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A GFP reporter system to assess gene transfer and expression in human hematopoietic progenitor cells

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Hematopoietic stem cells are widely recognized as attractive targets for gene therapy but current protocols to transduce these cells using recombinant retroviral vectors are inefficient. To evaluate optimization of retroviral transduction of hematopoietic stem cells and stability of gene expression in their progeny, the green fluorescent protein (GFP) was explored as a reporter. We first improved sensitivity of detection >100-fold over that achieved previously by using a novel retroviral vector (termed MGIN) expressing a high level of an enhanced GFP gene. Primitive human hematopoietic cells bearing the CD34 surface antigen and lacking lineage differentiation markers (CD34$^+$Lin$^-$) were transduced with the MGIN vector using a clinically applicable supernatant procedure. Under the conditions employed, >75% of the target cells retained the CD34$^+$Lin$^-$ primitive phenotype after 4–5 days in culture; of those ⩾25% expressed a high level of GFP detectable by both flow cytometric analysis and fluorescence microscopy. When transduced cells were cultured in clonogenic progenitor assays, GFP fluorescence was readily detected in situ, indicating that GFP expression was stable and not detrimental to the differentiative potential of the transduced CD34$^+$Lin$^-$ cells. We conclude that GFP is effective as a vital marker to quantify retrovirus-mediated gene transfer into human hematopoietic and perhaps other types of stem/progenitor cells, and monitor gene expression during their subsequent cell lineage determinations.

Keywords: *gene therapy; gene transfer; gene expression; retroviral vectors; GFP; hematopoietic stem cells*

Introduction

Hematopoietic stem and progenitor cells (HSPC) provide an attractive target for gene therapy because they have the potential to continue producing progeny cells containing a therapeutic gene indefinitely. Hematological diseases potentially benefiting from HSPC-based gene therapy approaches include hereditary hemoglobinopathies, immune deficiencies and disorders of phagocytic cells, as well as other diseases such as acquired immunodeficiency syndrome and cancer.[1] Retroviral vectors, which are being used in the majority of current clinical trials, are a primary choice as the vehicle for gene delivery since they are capable of integrating into cellular chromosomes, resulting in stable transmission to every progeny cell derived from transduced HSPC.[2–4] It has become clear, however, that current protocols for transducing human HSPC with retroviral vectors are inefficient.[5,6] Although transgenes in engrafting cells have been detected using sensitive assays such as polymerase chain reaction (PCR), they have rarely been found in long-term repopulating cells.[7] These results are in striking contrast to the efficient transduction of murine HSPC achieved under laboratory conditions.[8]

In addition to inefficient gene transfer into human HSPC, the second problem associated with retroviral vector-based gene therapy is low levels of sustained gene expression. Retroviral vectors derived from Moloney murine leukemia virus (MoMLV) are the most commonly used retroviral vectors in clinical trials. In a standard configuration, the gene of interest is placed under the transcriptional control of the viral long terminal repeat (LTR) since gene expression driven by LTR is generally higher than by an internal promoter.[9,10] However, it has been reported that MLV LTR-mediated gene expression is frequently down-regulated during differentiation of HSPC.[11,12] Because the LTR of the murine stem cell virus (MSCV) retroviral vector is permissive for expression in murine HSPC,[8,13] we were interested in examining the performance of the MSCV vector in HSPC as well as their differentiated progeny. With a view toward optimizing retrovirus-mediated gene transfer and expression in CD34$^+$ cell populations, we decided to investigate the utility of a reporter gene which encodes a product that can be readily monitored as HSPC progress along their developmental pathway.

The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* has emerged as an important reporter molecule for non-invasively monitoring gene expression and protein localization within cells.[14–16] Unlike other bioluminescent reporters, the chromophore in GFP is intrinsic to the primary structure of the protein, and GFP fluorescence does not require substrates or co-factors. We and others have demonstrated that the first generation of improved GFP molecules (such as S65T and RSGFP4

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variants which have fluorescein-like fluorescence spectra and brighter chromophores than the wild-type GFP) are functional in a variety of cultured mammalian cells.[16–19] GFP signals are readily detectable in many transiently transfected cells which harbor hundreds of copies of the transgene. Although GFP signals can frequently be detected in selected cell lines and transformed cells which contain a few copies of integrated retroviral vectors expressing S65T or RSGFP4,[17,18] green fluorescence from these GFP variants in transduced primary cells is not always bright enough above background levels of cellular autofluorescence to be useful. Recently, an enhanced GFP (EGFP) gene has been developed based on the mut1GFP fluorescein-like variant (F64L/S65T) which contains an additional mutation (F64L) and is 35-fold brighter than the wild-type GFP.[20,21] To obtain higher levels of GFP product in mammalian cells, the mut1GFP coding region was re-engineered by using codons preferentially found in highly expressed primate proteins in addition to incorporating a Kozak sequence (the consensus translation initiation site).[21] It has been shown that these combinatorial improvements make EGFP >17-fold brighter than the commonly used S65T GFP variant containing a Kozak sequence.[21] We report the development of a reporter system using EGFP for the analysis of conditions leading to optimal retrovirus-mediated gene transfer into human primitive hematopoietic progenitors.

Results

Testing novel GFP-encoding retroviral vectors

We first constructed a derivative of MSCV containing the EGFP variant, MPGN2, by replacing the RSGFP4 gene in the MGPN retroviral vector described previously[17] (Figure 1). Because LTR-mediated expression is partially inhibited by adenovirus E1A gene products in human 293 embryonic kidney cells,[22] as a stringent test to determine whether EGFP is superior to RSGFP4 in the context of the MSCV vector backbone, 293-based BOSC23 cells were transiently transfected with MGPN2 and MGPN vector plasmid DNA. Twenty hours after transfection, the cells were harvested and green fluorescence in live cells was analyzed by a fluorescence-activated cell sorter (FACS). As shown in Figure 2a, approximately 12% of the cells transiently transfected with the MGPN vector displayed green fluorescence slightly above the background fluorescence observed in mock-transfected cells, consistent with our previously published results.[17] By comparison, a fluorescent signal could be readily detected in approximately 60% of the MGPN2-transfected cells, which was at least five-fold brighter on average than that detected in the MGPN-transfected population (Figure 2b). MGPN and MGPN2 also contain a bacterial neomycin phosphotransferase (*neo*) gene under the control of an internal murine PGK-1 promoter for selection of stably transduced cells by growth in G418-supplemented medium (Figure 1). Although pilot studies evaluating the potential of MSCV-based retroviral vectors with this configuration for exogenous gene expression in human HSPC have been encouraging,[12,23] there was reason to believe that removal of the PGK-1 promoter may have a beneficial effect on long-term MSCV LTR-directed gene expression.[24,25] We therefore constructed a second retroviral vector, MGIN, in which an internal ribosome entry site (IRES) was used to coexpress the *neo* gene with the EGFP gene on a bicistronic transcript emanating from the MSCV LTR (Figure 1).[26,27] As can be seen in Figure 2c, while approximately 60% of BOSC23 cells transiently transfected with either MGIN or MGPN2 plasmid DNA displayed green fluorescence, MGIN yielded >10-fold brighter mean fluorescence intensity relative to MGPN2. The phenomenon that GFP expression was increased >10-fold by the MGIN vector was also observed in a variety of cells either by transfection (Figure 2) or retroviral transduction (see below in Figures 3 and 4).

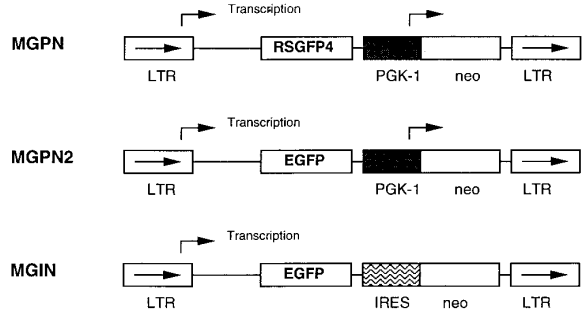

Figure 1 *Schematic representation of GFP-encoding retroviral vectors. Murine stem cell virus (MSCV) with a selectable neo gene driven by an internal promoter (from the murine PGK-1 gene) was used as the parental vector backbone. MGPN containing the RSGFP4 gene has been described previously.[17] MGPN2 is analogous to MGPN except that it contains the EGFP variant instead of RSGFP4. In the MGIN vector, the PGK-1 promoter was replaced by an internal ribosome entry site (IRES), permitting translation of the neo coding region from the same LTR-directed RNA transcript as the EGFP gene. Initiation and direction of transcription are indicated by arrows.*

Transduction of a human CD34$^+$ hematopoietic cell line

To transduce primary human hematopoietic cells more efficiently, we used a new packaging cell line ProPak-A to produce viral supernatants of the three vectors.[28] A CD34$^+$, cytokine-dependent cell line TF1 was first used to evaluate these amphotropic viral stocks.[29] As shown in Figure 3a, GFP fluorescence directed by the MGPN vector was only slightly above the high level of TF1 background cellular autofluorescence. However, over half of the TF1 cells transduced with the MGPN2 vector displayed a medium to high level of fluorescence intensity (Figure 3b and b′). Moreover, a similar percentage of MGIN-transduced TF1 cells displayed an even higher level of GFP fluorescence (Figure 3c and c′). The high level of GFP fluorescence achieved with MGIN was nearly off the scale so that it was necessary to lower the detection sensitivity in order to resolve the fluorescent signals proportionally at the high end (Figure 3b′ and c′). When this was done, it was possible to estimate that the GFP signal intensity mediated by the MGIN vector was more than 10-fold and 100-fold greater, respectively, than that obtained with the MGPN2 and MGPN vectors. GFP expression in MGIN-transduced TF1 cells was stable since GFP-expressing TF1 cells (which were selected either by resistance to G418 or by FACS for GFP fluorescence) continued expressing EGFP at a high level for more than 2 months in the absence of G418 selection. In addition, these GFP-expressing TF1 cells were indistinguishable from parental TF1 cells with respect to cell growth kinetics and responsiveness to various cytokines,

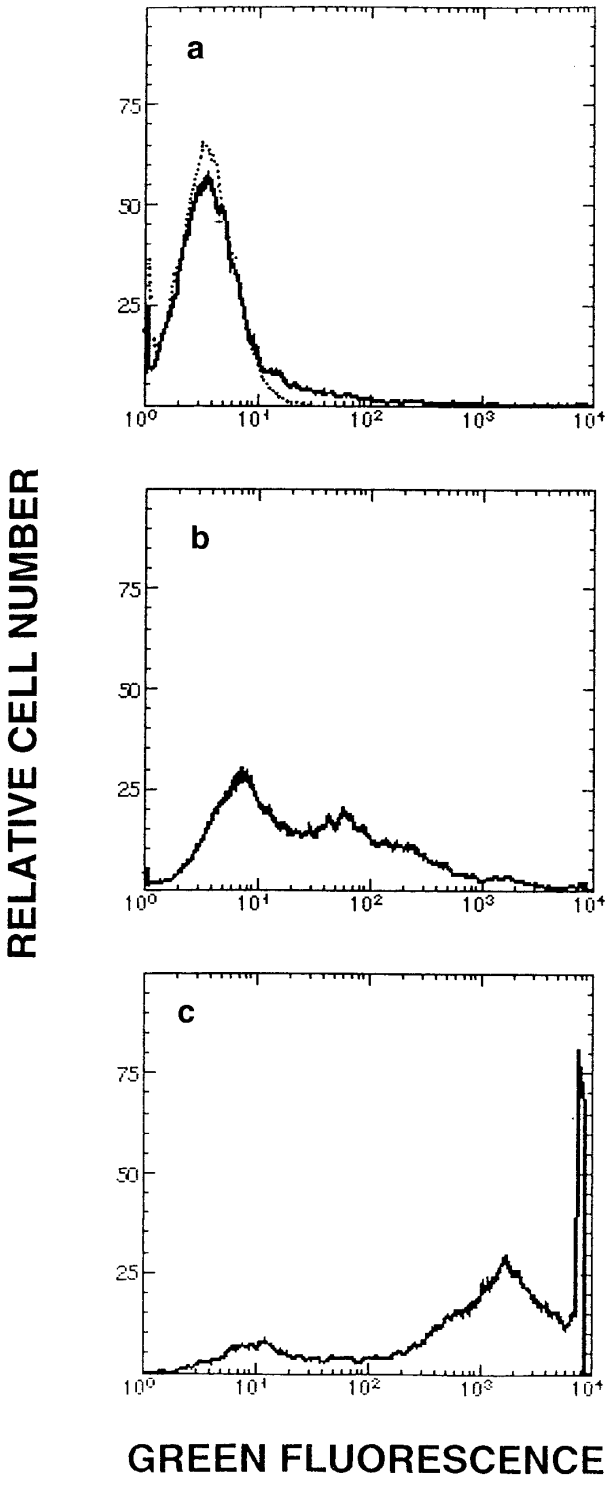

Figure 2 *FACS analysis of GFP expression in transfected human fibroblasts. Plasmid DNAs of the retroviral vector MGPN containing the RSGFP4 gene (a), MGPN2 (b) and MGIN (c) which contain the EGFP variant, were used to transfect BOSC23 cells. Twenty hours after transfection, adherent cells were harvested and green fluorescence in transfected cells was analyzed by a flow cytometer. The relative numbers of viable cells were then plotted as a function of intensity of green fluorescence from individual cells. The profile of mock-transfected cells (dotted line) is also overlaid in (a) for comparison. Approximately 12% (a), 60% (b) and 58% (c) of cells displayed green fluorescence over background levels.*

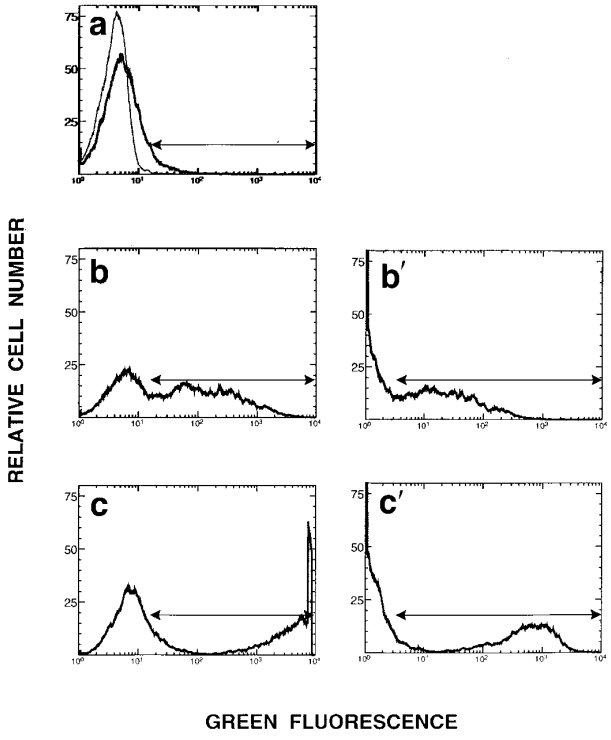

Figure 3 *FACS analysis of GFP gene transfer and expression in retrovirally transduced TF1 cells. Proliferating TF1 cells were exposed for 4 h to amphotropic retroviral supernatants of MGPN (a), MGPN2 (b) or MGIN (c) produced by ProPak-A packaging cells. GFP expression 4 days after transduction was analyzed as in Figure 2. The profile of nontransduced TF1 cells (a, thin line) was overlaid to distinguish positive populations emitting GFP fluorescence. Approximately 5, 58 and 48% of TF1 cells transduced with MGPN, MGPN2 and MGIN retroviral vectors, respectively, displayed GFP fluorescence over background (0.2%). To resolve the saturated brighter GFP fluorescence in MGIN-transduced cells, the detection sensitivity of the FL1 emission channel was reduced. The total 1024 recording channels were shifted to resolve the brightest fluorescent signals (on the right) proportionally. The same MGPN2 and MGIN-transduced cells were then reanalyzed using the new setting (b′ and c′, respectively). The mean fluorescence of GFP-expressing TF1 cells transduced with the MGIN vector was >11-fold brighter than that for cells transduced with the MGPN2 vector.*

indicating that GFP expression was not detrimental to cellular properties (data not shown).

Transduction of primary human hematopoietic progenitors

We next set out to transduce human HSPC isolated from normal bone marrow (BM) aspirates or mobilized peripheral blood (mPB). Selected CD34-expressing ($CD34^+$) cells were further enriched by FACS to reach ⩾95% purity with respect to CD34 expression and to eliminate cells expressing one or more lineage-specific differentiation markers (CD2 and CD4 for T cells, CD16 for NK cells, CD19 for B cells, CD14 and CD15 for myeloid cells, and glycophorin A for erythroid cells). These highly purified HSPC (denoted as $CD34^+Lin^-$) were first activated overnight in a cytokine cocktail before being exposed to retroviral supernatants. The transduction protocol was repeated on day 2, and the cells were cultured for 2–3 more days to allow GFP expression. Gene transfer efficiency and GFP expression level in live cells were subsequently analyzed by FACS (Figure 4). More than 75%

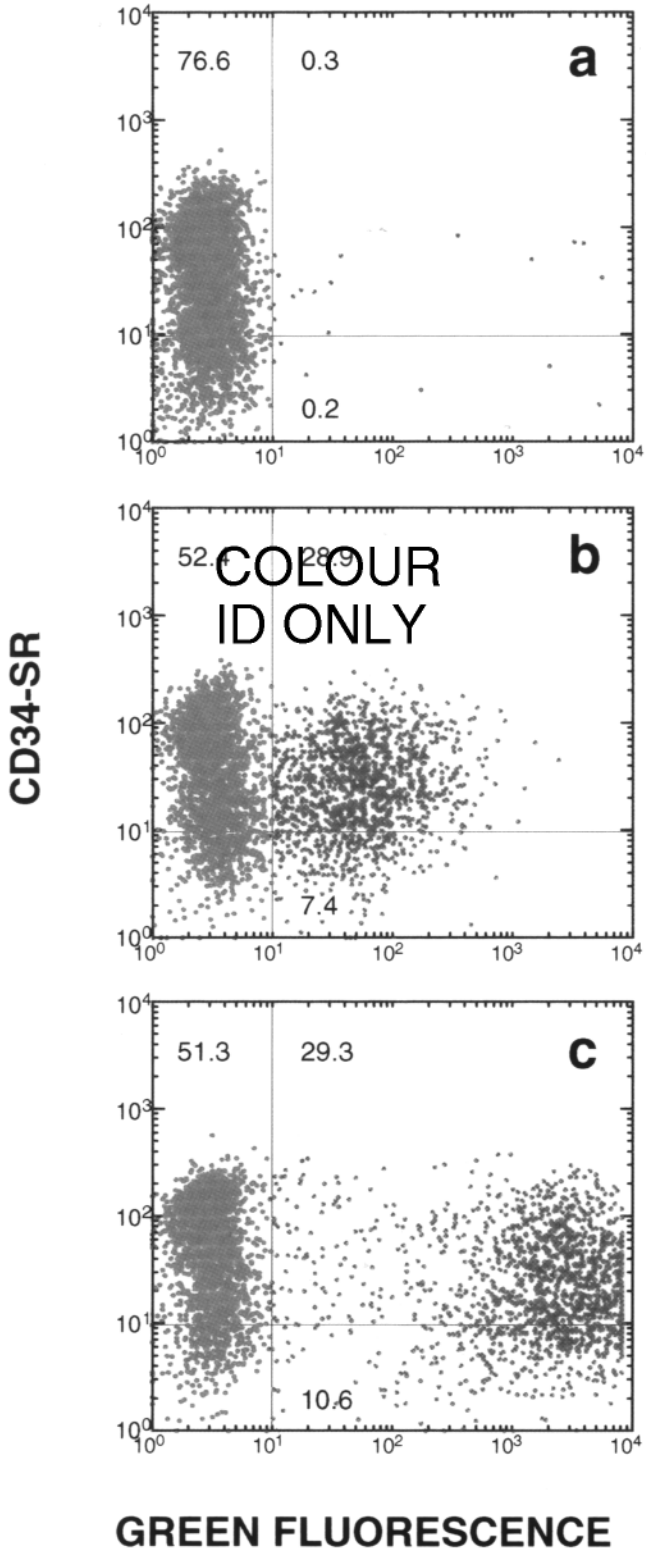

Figure 4 *FACS analysis of GFP gene transfer and expression in transduced human hematopoietic progenitors which retained the CD34⁺Lin⁻ phenotype. Sorted CD34⁺Lin⁻ human mPB progenitors were activated and transduced with amphotropic retroviral supernatants. (a) Conditioned medium from ProPak-A parental cells used as mock control, (b) MGPN2 and (c) MGIN viral supernatants. GFP expression was analyzed 5 days after ex vivo transduction. Expression of CD34 (stained with a SR-conjugated antibody) and the Lin markers (stained with PE-conjugated antibodies) were also analyzed simultaneously. Live cells which did not express Lin markers were then gated and plotted for GFP as well as CD34 expression. The percentages of cells which expressed CD34 and/or GFP (in green) are indicated among the gated Lin⁻ populations. The mean fluorescence of GFP-expressing cells transduced with the MGIN vector is >30-fold brighter than that with the MGPN2 vector.*

cells retained the CD34⁺Lin⁻ progenitor phenotype, while total cell numbers increased approximately four-fold and two-fold, respectively, from the input BM or mPB CD34⁺Lin⁻ cells (data not shown). Among mPB cells which retained the progenitor phenotype, approximately 35% of CD34⁺Lin⁻ cells transduced by MGPN2 or MGIN expressed GFP at day 5 (Figure 4). Notably, however, MGIN-transduced cells exhibited >30-fold brighter GFP fluorescence than those transduced with MGPN2 vector. Similar results were observed with transduced CD34⁺Lin⁻ cells of BM origin (data not shown).

We also confirmed that GFP expression in MGIN-transduced HSPC could be detected by fluorescence microscopy. A portion of the cell population transduced with the MGIN vector was stained for CD34 expression and then deposited on to a microscope slide on day 4; as can be seen in Figure 5, the majority of MGIN- or mock-transduced cells retained the CD34 antigen (in red) on the cell surface. In addition, approximately one quarter of the cells in the MGIN-transduced sample expressed cytoplasmic and nuclear GFP green fluorescence, whereas <1% of the mock-transduced cells displayed a detectable signal. Taken together with the flow cytometric data, these experiments demonstrated that the EGFP gene driven by the MSCV LTR in the MGIN retroviral vector is fully functional in primitive human hematopoietic cells which retained a CD34⁺Lin⁻ progenitor phenotype during *ex vivo* gene transduction.

Effect of GFP expression on biological properties of transduced HSPC

In addition to using GFP fluorescence to evaluate gene transfer and expression in primitive human hematopoietic progenitors which retained the CD34⁺Lin⁻ phenotype, we also analyzed GFP expression in live progeny cells derived from transduced CD34⁺Lin⁻ cells in two bioassays. The first was an *in vitro* colony forming cell (CFC) assay which, depending on the cytokine combination employed, detects clonogenic myeloid and erythroid progenitors in semi-solid medium. To estimate transduction efficiency of CFC on the basis of GFP expression, MGIN- or MGPN2-transduced cells were plated in methylcellulose cultures under conditions stimulating the growth of CFU-GM progenitors which give rise to colonies of granulocytes and macrophages or BFU-E progenitors which give rise to colonies of erythroid cells. After 2 weeks, total and GFP-expressing colonies were enumerated by fluorescence microscopy. Similar types and numbers of CFC (approximately 22% of total input cells) were obtained in mock-, MGIN- or MGPN2-

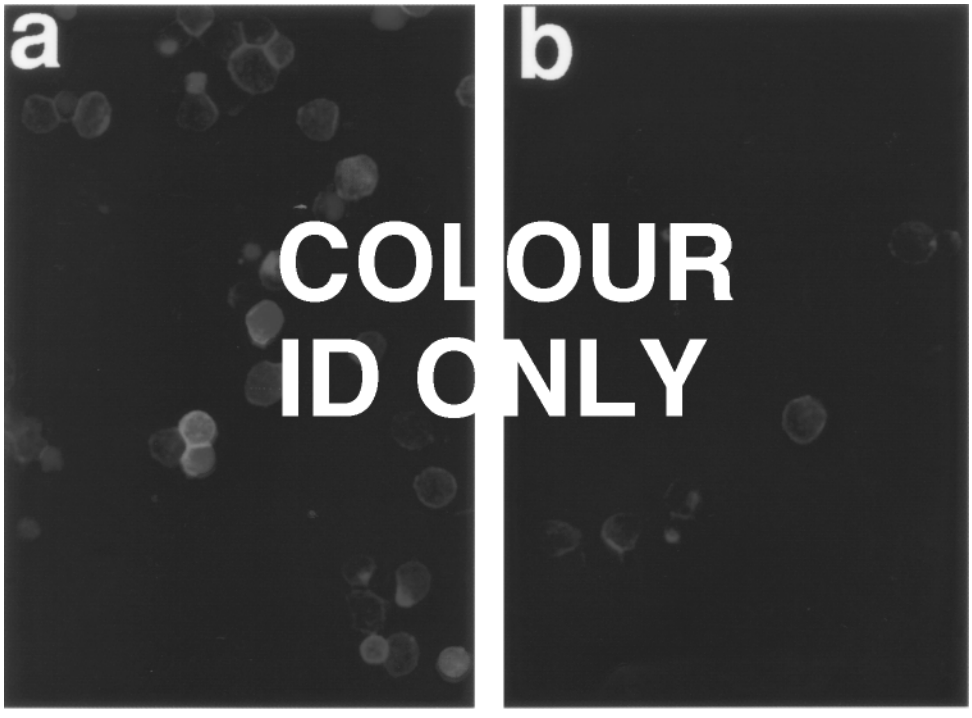

Figure 5 *Fluorescence microscopy of CD34 and GFP expression in transduced human hematopoietic cells. CD34⁺Lin⁻ human progenitors transduced with the MGIN retroviral vector (a) or mock-transduced cells (b) were analyzed 4 days after ex vivo transduction. Cells were first stained with the CD34-SR antibody and then fixed. The fixed cells were then spun on to cytospin slides and analyzed by confocal fluorescence microscopy. Green (GFP expression) and red (CD34 expression) fluorescent signals from the same field were recorded sequentially through a ×20 objective lens.*

transduced cells. Approximately 28% of colonies derived from MGIN-transduced progenitors expressed GFP (Figure 6 and Table 1), whereas some of the MGPN2-transduced cells displayed a weak signal which was not well above background fluorescence (background autofluorescence was particularly high in BFU-E colonies). GFP fluorescence in the case of MGIN-transduced cell populations was observed in both CFU-GM and BFU-E colonies (Figure 6b and d), although some larger BFU-E colonies were not uniformly GFP positive.

We also compared the transduction efficiency of CFC by using two conventional methods. In the first method, the presence of transgene-specific DNA sequences in individually picked colonies was investigated by PCR. The sensitive PCR analysis indicated that approximately 40% of CFC arising from MGIN-transduced progenitors contained the MGIN transgene, and similar results were obtained for MGPN2-transduced cells (Table 1). These data indicated that the majority of progenitors containing the integrated MGIN vector could express the transgene at a detectable level (28% GFP-expressing *versus* 40% gene-marked measured by PCR). A second conventional method to monitor functional gene transduction is enumeration of G418-resistant colonies. We therefore plated transduced cells in various concentrations of G418 and determined the number of drug-resistant colonies after 2 weeks. A G418 concentration $\geqslant$0.85 mg/ml was required to eliminate essentially all the colonies from mock-transduced mPB cells (Table 1). We noticed that at this concentration the size as well as the number of colonies (as compared with those arising in the absence of G418) were significantly reduced in the case of MGIN-transduced cells, and more profoundly in the case of MGPN2-transduced cells. Approximately 31% of MGIN-transduced small CFC colonies were G418 resistant compared with 21% of the MGPN2-transduced cells (Table 1). These results indicated that neomycin phosphotransferase activities as well as GFP levels mediated by the MGIN vector were higher than those achieved with MGPN2.

The second HSPC assay we utilized was the cobblestone area-forming cell (CAFC) assay, in which test cells are plated on a bone marrow-derived stromal cell monolayer and progenitors form cobblestone-like colonies in long-term cultures.[30] Late-appearing CAFC (after 4–5 weeks) are considered more primitive than CFC progenitors.[30,31] Accordingly, we plated MGIN-transduced CD34⁺Lin⁻ cells on the SyS-1 clonal stromal cell line to monitor gene transfer and expression in these primitive progenitors. GFP expression was observed as expected in early-appearing myeloid cells in the cultures as well as in CAFC colonies which appeared from 2 to 5 weeks. At week 5, GFP fluorescent signals were observed in approximately 15–20% of CAFC colonies by fluorescence microscopy (data not shown). Examples of CAFC colonies expressing GFP at week 5 are shown in Figure 7 (a–d); no green fluorescent CAFC colonies were observed in cultures of mock-transduced cells (Figure 7e and f).

Discussion

We have described in this article a combination of the EGFP variant and an improved MSCV-based retroviral vector that results in >100-fold increase in GFP fluorescence in transduced mammalian cells, and have demonstrated the utility of this methodology for gene transfer studies involving primitive human hematopoietic cells. We have estimated by semi-quantitative PCR the number of GFP transgenes in GFP-expressing, CD34⁺Lin⁻ pro-

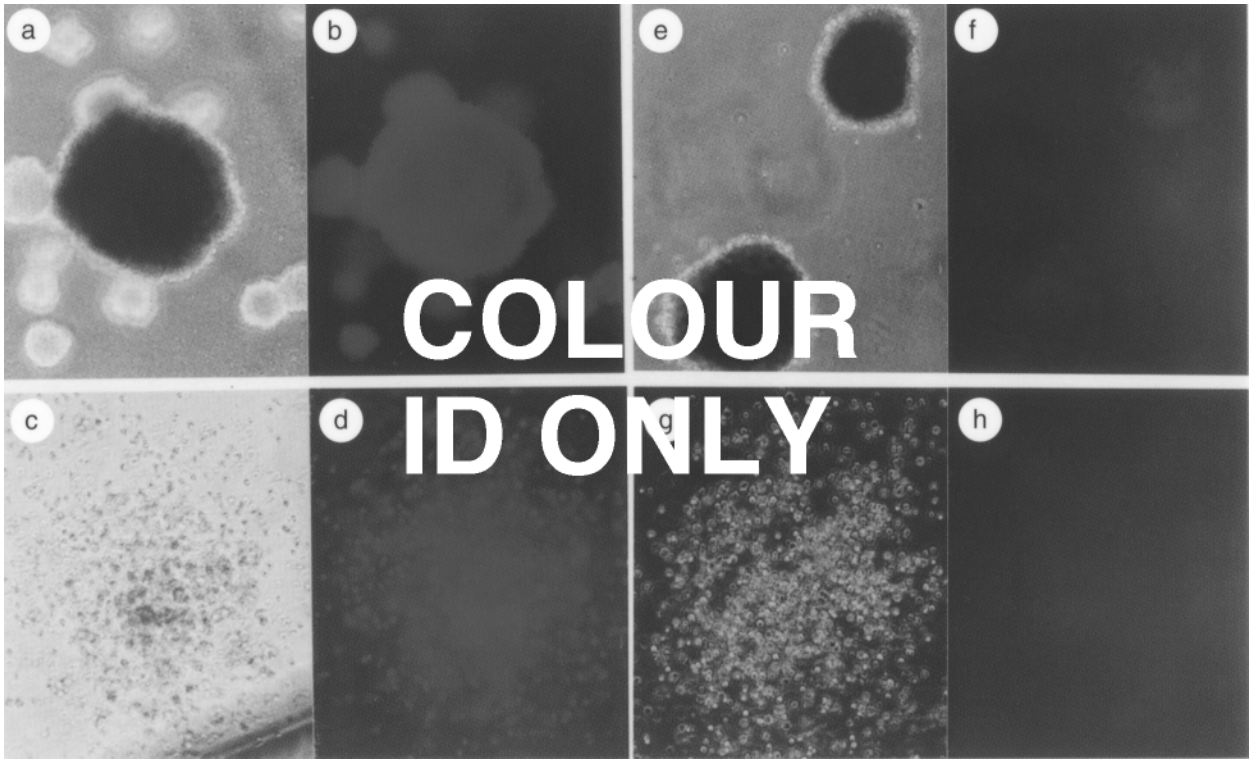

Figure 6 *Fluorescence microscopy of GFP expression in clonogenic cells. CD34⁺Lin⁻ cells transduced with MGIN (a-d) or mock supernatants (e–h) were plated in semi-solid medium to measure BFU-E and CFU-GM progenitor activities. Fourteen days later, plates were examined for colonies by a fluorescence microscope with a ×10 objective lens. The upper row are samples of BFU-E colonies and the bottom row are CFU-GM colonies. The diameter of the central BFU-E colony is approximately 200 mm. Bright field images (a, c, e and g) were taken without any filter and the corresponding green fluorescent images (b, d, f and h, respectively) of the same colonies were taken with a FITC filter.*

Table 1 Evaluation of gene transduction efficiency in CFC

	Mock-transduced cells		*MGIN-transduced cells*		*MGPN2-transduced cells*	
	–G418	*+G418*[a]	*–G418*	*+G418*[a]	*–G418*	*+G418*[a]
No. of total colonies	87.3 (11.3)	0.3 (0.5)[b]	88.6 (4.7)	27.4 (0.9)	84.7 (6.3)	18.0 (1.3)
Relative No. of colonies %	100	0.3	101.5	31.3	97.0	20.6
No. of GFP⁺ colonies	0/200	0/1	56/200 (28%)	49/50 (98%)	10/60 (17%)[c]	19/54 (35%)[c]
No. of PCR⁺ colonies	ND	ND	16/40 (40%)	21/21 (100%)	17/40 (43%)	17/18 (94%)

Transduced mPB cells (4×10^2) 5 days after *ex vivo* activation/transduction were plated in methylcellulose medium in the presence or absence of G418. After 14 days, total or green fluorescent (GFP⁺) colonies were counted by microscopy. Colonies of MGIN- or MGPN2-transduced cells were individually picked and the presence of the GFP transgene was assessed by PCR. The mean and standard variation (in brackets) of the total numbers of colonies from each of three triplicates are listed in the first row. Relative numbers of colonies were calculated by defining the colony number of mock-transduced cells (in the absence of G418) as 100%. GFP⁺ or transgene positive (PCR⁺) colonies listed are actual numbers divided by the total numbers of colonies examined (followed by calculated percentages in brackets). ND, not done. Similar results were obtained from transduction of two other mPB samples and one BM sample. See Materials and methods for more details.

[a]The G418 (0.85 mg/ml) selection yielded smaller sized colonies.

[b]A single small colony out of three plates was observed which did not exhibit green fluorescence.

[c]Some CFU-GM and smaller BFU-E colonies displayed weak green fluorescence over background and were counted as GFP⁺.

genitors sorted 3 days after transduction (5 days in culture). By limiting dilution analysis of cells and MGIN plasmid DNA, we determined that one to 10 copies of GFP transgene (integrated and unintegrated DNA or RNA) were present on average per single sorted GFP-expressing cell (unpublished observations). Although we have not determined the absolute GFP reporter sensitivity, which is dependent upon background cellular autofluorescence and inherent detection limits of the particular instrument used, we have shown that GFP fluorescence can now be reproducibly detected by fluorescence microscopy in individual viable human HSPC harboring low copy numbers of integrated transgenes.

With this improved sensitivity, GFP has obvious advantages over cell surface or enzymatic markers to evaluate and optimize gene transfer and expression in

Figure 7 *Fluorescence microscopy of GFP expression in transduced CAFC formed on stromal cells. CD34$^+$Lin$^-$ cells transduced with MGIN (a–d) or mock supernatants (e and f) were plated on SyS-1 stromal cell monolayers. Cobblestone area formation and GFP expression were examined weekly up to 5 weeks by a fluorescence microscope. Bright field images (a, c and e) were taken at week 4 with a ×20 objective lens in the absence of any filter. The corresponding fluorescence images (b, d and f, respectively) of the same colonies were taken with a FITC filter.*

human hematopoietic (and other target) cells.[32] In addition to eliminating specific antibody staining or enzymatic reactions (eg for *lacZ*) to detect the reporter by flow cytometric analysis,[33] autonomous fluorescence of GFP enables facile monitoring of reporter expression *in situ* as illustrated herein. Although G418 resistance provides an estimation of gene transfer and expression in cells derived from transduced progenitors, G418 evidently retarded cell proliferation and/or differentiation in the CFC assays since the sizes of the resulting colonies were much smaller in the presence of the drug (Table 1). In contrast, GFP expression mediated by the MGIN vector had no detectable detrimental effects on either freshly transduced cells or their differentiative potentials in the progenitor bioassays. Moreover, selection for GFP expression has additional advantages over G418 and other selection schemes based on drug resistance in that an optimal drug selection window for the progenitor cells being assayed does not have to be determined *a priori* (indeed, this is not always readily achievable) and, as in the case of the CAFC assay, detection of GFP expression does not require the existence of a drug-resistant stromal cell line. While sensitivity of detection of gene transfer based on GFP expression is no doubt still lower than that achievable by PCR, we believe that the GFP reporter strategy is complementary to this DNA-based technique and, for the intended purpose, arguably more relevant, since documentation of sustained retrovirus-mediated gene expression is ultimately the goal of these studies.

In this regard, it is important that stable EGFP expression mediated by the MGIN retroviral vector did not interfere with the biological properties of HSPC, as

exemplified by their ability to give rise to progeny assessable in the CFC and CAFC assays. It remains to be demonstrated how this GFP expression system will perform *in vivo*. Because several groups have reported that GFP is functional in mice and rats as well as in a variety of lower organisms,[16,34–37] we anticipate that this marker will prove useful to monitor engraftment of HSPC in immune-deficient mice and perhaps in clinical trials.[7,38]

The simple and rapid GFP detection scheme enabled us to determine that the MGIN vector expressed the reporter >10-fold higher than by the MGPN2 vector, although EGFP expression was under the transcriptional control of the MSCV LTR in both vectors. We also found that in CFC assays the neomycin phosphotransferase activity originating from the *neo* gene via an IRES in the MGIN vector was higher than that obtained from the MGPN2 vector in which the *neo* gene is driven by an internal PGK-1 promoter. For integrated vector sequences, promoter interference, where the establishment of a transcription complex at one promoter changes the DNA topology such that another nearby transcription complex is not efficiently formed, is presumably the explanation for this phenomenon.[39] It was of interest, however, that lower levels of MSCV LTR-directed EGFP expression from the MGPN2 vector were also observed in the transient transfection experiments. It is possible that the latter effect is due to transcriptional interference reported in episomal plasmids containing tandem promoters.[40] In any event, these findings suggest the use of retroviral vectors containing IRES sequences when a high-level expression of more than one exogenous gene is required in gene therapy.[41,42]

Materials and methods

Construction and detection of retroviral expression vectors

The MGPN retroviral vector containing the RSGFP4 variant has been described previously.[17] MGPN2 was generated by replacing the RSGFP4 gene in MGPN with the EGFP variant.[21] The MGIN vector was constructed by inserting the EGFP gene into the MINV retroviral vector which contains an internal ribosome entry site (IRES) from the encephalomyocarditis virus.[26,27] All the plasmids were purified and used for cell transfection as described.[19] The primers used to amplify EGFP transgenes were: upstream 5′-TGAGCAAGGGCGAGGAGCTG-3′ and downstream 5′-ACGAACTCCAGCAGGACCAT-3′; control primers (upstream 5′-ACACAACTGTGTTCACTAGC-3′ and downstream 5′-CAACTTCATCCACGTTCACC-3′) were used to amplify the endogenous β-globin sequence. Forty-cycle PCR reactions for both genes were performed with an annealing temperature of 62°C. PCR products (a 580-bp fragment for EGFP and 220-bp fragment for β-globin) were separated by 4% agarose gel electrophoresis as described.[10]

Cell lines, production of retroviral supernatants and transduction protocol

Culture medium such as DMEM, IMDM and RPMI 1640 were purchased from GIBCO/BRL (Gaithersburg, MD, USA) and fetal calf serum (FCS) from Hyclone (Logan, UT, USA). TF1 cells[29] were maintained in RPMI 1640 plus 10% FCS and 2 ng/ml GM-CSF. PA317 amphotropic packaging cells[43] were maintained in DMEM plus 5% FCS. BOSC23 ecotropic packaging cells[44] were maintained in DMEM plus 10% FCS and transfected with GFP-containing plasmids as described.[17] Amphotropic supernatants produced by PA317 packaging cells were made from selected producers after infection by BOSC23 ecotropic viral stocks.[17] Amphotropic supernatants produced by a human 293 cell-based ProPak-A packaging line were made through transduction with pseudotyped viral stocks.[28] Briefly, ProGag cells which constitutively produce the MLV gag/pol protein were transiently cotransfected with vectors and a plasmid expressing the VSV-G envelope protein.[45] Then the VSV-G pseudotyped retroviral supernatants collected at day 3 were used to transduce ProPak-A packaging cells. ProPak-A producers making high levels of MGPN, MGPN2 or MGIN viruses were further selected based on G418 resistance or GFP expression. Amphotropic supernatants were then made from stable ProPak-A producers incubated at 32°C, filtered and stored at −80°C until use. For transduction, fresh or previously frozen vector supernatants were mixed at a 1:1 ratio with medium containing target cells in the presence of 8 μg/ml polybrene (Sigma, St Louis, MO, USA). The transduction mixture was then centrifuged at 1800 *g* at 32–35°C. After a 4-h 'spinoculation', cells were washed once and cultured in appropriate medium.[46] The end-point titers of three vector supernatants were similar ($2–4 \times 10^5$ c.f.u./ml), based on G418 resistance of NIH3T3 cells according to standard methods as described.[17] The G418 antibiotics from GIBCO/BRL were used at 1 mg/ml (active) for selecting transduced 3T3, PA317, ProPak-A and TF1 cells. Although the end-point titers of ProPak-A supernatants were equal or slightly lower than those made from PA317 producers, up to 10-fold more cells can be transduced by spinoculation and expressed GFP reporter genes if ProPak-A supernatants (of either MGPN2 or MGIN) were used. These observations held for transductions of cultured cells (murine NIH3T3 fibroblasts and human TF1 cells) as well as primary human $CD34^+Lin^-$ progenitor cells (unpublished data), and were consistent with results obtained in a previous report using other types of cells.[28] All the results presented for retroviral transductions were obtained using viral stocks produced by ProPak-A packaging cells.

Isolation and processing of human hematopoietic progenitors

Human BM aspirates and aphoresed mPB (collected at day 4 or 5 after G-CSF treatment) were obtained from healthy donors in compliance with regulations established by the federal and state governments. Low-density (<1.077 g/cm^3) mononuclear BM cells after Ficoll–Hypaque gradient (Pharmacia, Piscataway, NJ, USA) or mPB were stained with a CD34 antibody included in the Isolex kit (Baxter Biotech Immmunotherapy Division, Irving, CA, USA). $CD34^+$ cells were magnetically isolated by Isolex using modified protocols developed at Systemix. The purity of $CD34^+$ cells from both BM and mPB was usually ⩾90%. These isolated cells were then stained with a CD34 antibody (Tuk3) conjugated with sulfo-rhodamine (SR), and a panel of FITC-conjugated mouse monoclonal antibodies against lineage differentiation markers. This lineage panel (collectively called Lin) comprised CD2, CD4, CD14, CD15, CD16, CD19 (Becton Dickinson,

San Jose, CA, USA) and glycophorin A (Immunotech, Westbrook, ME, USA). $CD34^+Lin^-$ cells were then sorted by FACS and activated *ex vivo* for gene transduction. Cells ($\leqslant 10^6$/ml) were cultured overnight in IMDM/RPMI 1640 (1:1) medium plus 10% FCS supplemented with 10 ng/ml IL-3 and IL-6, and 100 ng/ml SCF. These cytokines as well as GM-CSF and leukemia inhibitory factor (LIF) were obtained from Sandoz Pharma (Basel, Switzerland), and Epo was produced by Amgen (Thousand Oaks, CA, USA). The next day, cells were transduced with ProPak-A viral supernatants for 4 h as described above. The transduction procedure was repeated the following day, and then the medium was changed and the cells were cultured for an additional 2–3 days to allow gene expression.

FACS analysis

Propidium iodide (0.5 mg/ml) was added to cell suspensions to exclude dead/dying cells from the FACS analyses. FACStar^{Plus} or FACSVantage cell sorters (Becton Dickinson) equipped with Argon ion lasers tuned at 488 nm were used, and green fluorescence was recorded in the FL1 emission channel (with a 515/20 nm emission filter). All cells transfected or transduced with different GFP-encoding retroviral vectors were analyzed similarly. When CD34 and lineage marker expression as well as GFP expression were analyzed simultaneously, R-Phycoerythrin (PE)-conjugated monoclonal antibodies against the lineage marker panel (CD2, CD4, CD14, CD16, CD19 and glycophorin A, missing CD15) were used.

Fluorescence microscopy

An inverted Olympus fluorescence microscope with a mercury arc lamp (100 W) and a FITC filter set (consisting of a 480/40 nm excitation filter and a 535/50 nm emission filter) was routinely used to detect GFP in living cells or in cells fixed by treatment with 2% paraformaldehyde. Green fluorescence was readily detectable in cells transduced by MGPN2 or MGIN under sterile conditions in which live cells were cultured in plastic dishes (in complete medium) with covers. Portions of transduced progenitors were collected on day 4 and stained with the anti-CD34-SR antibody. The stained cells were then fixed to preserve the CD34 antigen. After centrifugation on to cytospin slides, cells were analyzed for CD34 and GFP expression, either with the Olympus (Japan) microscope or with a confocal fluorescence microscope (MRC; Bio-Rad, Hercules, CA, USA). Microphotographs in Figure 5 were recorded separately and sequentially for FITC and SR channels by confocal microscopy, and the original green and red colors were restored by Adobe Photoshop computer software (Adobe Systems, San Jose, CA, USA). Microphotographs in Figures 6 and 7 were obtained with the Olympus microscope using Kodak slide film (ASA 400) (Eastman Kodak, Rochester, NY, USA). No filter was used when bright field images were taken.

In vitro progenitor assays

Methylcellulose and reagents for clonogenic progenitor assays were obtained from StemCell Technologies (Vancouver, Canada). Cells (4×10^2) were added to 1 ml methylcellulose medium supplemented with IL-3, IL-6, GM-CSF (10 ng/ml each), SLF (100 ng/ml) and Epo (2 units/ml). Cell mixtures were plated in 35 mm suspension culture dishes (Nunc, Naperville, IL, USA), and incubated at 37°C. After 2 weeks, total and fluorescent colonies were counted in each of three triplicate plates. Individual colonies were subsequently picked from some plates and lysed for PCR analysis.[12]

For stromal-dependent CAFC assays,[30] a stromal cell line (SyS-1) derived from murine bone marrow was used.[31] Cultures contained IMDM/RPMI 1640 (1:1) medium plus 10% FCS supplemented with 10 ng/ml IL-6 and 50 ng/ml LIF. Up to 100 cells were cultured on subconfluent monolayers of SyS-1 stromal cells in each well of 96-well plates. Half of the medium was replaced weekly, and the cultures were monitored for 5 weeks.

Note

Requests for retroviral vectors should be addressed to: RG Hawley, The Toronto Hospital, CRCS 424, 67 College Street, Toronto, Ontario M5G 2M1, Canada.

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