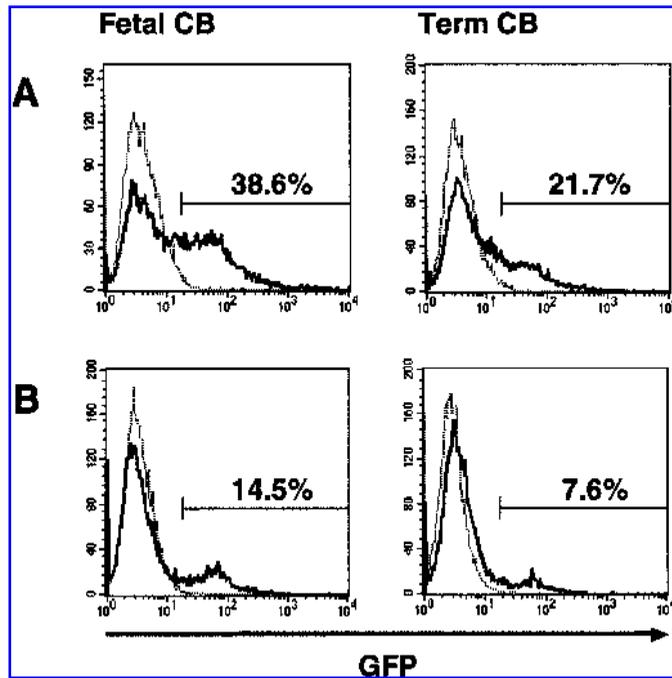


FIG. 1. GFP expression by fetal CB progenitor cells transduced with lentiviral vectors. CD34⁺ cells from fetal (week 31) and term CB were transduced either after prestimulation for 3 days with IL-3, IL-6, SCF, and FL (A), or without prestimulation (B), as described in Materials and Methods, and GFP expression was analyzed by FACS (solid line). Cells transduced with a Δenv vector were used as a control (dotted line). The percentage of GFP⁺ cells is indicated.

than in term CD34⁺ cells observed after 3 days of culture (Table 1). GFP expression was found in all phases of the cell cycle: 7.1% of G₀, 12.1% of G₁, and 16.2% of S/G₂/M fetal CB cells were GFP⁺ (Fig. 2B). GFP expression in cells in the G₀ phase suggests that resting progenitors were transduced by lentiviral vectors. The proportion of GFP-expressing cells was higher in fetal than in term CB samples in every cell cycle phase.

Transduction of fetal CB-derived CFUs and LTC-ICs by lentiviral vectors

To determine the ability of lentiviral vectors to transduce colony-forming CB progenitors, CD34⁺ cells were exposed to the vector, as described above, and GFP expression was analyzed in colonies derived from committed CFU and primitive LTC-IC precursors. The transduction procedure did not impair colony growth, in comparison with nontransduced cells, in either type of culture, and no GFP expression was detected in cultures initiated with mock-transduced (Δenv) cells (not shown). The frequency of GFP⁺ colonies formed by fetal and neonatal progenitors, as determined by fluorescence microscopy, was similar despite the superior transduction efficiency of fetal cells seen at an earlier time point with flow cytometry. On average, 23% of fetal CFU-derived colonies expressed GFP when transduction was preceded by 3 days of prestimulation with IL-3, IL-6, SCF, and FL (Table 1). This was about 2-fold higher than the frequency of GFP⁺ colonies derived from nonprestimated cells, and also the total number of transduced colonies was higher, confirming the effect of cytokines observed by FACS analysis of CD34⁺ cells on day 3 after transduction. The average frequency of GFP-expressing

colonies scored in the LTC-IC assay was 11% (Table 1). This frequency, and the total amount of transduced LTC-ICs, was not markedly influenced by cytokine pretreatment, suggesting that progenitors detected in the assay represent cells remaining quiescent during transduction, in agreement with a significant enrichment in LTC-ICs in the G₀ compared with the G₁ compartment (Gothot *et al.*, 1998). Examples of GFP⁺ progeny of LTC-IC precursors from fetal CB are shown in Fig. 3. Notably, highly variable intensity of GFP expression was observed in cells belonging to the same colony defined as GFP positive by fluorescence microscopy, suggesting transgene silencing in some transduced cells. To investigate further the possibility of a "shutdown" of transgene expression during the culture period, we used PCR to assess the presence of the GFP gene in DNA from colonies scored as GFP negative by fluorescence microscopy. While none of the tested CFU-derived GFP-negative colonies contained the GFP-specific sequences, these were detected in 3 of 19 (16%) and 4 of 19 (21%) LTC-IC derived colonies from fetal and neonatal CB, respectively (Table 1). The presence of GFP sequences in the absence of GFP expression suggests that the frequency of transduced LTC-IC precursors was higher than estimated by fluorescence microscopy, and that expression of integrated transgene was downregulated during the prolonged culture period.

Expansion of GFP⁺ fetal CB progenitors transduced by lentiviral vectors

We have previously shown that precursors from fetal CB can be extensively expanded *ex vivo* in cultures containing MGDF and FL (Wyrsh *et al.*, 1999). To test the expansion capacity

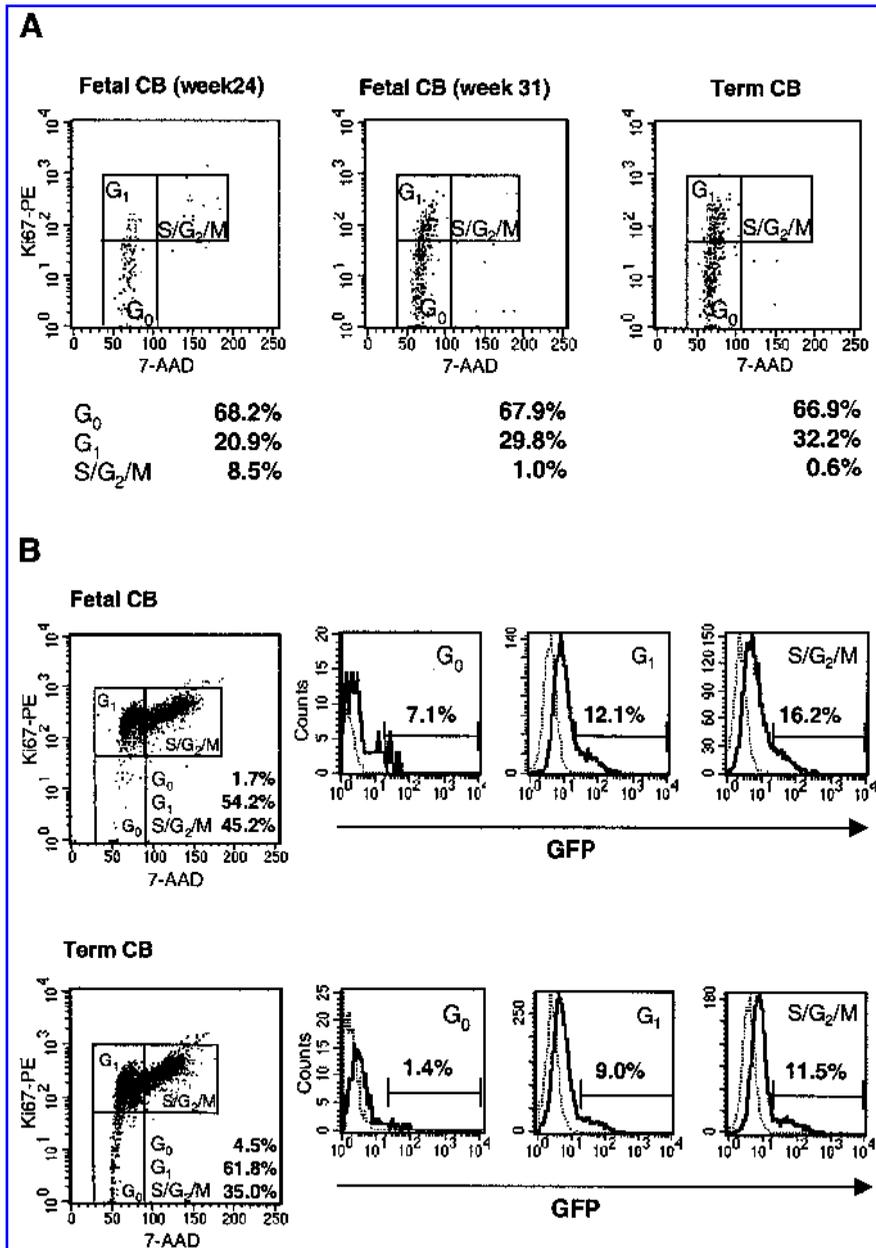


FIG. 2. Relationship between cell cycle status and transduction efficiency of fetal CB progenitors. **(A)** Cell cycle analysis of fetal and term CD34⁺ cells. CD34⁺ cells were stained with Ki67-PE and 7-AAD and analyzed as described in Materials and Methods. Gates were set according to the staining with IgG1-PE. Examples of CB from weeks 24 and 31 of gestation, and from a term pregnancy, are shown. The percentage of cells in the G₀, G₁, and S/G₂/M phases of the cell cycle is indicated beneath each dot plot. **(B)** Cell cycle status and efficiency of transduction with lentiviral vectors. Fetal (week 31) and term CD34⁺ cells were transduced for 48 hr (see Materials and Methods) and stained with Ki67-PE and 7-AAD as described above, and three-color FACS analysis was performed. The percentage of cells in the G₀, G₁, and S/G₂/M phases of the cell cycle and corresponding GFP expression are indicated.

of transduced CD34⁺ cells, analogous long-term liquid cultures were initiated after exposure to lentiviral vector. In agreement with our earlier results, fetal and neonatal CB CD34⁺ cells grew with the same kinetics (not shown) and an amplification of CD34⁺ cells and CFUs of about 1000-fold was observed after 10 weeks (Fig. 4A). At this time point, the content of CD34⁺ cells in liquid cultures was $4.1 \pm 2.0\%$, of which $2.9 \pm 1.4\%$ were CD34⁺CD38⁻ (not shown). Pretreatment with IL-3, IL-

6, SCF, and FL for 3 days prior to exposure to lentivirus had no influence on the total output of the progenitors in long-term cultures with MGDF and FL (Fig. 4A). However, this treatment had a negative effect on long-term maintenance of GFP expression by both fetal and term CB cells (Fig. 4B). An initial advantage of prestimulation with cytokines for the transduction efficiency (see also Fig. 1 and Table 1) was observed only until week 3, when about 45% of fetal CFUs were GFP⁺. There-

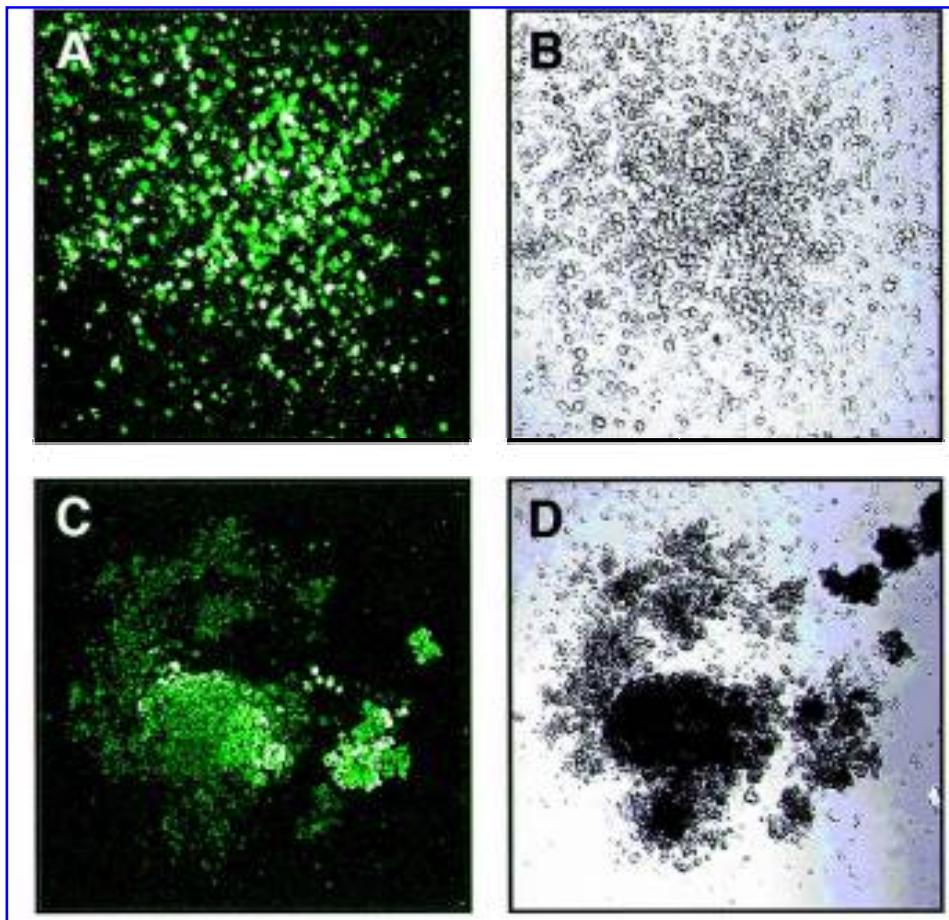


FIG. 3. GFP expression by LTC-IC-derived colonies from fetal CB progenitors, assessed by fluorescence microscopy. LTC-IC cultures with transduced cells were initiated on M210B4 fibroblasts as described in Materials and Methods. After 5 weeks, cells were plated into methylcellulose cultures, and the resulting colonies were assessed after 14 days with a fluorescence (A and C) and an inverted light (B and D) microscope. Colonies derived from a granulocyte-macrophage (GM-CFU; A and B) and a granulocyte-macrophage erythroid megakaryocyte (GEMM-CFU; C and D) precursor from fetal CB (week 32) are shown.

after, the content of GFP⁺ colonies declined rapidly to $1.7 \pm 1.0\%$ by week 10. In contrast, an initially lower frequency of GFP⁺ colonies in cultures generated from cells transduced without cytokine pretreatment gradually increased and GFP expression was detected in $40.5 \pm 11.7\%$ of CFUs present after 10 weeks of expansion. The maintenance of transgene expression in fetal cell cultures was similar to that of term CB progenitors. Expression of GFP was multilineage, observed in both myeloid and erythroid colonies formed by transduced fetal clonogenic progenitors. In addition, after expansion over 10 weeks, we could also obtain differentiation of the transduced progenitors to dendritic cells and natural killer cells under appropriate culture conditions (not shown).

To confirm the maintenance of GFP⁺ cells during expansion, and to evaluate the intensity of GFP expression in transduced fetal cells, flow cytometry was used (Fig. 5). The GFP⁺ population was maintained in cultures not preexposed to growth factors, whereas it was progressively lost in cultures of pre-stimulated cells, in agreement with the outcome in clonogenic GFP⁺ cells (see Fig. 4B). Interestingly, in both growth factor-prestimulated and nonprestimulated cells, the intensity of GFP

fluorescence decreased as a function of time (Fig. 5A and B). Reduction in signal intensity was evident at week 3, and the fluorescence became dull by week 10, despite the presence of a well-pronounced population of GFP⁺ cells in nonprestimulated cultures (Fig. 5A). A similar decline in GFP fluorescence intensity was also seen in cultures of term CB (not shown). These results speak for a gradual downregulation of transgene expression during the prolonged culture period, as suggested before by detection of integrated GFP sequences in LTC-IC-derived colonies without detectable transgene expression (see Table 1).

To explore further the maintenance of GFP expression in transduced progenitors, we analyzed LTC-ICs after 10 weeks of expansion in cultures with MGDF and FL. Approximately 50,000 to 110,000 LTC-ICs derived from 10^5 fetal CB CD34⁺ cells were measured (not shown), representing a 40- to 60-fold amplification of LTC-ICs during expansion. However, none of the analyzed colonies displayed GFP expression detectable by fluorescence microscopy, consistent with a downregulation of transgene expression during the prolonged culture period. This was further confirmed by the presence of integrated GFP-spe-

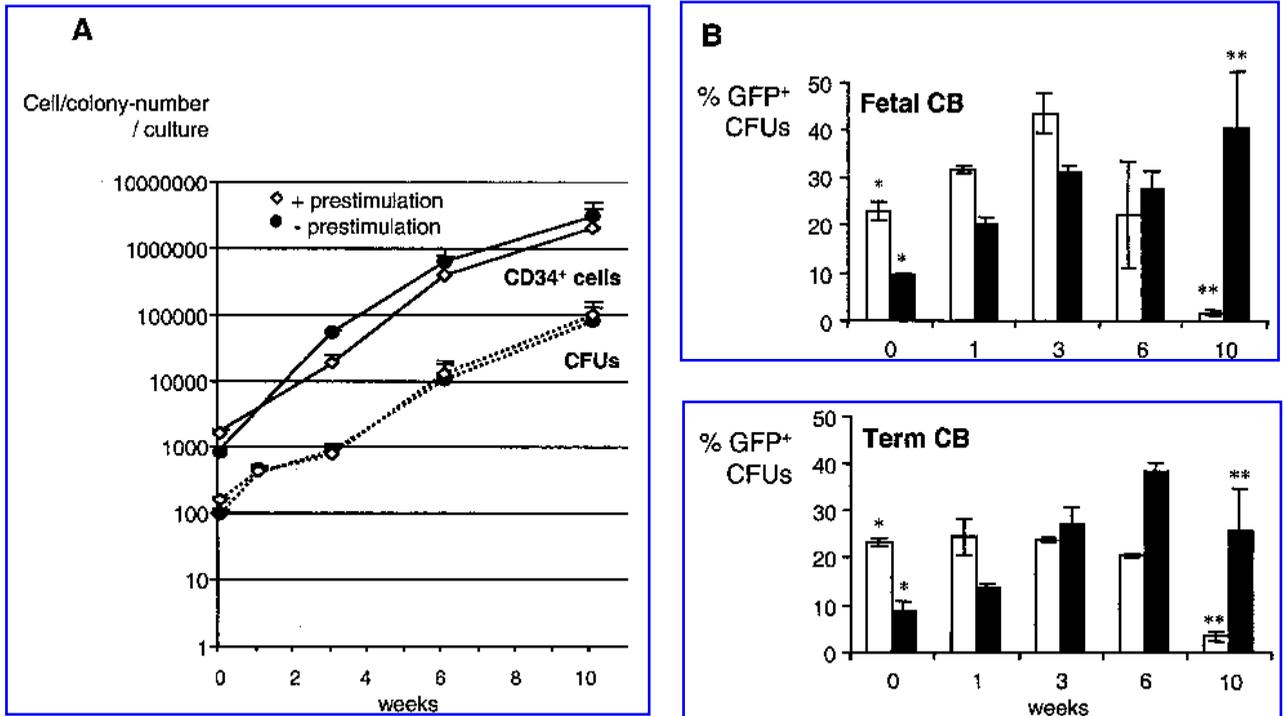


FIG. 4. Long-term expansion of fetal CB progenitors transduced with lentiviral vectors. CD34⁺ cells, either prestimulated for 3 days with IL-3, IL-6, SCF, and FL or nonprestimulated, as indicated, were transduced with lentiviral vectors. Subsequently, cells were cultured in the presence of MGDF and FL for 10 weeks. Aliquots of cells were analyzed by FACS for CD34 and GFP expression and assayed for CFUs at weeks 0, 1, 3, 6, and 10. **(A)** Amplification of CD34⁺ cells (solid line) and CFUs (dotted line) in cultures with MGDF and FL. The total output of CD34⁺ cells and CFUs + SEM per culture initiated with 5000 cells is shown. **(B)** GFP expression by CFUs derived from fetal and term CB progenitors cultured in the presence of MGDF and FL. Shown is the percentage of GFP⁺ CFUs derived from cells transduced after 3 days of prestimulation (open columns) or without prestimulation (solid columns). The significance of the differences between prestimulated and nonprestimulated cells was * $p < 0.04$ in favor of prestimulated cells at week 0, and ** $p < 0.01$ in favor of nonprestimulated cells at week 10, for both fetal and term CB.

cific sequences found by PCR in DNA isolated from 7 of 20 (35%) and 4 of 10 (40%) fetal and neonatal CB LTC-IC-derived colonies, respectively (Fig. 6). The frequency of PCR⁺/GFP⁻ LTC-IC colonies was not influenced by growth factor stimulation prior to transduction. The presence of the GFP gene in colonies formed after 10 weeks of expansion and the additional 7 weeks needed to enumerate LTC-ICs provide evidence that the transgene had been initially transferred to immature progenitors with a high potential for self-renewal. This is in accordance with the results of cell cycle analysis that demonstrated GFP expression in resting G₀-phase cells.

DISCUSSION

Many genetic diseases of the lymphohematopoietic system can now be diagnosed early in gestation and technologies of noninvasive sampling and reliable molecular screening are being developed. Prenatal diagnosis is the basis for *in utero* gene therapy with autologous genetically corrected hematopoietic stem cells (Surbek *et al.*, 1999). If sufficient cells could be collected by cordocentesis during pregnancy, genetically modified *ex vivo* and transplanted *in utero*, clinical manifestations of the disease could be prevented before birth (Flake and Zanjani,

1999; Schneider and Coutelle, 1999; Zanjani and Anderson, 1999). We have demonstrated that fetal CB obtained from human fetuses in the second and early third trimester of pregnancy is rich in hematopoietic precursors with high self-renewal capacity (Wyrsh *et al.*, 1999). Here, we describe the use of a lentivirus-based gene transfer vector for efficient transduction of human CD34⁺ progenitor cells from CB of early gestational stages with the GFP marker gene.

The results demonstrated that 42 ± 9% of CD34⁺ from CB at weeks 24–34 of pregnancy were transduced by the lentiviral vector. This efficiency was higher than that of CB cells from full-term pregnancies, perhaps as a result of the higher proliferative capacity of fetal cells, also reflected in the formation of larger colonies and higher replating potential, compared with term CB cells (Migliaccio *et al.*, 1996; Shields and Andrews, 1998). Analysis of the dependence of transgene expression on cell cycle activity showed that freshly isolated fetal CD34⁺ cells were largely residing in G₀ phase and, on cytokine stimulation, rapidly entered G₁ phase. GFP expression was found in a small proportion of residual dormant G₀-phase cells, and the bulk expression was evenly distributed among cells that progressed to G₁ phase and actively dividing S/G₂/M-phase cells. These findings are in accordance with the properties of lentiviral vectors, which have been shown to transduce both quiescent and pro-

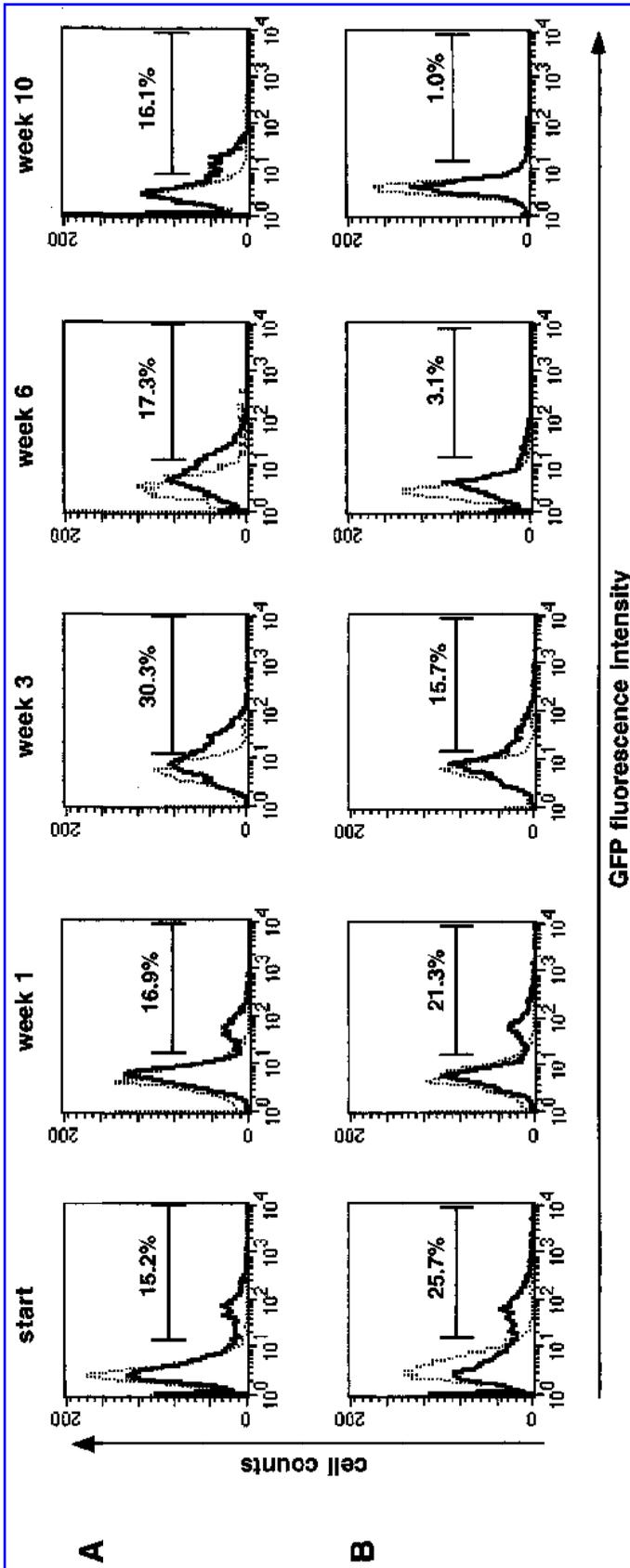


FIG. 5. Time-dependent changes in the content of GFP⁺ cells and intensity of GFP expression by fetal CB progenitor cells in long-term cultures. Fetal CD34⁺ cells were transduced without prestimulation (A) or after 3 days of stimulation with IL-3, IL-6, SCF, and FL (B) and subsequently cultured in the presence of MGDF and FL (see legend to Fig. 4). GFP expression was analyzed by FACS at the indicated time points. Transduced cells (solid line); nontransduced cells (dotted lines). The percentage of GFP⁺ cells is indicated.

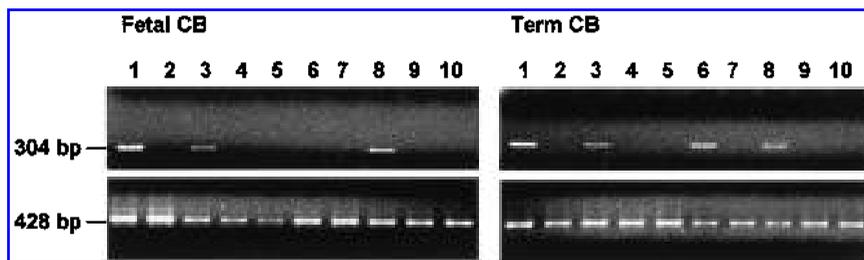


FIG. 6. PCR analysis of GFP gene integration in DNA of LTC-IC-derived colonies from fetal and term progenitors after 10 weeks of culture in MGDF and FL. Colonies from cultures of cells transduced without prestimulation and scored negative for GFP expression with the fluorescence microscope were plucked from the culture dishes. Cells were lysed, and aliquots were subjected to PCR amplification of a fragment of the GFP gene (304 bp) and of the human β -actin gene (438 bp). Results from 10 fetal and term CB-derived colonies are shown.

liferating hematopoietic progenitors (Uchida *et al.*, 1998; Case *et al.*, 1999), however, at greater efficiency once the cells exit G_0 and enter G_1 phase (Sutton *et al.*, 1999). Transduction rates were higher after preculture with IL-3, IL-6, SCF, and FL, as also previously reported for term CB progenitors (Evans *et al.*, 1999; Sutton *et al.*, 1999). Likewise, we have previously reported that activation of resting T cells with CD3 and CD28 antibodies, resulting in cell cycling prior to transduction with lentiviral vectors, increased gene transfer efficiency (Costello *et al.*, 2000). Cell stimulation may have a positive effect on transduction by raising pools of nuclear deoxynucleoside triphosphates for reverse transcription of viral RNA (Sutton *et al.*, 1999). When applying identical transduction conditions to fetal and term CD34⁺ cells, the number of fetal cells remaining in G_0 phase was reproducibly lower, indicating that they responded more readily to cytokine stimulation. This effect may contribute to the initially higher transduction efficiency of fetal CB cells. In colony assays, GFP expression was observed in about 23% of CFU progeny and 11% of more primitive LTC-IC-derived colonies and was equal for both fetal and term CB cells.

Interestingly, long-term maintenance of transgene expression was clearly superior when freshly isolated CD34⁺ cells were subjected to transduction directly, omitting the cytokine prestimulation step. While efficiency of GFP gene transfer was initially increased 2- to 3-fold by preculture with IL-3, IL-6, SCF, and FL, induction of cell cycling prior to transduction had a negative effect on GFP expression by cells maintained in long-term cultures. Only $1.7 \pm 1.0\%$ of GFP-expressing cells was present after 10 weeks of culture in MGDF and FL. In contrast, when the cytokine stimulation step prior to transduction was omitted, the proportion of fetal GFP⁺ CFUs increased gradually to $40.5 \pm 11.7\%$. A proportional increase in the content of transgene-expressing cells has also been reported on culturing of cell cycle-arrested fibroblasts transduced with lentiviral vectors (Naldini *et al.*, 1996). In cultures of hematopoietic cells, an increase in transgene expression may reflect changes in the cell population during long-term expansion; indeed, an increasing contribution by more primitive CD34⁺CD38⁻ cells under these culture conditions was described (Piacibello *et al.*, 1997).

In long-term cultures, GFP expression was subject to gradual downregulation, as evidenced by a progressive loss of fluorescence intensity by a GFP⁺ cell population, irrespective of

the conditions of transduction and further propagation of cells. In agreement, the frequency of colonies containing GFP-specific sequences integrated into cellular DNA but lacking GFP expression increased over time. After 10 weeks of liquid culture with MDGF and FL, and a further 7 weeks on stroma support, 35% of transduced LTC-ICs in fetal CB contained the transgene, but expression of GFP was undetectable by fluorescence microscopy. It is conceivable that loss of GFP expression without the loss of the expression construct was due to transgene silencing during the prolonged culture. Gradual downregulation of expression of a transferred gene in the case of retroviral delivery vectors is a phenomenon recognized as a threat to the efficacy of gene therapy (Bestor, 2000; Trono, 2000). Although the precise mechanism accounting for gene shutoff in hematopoietic cells is not understood, *de novo* methylation is likely to play a role in the transcriptional silencing of transduced genes (Bestor, 2000). Activity of CpG methyltransferases is upregulated in response to nitric oxide, the production of which is known to increase in response to hematopoietic growth factors (Punjabi *et al.*, 1992; Hmadcha *et al.*, 1999). Silencing may particularly strongly affect transgenes under the control of CpG-rich promoters, such as the human PGK promoter used in this study (Pfeifer *et al.*, 1990). Therefore, choice of promoters (Fahlmann *et al.*, 1999) or inclusion of insulator regions preventing spread of methylation (Pikaart *et al.*, 1998; Rivella *et al.*, 2000) is an important consideration in designing vectors for gene transfer.

Clinical experience with *in utero* stem cell transplantation showed that failure of engraftment was not only due to immune-mediated rejection of allogeneic grafts, but also to a lack of selective growth advantage for normal relative to defective cells in the bone marrow of the recipient (Flake and Zanjani, 1999). This latter obstacle is also likely to restrain repopulation with autologous genetically corrected cells. Because myeloablative treatment to create space in the bone marrow is not feasible in the fetus, an alternative is to increase the number of transplanted cells to enhance engraftment. This work demonstrated that an extensive amplification of transduced fetal CD34⁺ clonogenic cells capable of giving rise to multiple hematopoietic lineages expressing GFP is possible. Although the functional characterization of fetal cells in all available *in vitro* assays, including current results on susceptibility to lentiviral transduction, revealed a growth behavior equal to that of progenitors from term CB, the repopulation ability of lentiviral vector-transduced and

expanded human fetal CB needs to be confirmed *in vivo* in the NOD/SCID mouse. Importantly, under conditions analogous to those used here for expansion of transduced fetal CB progenitors, efficient engraftment has been reported with term CB cells expanded for up to 10 weeks in long-term cultures containing MGDF and FL (Piacibello *et al.*, 1999). Also, other growth factor combinations allowed maintenance or moderate expansion of repopulating cells over shorter time periods (Conneally *et al.*, 1997; Dorrell *et al.*, 2000; Ueda *et al.*, 2000).

Lentiviral vectors are capable of efficient and stable transduction of primitive progenitors from bone marrow, "mobilized" peripheral blood, and CB (Sutton *et al.*, 1998, 1999; Uchida *et al.*, 1998; Case *et al.*, 1999; Evans *et al.*, 1999; Miyoshi *et al.*, 1999). Direct comparison between the transduction efficiency of lentivirus- and oncoretrovirus-derived vectors provided evidence that lentiviral vectors are superior for gene delivery to primitive quiescent hematopoietic cells (Uchida *et al.*, 1998; Case *et al.*, 1999; Evans *et al.*, 1999). Since in lentiviral vector transduction, the transgene can integrate without growth factor stimulation, depletion of marrow-repopulating cells can be minimized and stem cells capable of long-term expression of the therapeutic gene can be preserved. The important issue of optimizing the biological safety of this system prior to clinical application has led to the design of vectors that exclude the possibility of recombination of infective viral particles (Zufferey *et al.*, 1997, 1998; Buchschacher and Wong-Staal, 2000). We have found that lentiviral vectors, in which most accessory HIV genes have been deleted and self-inactivating mutations have been introduced (a gift of D. Trono, Geneva University, Switzerland), thus being designed for a high degree of biosafety (Zufferey *et al.*, 1998), can transduce fetal CB progenitors with the same efficiency as the vectors in this study (A. Luther-Wyrsh, unpublished results).

While postnatal gene therapy is already possible for some diseases (Kohn *et al.*, 1998; Cavazzana-Calvo *et al.*, 1999), therapy with autologous CB stem cells harvested during pregnancy and genetically modified *ex vivo* has not yet been attempted. Although fetal gene therapy first needs to be established experimentally in animal models, medical and ethical aspects of this novel treatment option in humans are currently under consideration (Zanjani and Anderson, 1999). Risks and benefits to the mother and child will also determine the choice of optimal time for gene therapy *in utero*. One study has documented circulating progenitor cells already in the first trimester of non-continuing pregnancies (Campagnoli *et al.*, 2000), but early fetal CB sampling is inefficient and may pose a significant risk to the fetus (Orlandi *et al.*, 1990; Wyrsh *et al.*, 1999). Since autologous transplantations do not depend on the permissive environment of the immunologically immature fetus, gene therapy later in pregnancy seems more appropriate. The results of our experiments show that CB progenitors obtained in the second and early third trimester can be targeted for gene transfer by lentiviral vectors and may, therefore, prove useful for autologous gene therapy of genetic diseases amenable to prenatal stem cell transplantation.

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