

# High Efficiency Retroviral Mediated Gene Transduction into Single Isolated Immature and Replatable CD34<sup>3+</sup> Hematopoietic Stem/Progenitor Cells from Human Umbilical Cord Blood

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## Summary

Umbilical cord blood is rich in hematopoietic stem and progenitor cells and has recently been used successfully in the clinic as an alternative source of engrafting and marrow repopulating cells. With the likelihood that cord blood stem/progenitor cells will be used for gene therapy to correct genetic disorders, we evaluated if a TK-*neo* gene could be directly transduced in a stable manner into single isolated subsets of purified immature hematopoietic cells that demonstrate self-renewed ability as estimated by colony replating capacity. Sorted CD34<sup>3+</sup> cells from cord blood were prestimulated with erythropoietin (Epo), steel factor (SLF), interleukin (IL)-3, and granulocyte-macrophage colony stimulating factor (GM-CSF) and transduced with the gene in two ways. CD34<sup>3+</sup> cells were incubated with retroviral-containing supernatant from TK-*neo* vector-producing cells, washed, and plated directly or resorted as CD34<sup>3+</sup> cells into single wells containing a single cell or 10 cells. Alternatively, CD34<sup>3+</sup> cells were sorted as a single cell/well and then incubated with viral supernatant. These cells were cultured with Epo, SLF, IL-3, and GM-CSF ± G418. The TK-*neo* gene was introduced at very high efficiency into low numbers of or isolated single purified CD34<sup>3+</sup> immature hematopoietic cells without stromal cells as a source of virus or accessory cells. Proviral integration was detected in primary G418-resistant<sup>(R)</sup> colonies derived from single immature hematopoietic cells, and in cells from replated colonies derived from G418<sup>R</sup>-colony forming unit-granulocyte erythroid macrophage megakaryocyte (CFU-GEMM) and -high proliferative potential colony forming cells (HPP-CFC). This demonstrates stable expression of the transduced gene into single purified stem/progenitor cells with replating capacity, results that should be applicable for future clinical studies that may utilize selected subsets of stem/progenitor cells for gene therapy.

Foreign genes have been introduced into mammalian cells (1, 2), and gene therapy has been evaluated as a possible option in patients for treatment of certain inherited diseases (3, 4). Retroviral-vectors have been used most often to stably transduce genes into cells, and targets for these genes have included hematopoietic stem/progenitor cells (5–20), lymphocytes (21), and tumor cells (22). Hematopoietic stem and progenitor cells are found in low frequency in blood forming tissue, but it is these rare cells that present a viable target for gene therapy. In humans, although antigenic determinants specific for stem and progenitor cells have not yet been identified, these cells contain CD34 antigens and this antigenic determinant has been used successfully as a marker to highly enrich stem and progenitor cells in human adult bone marrow (23–25), blood (20), and umbilical cord blood (25).

Cord blood, which contains hematopoietic stem/progenitor cells at a frequency equal to or greater than that of adult bone marrow (26–29) and has a high quality for proliferation (26–29) and self-renewal (25, 30, 31) has shown promise clinically, as an alternative source of human transplantable and marrow repopulating cells (32–36). Recently, it has been demonstrated that cord blood progenitors and long-term culture initiating cells present in a relatively unseparated population of cells are more efficiently transduced by retroviral-mediated gene transfer than are these cells in adult bone marrow (37). For logistic reasons, including the amount of viral supernatant available for gene transduction, purified populations of human stem, and progenitor cells will most likely serve increasingly as targets for future clinical gene therapy procedures. In this context, cord blood is an interesting choice for this source

of cells especially for autologous transplantation for in utero-diagnosed genetic disorders. For these reasons we chose to evaluate the efficiency of retroviral-mediated gene transduction into highly enriched populations of human cord blood stem and progenitor cells. Future insights will no doubt allow for antigenic characterization of distinct subsets of stem and progenitor cells differing in capacity for self-renewal, proliferation, and lineage-specific differentiation that could potentially be used for selective transplantation. Since the growth of isolated single cell-sorted human stem/progenitor cells is possible (25, 38, 39), we also evaluated the capacity to transduce genes into single isolated CD34<sup>3+</sup> cord blood cells with extensive replating capacity (a measure of self-renewal). We show that populations of CD34<sup>3+</sup> cord blood high proliferative potential colony forming cells (HPP-CFC)<sup>1</sup>, and multipotential CFU-granulocyte erythroid macrophage megakaryocyte (GEMM), burst forming unit-erythroid (BFU-E), and CFU-granulocyte-macrophage (GM) progenitor cells can be retrovirally transduced with a TK-*neo* gene at very high efficiency. This is accomplished also at the single isolated CD34<sup>3+</sup> cell level, with the gene stably integrated into cells with high replating capacity.

## Materials and Methods

**Cells and Cell Separation.** Cells were obtained from normal human umbilical cord blood scheduled for discard after delivery of the infant and after prior need for samples for clinical study had been satisfied. CD34<sup>3+</sup> cells were obtained after sorting nonadherent low density T lymphocyte depleted (NALT<sup>-</sup>) cells on a Dual Laser Flow Cytometer (Epics 753; Coulter Corp., Hialeah, FL) (25). This population was >98% pure for cells expressing the CD34 antigen. CD34<sup>3+</sup> cells included the 20% of CD34 antigen expressing cells with the highest density distribution of CD34 antigens. This fraction is richest in stem/progenitor cells (23, 25). In some experiments, CD34<sup>3+</sup> cells were sorted or resorted using an auto-clone device (Coulter Corp.) into single wells, as a single cell or as 10 cells, containing 0.1 ml semisolid or liquid culture medium (25, 39).

**Colony Assay and Replating Experiments.** Cultures contained IMDM (GIBCO BRL, Gaithersburg, MD), 1% methylcellulose, 30% FCS (Hyclone Laboratories, Inc., Logan, UT), 0.1 mM hemin (Eastman Kodak Co., Rochester, NY) and recombinant human (rhu) erythropoietin (Epo) (Amgen Corp., Thousand Oaks, CA), rhu steel factor (SLF), rhu IL-3, and rhu-GM-CSF (gifts from Immunex Corporation, Seattle, WA). Colonies were scored after incubation at 37°C, lowered (5%) O<sub>2</sub>, and 5% CO<sub>2</sub> for 14 or 21 d. Replating experiments were performed after removing and dispensing a single colony into the same type of semisolid medium and growth factors as the primary culture (25). Results are given as the mean or mean ± 1 SEM.

**Retroviral Vector.** The N<sub>2</sub>/ZipTKNEO (TKNEO) vector used in these studies has been described previously (37). Neo phosphotransferase sequences are expressed in the sense orientation (rel-

ative to the 5' long terminal repeat-LTR) via the herpes simplex virus thymidine kinase promoter. GP+EnvAM 12-TKNEO producer cells containing the TKNEO retroviral plasmid were cultured in IMDM containing 10% FCS. Viral-containing supernatant was collected by adding 10 ml of IMDM plus 20% FCS to confluent plates overnight. Harvested medium was filtered through 0.45 μm filters and stored at -80°C until use. The TKNEO virus was titered at 1 × 10<sup>5</sup> G418-resistant CFU/ml on NIH 3T3 cells.

**Retroviral Transduction Protocol.** Two protocols were used in these studies. In the first protocol, NALT<sup>-</sup> or CD34<sup>3+</sup> cord blood cells (<5 × 10<sup>5</sup> cells/ml) were prestimulated with Epo (1 U/ml), SLF (50 ng/ml), IL-3 (200 U/ml), and GM-CSF (200 U/ml) at 37°C, 5% CO<sub>2</sub>, and 20% O<sub>2</sub> for 40 h. 2 ml viral supernatant and 8 μg/ml polybrene were added to cells 4× at 2-h intervals. Cells were washed twice and assayed for colony formation or resorted into single wells containing either a single cell or 10 cells in the presence of 1 U/ml Epo, 50 ng/ml SLF, 200 U/ml IL-3, and 200 U/ml GM-CSF ± 1.5 mg/ml or 12 μg/ml (dry weight) G418. In the second protocol, single CD34<sup>3+</sup> cord blood cells were sorted into single wells containing 0.1 ml methylcellulose culture in the presence of cytokines as described above (25). 3 or 4 d later, viral supernatant was added only once at 20 μl/well with polybrene (8 μg/ml). G418 was added at 1.5 mg/ml or 12 μg/ml 40 h after addition of viral supernatant.

To assay the temperature sensitivity of the virus preparation, 1000 NIH 3T3 cells were plated in 35-mm tissue culture dishes. Viral supernatant was added for 2.5 h with 8 μg/ml polybrene, cells were washed, and fresh medium added with 1.5 mg/ml (dry weight) G418.

**Polymerase Chain Reaction (PCR) Analysis.** Genomic DNA was isolated from individual colonies as described by others (40) with modification. Individual colonies were removed from primary or secondary methylcellulose culture or cells were removed after suspension culture and washed with 1 ml of PBS. Cell pellets were resuspended in the small volume of remaining PBS to which was added 200 μl of a chelex 100 solution (41). Cells were lysed by boiling for 5 min, chilled on ice for 5 min, and pelleted for 30 s at 2,800 g. 10 μl of supernatant from the lysate was used for PCR. As a positive control, DNA was obtained from cells incubated with viral supernatant that contained the TKNEO gene. As a negative control, DNA was obtained from cells incubated with supernatant not containing the TKNEO gene (mock control). Two 27-bp oligonucleotides were used at 20 pmol in the PCR reaction. The PCR generated a 792-bp fragment of the TKNEO gene beginning with the 5' oligo-[27-bp]5'CAAGATGGATTGCACGCAGGTTCTCCG and ending with the 3' oligo-[27-bp]5'CCAGAGTCCCGCTCAG-AAGAACTCGTC. Each sample was amplified for 30 cycles (94°C for 60 s to denature the DNA, 60°C for 2 min for primer annealing, and 72°C for 3 min for primer extension) as described (17). 20 μl of reaction mixture was electrophoresed on a 1% agarose gel and this was photographed after ethidium bromide staining. Electrophoresed DNA was then transferred to Gene Screen Plus (Dupont-NEN, Boston, MA) and hybridized with [<sup>32</sup>P]dCTP-labeled neo fragment. Hybridization was performed overnight at 42°C (17). Filters were washed with 0.1% SDS, 0.1× SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 55°C, dried, and exposed to Kodak XAR film at -80°C.

## Results

**Gene Transduction into Early Hematopoietic Cells as Evaluated by G418<sup>R</sup>-Colonies.** Before evaluating the transducing efficiency of TK-*neo* gene into purified populations of cord

<sup>1</sup> Abbreviations used in this paper: BFU-E, burst forming unit erythroid; Epo, erythropoietin; GEMM, granulocyte erythroid macrophage megakaryocyte; HPP-CFC, high proliferative potential colony forming cells; NALT<sup>-</sup>, nonadherent low density T lymphocyte depleted; rhu, recombinant human; SLF, steel factor; TKNEO, N<sub>2</sub>/ZipTKNEO.

**Table 1.** Colony Formation by G418<sup>R</sup>-NALT<sup>-</sup> or -CD34<sup>3+</sup> Cord Blood Cells\*

Cells infected with:	Target cell population	Plating cell No.	No. experiments	Total No. G418 <sup>R</sup> -colonies evaluated	Percent G418 <sup>R</sup> -colonies				
					Total	HPP-CFC	CFU-GEMM	BFU-E	CFU-GM
TK- <i>neo</i> gene	NALT <sup>-</sup>	5 × 10 <sup>4</sup> /ml	4	2849	25 ± 6 (0.5 ± 0.3) <sup>‡</sup>	0	18 ± 11	25 ± 8	50 ± 10
TK- <i>neo</i> gene	CD34 <sup>3+</sup>	250/ml	6	2557	24 ± 7 (0.5 ± 0.5)	2 ± 1	23 ± 8	20 ± 6	53 ± 11
TK- <i>neo</i> gene	CD34 <sup>3+</sup>	10/well	2	129	39 ± 19 (0.3)	8 ± 2	52 ± 16	27 ± 17	53 ± 21
TK- <i>neo</i> gene	CD34 <sup>3+</sup>	1/well	2	103	49 ± 6 (0.1)	0.3 ± 0.3	45 ± 5	25 ± 15	85 ± 5

\* Retroviral gene transfer was performed at <10<sup>6</sup> cells/ml. 2 ml of viral superantant with 8 µg/ml polybrene was added for transduction as described in Materials and Methods. Sorted CD<sup>3+</sup> cells were transduced and either plated at 250 cells/ml, or subjected to a second sort in which 10 or 1 CD34<sup>3+</sup> cells were sorted into a single well. Cells were assayed for colony formation in the presence of Epo, SLF, IL-3, and GM-CSF ± G418 at 1.5 mg/ml (dry weight).

<sup>‡</sup> The numbers in parentheses designate background growth of colonies from mock-infected cells growing in G418 and are based respectively on results of three, three, one, and one experiment with 5 × 10<sup>4</sup> NALT<sup>-</sup>, 250 CD34<sup>3+</sup>, 10 CD34<sup>3+</sup> and 1 CD34<sup>3+</sup> cell(s).

blood progenitor cells, progenitors present in a NALT<sup>-</sup> population of cells were used as targets. As shown in Table 1, 25% of total progenitor cell-derived colonies were G418<sup>R</sup>. Over 50 G418<sup>R</sup> colonies were separately removed and each one was found by PCR analysis to contain the TK-*neo* gene. Similar studies were performed using populations of very highly enriched progenitor cells present in CD34<sup>3+</sup> populations of sorted cells in which at least 50–75% of the cells are CFU-GEMM, BFU-E, CFU-GM, and HPP-CFC (23, 25). Incubation of CD34<sup>3+</sup> cells with the TK-*neo* gene, followed by washing and plating at 250 cells/ml resulted in a TK-*neo* gene transduction efficiency similar to that noted for the NALT<sup>-</sup> population of cells (Table 1). Interestingly, the apparent efficiency of gene transduction was even greater when the CD34<sup>3+</sup> cells that were incubated with viral supernatant and then washed were resorted as 10 or 1 CD34<sup>3+</sup> cell(s)/well (Table 1). With one CD34<sup>3+</sup> cell/well, a 49%

transduction efficiency was noted for G418<sup>R</sup> colonies with 45, 25, and 85% transduction apparent, respectively, for CFU-GEMM-, BFU-E-, and CFU-GM-derived colonies. Confirmation of the TK-*neo* gene in 96% of these G418<sup>R</sup> colonies (129 positive from 135 evaluated colonies) was obtained by PCR analysis of the cells from individual colonies. A breakdown of the different colony types showed that 98% (60/61) CFU-GEMM-, 89% (24/27) BFU-E-, and 97% (30/31) CFU-GM-G418<sup>R</sup> colonies contained the TK-*neo* gene. High efficiency transduction was also observed in cells growing in suspension from single sorted cells (8 of 8 wells positive by PCR). As seen in Table 1, only a small percentage of G418<sup>R</sup>-HPP-CFC derived colonies were observed; 7 of 7 G418<sup>R</sup>-HPP-CFC colonies were documented by PCR analysis to contain the TK-*neo* gene.

*Gene Transduction into Early Hematopoietic Cells as Evaluated by Plating Cells in the Absence of G418.* To evaluate the

**Table 2.** Detection of Neo Gene by PCR Analysis of Primary Colonies Growing from CD34<sup>3+</sup> Cells in the Absence of G418

Cells analyzed from:	Total No. experiments	Percent HPP-CFC- and CFU-GEMM-colonies or cells positive for Neo gene		
		Total	HPP-CFC	CFU-GEMM
Primary colonies of cells infected with TK- <i>neo</i> and plated at 250 cells/ml	4	83 (38/46)*	83 (29/35)	82 (9/11)
10 cells/well	3	65 (13/20)	67 (6/9)	64 (7/11)
1 cell/well	3	45 (9/20)	75 (6/8)	25 (3/12)
Suspension cultures produce from 1 cell/well	1	50 (4/8 wells)		
Primary colonies from cells cultured with mock supernatant and plated as 1 cell/well (background)	2	0 (0/8)	0 (0/5)	0 (0/3)

\* Numbers in parentheses represent the number of PCR positive colonies/total number of colonies evaluated.

possibility that the transduction of the TK-*neo* gene was greater than that observed in Table 1, especially for HPP-CFC derived colonies, CD34<sup>3+</sup> cord blood cells were incubated with TK-*neo* genes containing viral supernatant, washed, and plated as 250 cells/ml or resorted as 10 or 1 CD34<sup>3+</sup> cell(s)/well in the absence of G418. Colonies were then assessed for transduction of the TK-*neo* gene by PCR analysis. As noted in Table 2, HPP-CFC- and CFU-GEMM-colonies were transduced at high efficiencies, respectively, of 67–83% and 25–82%. Additionally four of eight wells seeded with a single CD34<sup>3+</sup> cell/well generated cells in suspension culture that contained the TK-*neo* gene. None of eight colonies derived from mock-infected CD34<sup>3+</sup> cells were found positive by PCR analysis for the TK-*neo* gene.

**Gene Transduction at the Single Isolated CD34<sup>3+</sup> Progenitor Cell Level.** The experiments shown in Table 1 and 2 in which CD34<sup>3+</sup> cells were first incubated with viral supernatant containing the TK-*neo* gene and then resorted as a single CD34<sup>3+</sup> cell/well demonstrated that progenitor cells transduced with the TK-*neo* gene could proliferate and differentiate in the presence of growth stimulating cytokines but in the absence of other progenitor or accessory cells at the start of the culture. We additionally wished to determine if a single isolated CD34<sup>3+</sup> cell could be transduced with the TK-*neo* gene. CD34<sup>3+</sup> cells were sorted as a single cell/well and viral supernatant containing the TK-*neo* gene added as described in the Materials and Methods with the subsequent addition to these single cell cultures of either 1.5 mg/ml or 12 µg/ml G418 (Table 3). Based on the findings that HPP-CFC in the absence of G418 could be transduced with the TK-*neo* gene at high efficiency (Table 2), but few HPP-CFC-G418<sup>R</sup> colo-

nies formed in the presence of 1.5 mg/ml G418 (Table 1), we had evaluated the sensitivity of HPP-CFC to inhibition by G418. This had not been previously done. HPP-CFC were found to be ultrasensitive to inhibition by G418 such that as little as 12 µg/ml G418 was sufficient to greatly reduce colony formation by mock-infected HPP-CFC, while this concentration of G418 had little or no effect on CFU-GEMM, BFU-E, or CFU-GM. At 1.5 mg/ml G418, 4.2, 72.0, 82.0, and 97%, respectively, of HPP-CFC, CFU-GEMM, BFU-E, and CFU-GM formed colonies (Table 3 A). 26 of 27 of these G418<sup>R</sup>-colonies were positive by PCR analysis for the TK-*neo* gene (Table 3 B, Fig. 1). At 12 µg/ml G418, 85% of the HPP-CFC survived to form colonies (Table 3 A) and 11 of 12 of these colonies were positive to PCR analysis for the TK-*neo* gene (Table 3 B, Fig. 1). Also, as shown in Table 3 B, suspension cultures derived from single progenitors incubated with virus supernatant containing the TK-*neo* gene and grown in the presence of 1.5 mg/ml G418 were positive in 6 of 6 wells for the TK-*neo* gene by PCR analysis.

Since we found that it was not possible to wash single cells after sorting the cells into single wells without losing the cells, the single sorted cells were not washed free of the viral supernatant containing the TK-*neo* gene. Other means were thus used to evaluate whether the virus added to the single cells was transducing the TK-*neo* gene into the original sorted progenitor cell, or whether the possibility existed that immediate or subsequent daughter cells were being infected after the progenitor or daughter cells had divided. To this end, we first evaluated the transducing stability of the viral supernatant containing the TK-*neo* gene at 37°C (incubation temperature). Viral supernatant was incubated at 37°C for 6, 24,

**Table 3.** G418<sup>R</sup>-Colonies Derived from Single CD34<sup>3+</sup> Cord Blood Cells Transduced with Neo Gene at the Single Cell Level and Neo Gene Detection by PCR Analysis of Individual Primary G418<sup>R</sup>-Colonies\*

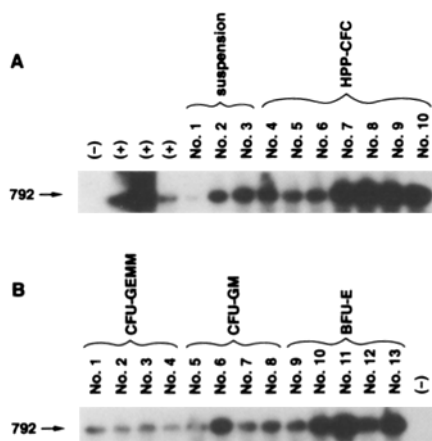
A Primary colonies or cells growing with G418 at concentrations of:	Percent G418 <sup>R</sup> -colonies				
	Total	HPP-CFC	CFU-GEMM	CFU-GM	BFU-E
1.5 mg/ml	58 (85/147) <sup>†</sup>	4.2 (2/48)	72 (26/36)	97 (35/36)	81 (22/27)
1.2 µg/ml	80 (117/147)	85 (41/48)	69 (25/36)	86 (31/36)	74 (20/27)
B Cells analyzed from	Percent colonies positive for Neo gene by PCR analysis				
Primary colonies growing with G418 at 1.5 mg/ml	96 [26/27] <sup>§</sup>	ND <sup>  </sup>	100 [9/9]	100 [9/9]	89 [8/9]
Primary colonies growing with G418 at 12 µg/ml	92 [11/12]	92 [11/12]	ND	ND	ND
Suspension cultures with G418 at 1.5 mg/ml	100 [6/6]				

\* Single CD34<sup>3+</sup> cells were sorted into single wells in either methylcellulose culture or suspension culture with Epo, SLF, IL-3, and GM-CSF and incubated for 3 or 4 d. Viral supernatant was then added as 20 µl/well with polybrene. G418 was added at either 1.5 mg/ml or 12 µg/ml 24 h after gene transduction. Neo gene was detected by PCR analysis of G418<sup>R</sup> colonies cells.

<sup>†</sup> The numbers in parentheses designate the number of colonies growing in G418 per total number of colonies growing without G418.

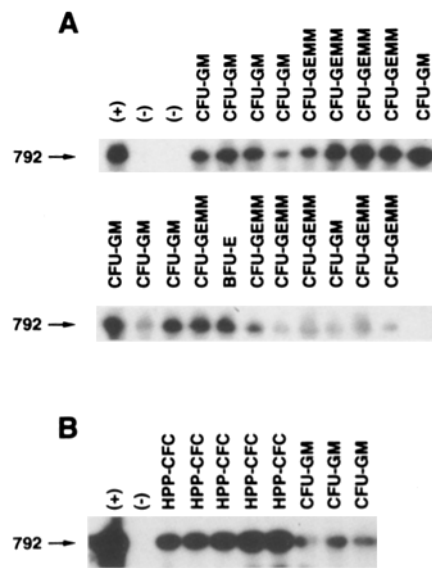
<sup>§</sup> The numbers in brackets designate the number of colonies positive for the new gene per total number of colonies evaluated.

<sup>||</sup> ND, not done.



**Figure 1.** Neo gene expression determined by PCR analysis of individual colonies derived from single CD34<sup>+</sup> cord blood cells transduced with TK-*neo* gene directly at the level of 1 cell/well. (A) Cells that grew in suspension culture with G418 at 1.5 mg/ml and primary HPP-CFC colonies growing with G418 at 12  $\mu$ g/ml; (B) Primary colonies from CFU-GEMM, CFU-GM, and BFU-E derived from CD34<sup>+</sup> cells growing in presence of G418 at 1.5 mg/ml. Products of a standard amplification reaction of 30 cycles separated by electrophoresis on a 1% agarose gel, transferred to Gene Screen Plus, and hybridized with <sup>32</sup>P-labeled neo fragment formed 1 band of 792 bp. (+) Indicates DNA extracted from NIH 3T3 cells infected with TK-*neo* gene as a positive control. (-) Indicates DNA extracted from NIH 3T3 cells without TK-*neo* infection as a negative mock control.

and 48 h and these treated viral samples were compared to viruses not subjected to 37°C for their capacity to transduce NIH 3T3 cells as assessed by the growth of G418<sup>R</sup> (1.5 mg/ml)-NIH 3T3 colony forming cells. Virus incubated for 6, 24, and 48 h at 37°C, respectively, produced 50, 0, and 0 G418<sup>R</sup> colonies compared with the >150 colonies formed from NIH 3T3 cells incubated with nonheat-treated virus. Thus, the transducing capacity of the virus was completely lost between 6 and 24 h incubation of the virus at 37°C. Examination of CD34<sup>+</sup> cells sorted as a single cell/well demonstrated that at a time that was equivalent to 24 h after



**Figure 2.** (A) Neo gene expression determined by PCR analysis of cells in secondary colonies derived from G418<sup>R</sup>-CFU-GEMM. Primary CFU-GEMM colonies growing from single CD34<sup>+</sup> cells with G418 at 1.5 mg/ml and were replated into secondary cultures without G418. (B) Neo gene expression determined by PCR analysis of secondary colonies derived from G418<sup>R</sup> HPP-CFC. Primary HPP-CFC-colonies growing from a single CD34<sup>+</sup> cell with G418 at 12  $\mu$ g/ml were replated into secondary cultures without G418. Individual secondary colonies were picked for PCR analysis. (+) Indicates DNA extracted from NIH 3T3 cells infected with TK-*neo* gene as a positive control. (-) Indicates DNA extracted from NIH 3T3 cells without TK-*neo* infection as a negative mock control.

the addition of virus to the single cells, >97% of the wells still contained only single cells. The remainder of the wells contained doublets. Together, the above information suggests that in >97% of the cases, the TK-*neo* gene was likely transduced into a single isolated CD34<sup>+</sup> progenitor cell.

**Stable Gene Transduction into Replatable HPP-CFC and CFU-GEMM.** We have previously demonstrated that colonies derived from single isolated CD34<sup>+</sup> cord blood HPP-CFC/well have extensive replating capacity (25) and that cord blood

**Table 4.** Neo Gene Detection by PCR Analysis of Secondary Colonies Deriving from Primary G418<sup>R</sup>-HPP-CFC and -CFU-GEMM Colonies\*

No. of secondary colonies from:	Total No. experiments	No. 2 <sup>o</sup> colonies evaluated	Number of secondary colonies with Neo gene integrated				
			Total	HPP-CFC	CFU-GM	BFU-E	CFU-GEMM
HPP-CFC growing with G418 at 12 $\mu$ g/ml	1	8	8	5	3	NA <sup>†</sup>	NA
CFU-GEMM growing with G418 at 1.5 mg/ml	2	30	30	NA	13	5	12

\* CD34<sup>+</sup> cells transduced with the Neo gene were sorted at 1 cell/well in the presence of the above concentrations of G418. G418<sup>R</sup>-HPP-CFC and -CFU-GEMM colonies were replated into 2<sup>o</sup> dishes in the absence of G418 and the resultant 2<sup>o</sup> colonies were analyzed for the Neo gene. <sup>†</sup> NA, not applicable because HPP-CFC colonies did not give rise to BFU-E or CFU-GEMM colonies and CFU-GEMM colonies did not give rise to HPP-CFC colonies when replated into 2<sup>o</sup> dishes.

CFU-GEMM can be replated with high efficiency (30, 31). Single colonies in primary plates derived from single CD34<sup>3+</sup> HPP-CFC/well and incubated with viral supernatant containing the TK-*neo* gene and cultured with 12 µg/ml G418, and single colonies from single CD34<sup>3+</sup> CFU-GEMM/well incubated with viral supernatant containing the TK-*neo* gene and cultured with 1.5 mg/ml G418 were replated into secondary culture plates. 50 primary HPP-CFC-colonies were individually placed into secondary dishes with a replating efficiency of 62%. Secondary plates contained HPP-CFC-colonies, which were at least as large as the primary colonies from which they were derived, containing tens of thousands of cells, and also CFU-GM colonies. 98 primary CFU-GEMM colonies were individually placed into secondary dishes with a replating efficiency of 69%. Secondary plates included CFU-GEMM-, BFU-E-, and CFU-GM-colonies. Results in Table 4 and Fig. 2 demonstrated that the TK-*neo* gene was detected in 100% of the secondary colonies demonstrating stable integration into subsets of stem/progenitor cells that have replating capacity.

## Discussion

Gene therapy may be an option for the treatment of certain genetic disorders (3, 4) and in this context retroviral containing vectors have been used to transduce genes into hematopoietic stem and progenitor cells (1, 2, 5–20, 37). Cord blood stem/progenitor cells have been used to clinically transplant and correct a number of blood disorders (32–36), and this source of cells may be especially useful for gene therapy in an autologous situation for newborns and young children. The use of cord blood cells for such purposes is highlighted by the enhanced frequency and quality of immature stem/progenitor cells in cord blood compared to adult bone marrow (25–31), and the more efficient transduction of progenitors and long-term culture initiating cells in relatively unseparated cord blood by retroviral-mediated gene transfer (37). It is our feeling that the future of gene therapy in the setting of stem/progenitor cells will not only entail the use of highly enriched fractions of these cells, but also, as technology for characterizing and isolating subsets of these cells based on their self-renewal, proliferative, and lineage differentiation capacities becomes available, it will be these isolated and distinct subsets that will be used as target cells for this purpose. Currently, antigenic profiles available for human stem-progenitor cells only allow for very small degrees of separation of subsets of these cell populations (23, 25).

In the present study, we have not only demonstrated a very high degree of efficiency of stable gene transduction into very highly purified stem/progenitor cells from cord blood, but

we have shown this capability at the level of a single isolated CD34<sup>3+</sup> cell. This opens up the possibility in the future that as different subtypes of stem/progenitors become recognizable antigenically, single such cells can be efficiently transduced with genes. That the genes were put into immature cells is suggested by the fact that the cells containing the genes were stimulated to proliferate by multiple growth factors that are necessary to induce early subsets of cells to grow (25, 39, 42), and by the extensive replating capacity of the transduced HPP-CFC and CFU-GEMM. The high efficiency of gene transduction may relate to the combination of potent growth factors used in the pretreatment phase, and also the use of few cells per volume of vector-containing supernatant. An especially high transduction efficiency was apparent when single CD34<sup>3+</sup> cells were used. Interestingly, we had to use no G418, or low amounts of G418 to demonstrate the high rate of gene transduction of HPP-CFC, due to the hypersensitivity of HPP-CFC to inhibition by G418. Our studies demonstrate that high efficiency gene transduction is feasible in the absence of vector-producing stromal cells, an extremely important consideration if one wishes to use the transduced cells for transplantation without worry of potential graft vs. host reactions that may occur from infusion into patients of contaminating vector-producing stromal cells. Also, our studies demonstrate high efficiency gene transduction in the complete absence of accessory cells as rigorously determined by the single cell studies.

Engraftment and repopulation of the hematopoietic system appears to require marrow repopulating cells as well as more mature short-term engrafting cells. Unfortunately, there is no assay yet available that definitively characterizes human marrow repopulating cells. Stem as well as progenitor cell populations entail a hierarchy of cells within each category from more immature to more mature. A human cell that has the expected characteristics of early subsets of stem cells has been identified (43) but whether this human cell population contains long-term marrow repopulating cells remains to be determined. The cells assessed in this current report are not considered to be long-term marrow repopulating cells, but HPP-CFC and CFU-GEMM can be considered to be at least subsets of more mature stem cells based on the extensive replating capacity of the primary colonies derived from these cells (25, 30, 31). From bone marrow transplantation studies using CD34<sup>3+</sup> cells (34) it is likely that our population of sorted CD34<sup>3+</sup> cells contain long-term marrow engrafting cells. It remains to be determined, as soon as an appropriate assay is available, whether the human marrow repopulating cells are also transducible with genes at the efficiency noted in our study for HPP-CFC, CFU-GEMM, BFU-E, and CFU-GM.

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