Lentiviral Vectors with Two Independent Internal Promoters Transfer High-Level Expression of Multiple Transgenes to Human Hematopoietic Stem-Progenitor Cells

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Lentiviral vectors (LVs) offer several advantages over traditional oncoretroviral vectors. LVs efficiently transduce slowly dividing cells, including hematopoietic stem-progenitor cells (HSCs), resulting in stable gene transfer and expression. Additionally, recently developed self-inactivating (SIN) LVs allow promoter-specific transgene expression. For many gene transfer applications, transduction of more than one gene is needed. We obtained inconsistent results in our attempts to coexpress two transgenes linked by an internal ribosomal entry site (IRES) element in a single bicistronic LV transcript. In more than six bicistronic LVs we constructed containing a gene of interest followed by an IRES and the GFP reporter gene, GFP fluorescence was undetectable in transduced cells. We therefore investigated how to achieve consistent and efficient coexpression of two transgenes by LVs. In a SIN LV containing the elongation factor 1α promoter, we included a second promoter from cytomegalovirus, the phosphoglycerate kinase gene, or the HLA-DRα gene. Using a single LV containing two constitutive promoters, we achieved strong and sustained expression of both transgenes in transduced engrafting CD34+ HSCs and their progeny, as well as in other human cell types. Thus, such dual-promoter LVs can coexpress multiple transgenes efficiently in a single target cell and will enable many gene transfer applications.

Key Words: lentiviral vectors, gene transfer, gene therapy, hematopoietic stem-progenitor cells, bicistronic vectors, IRES, NOD/SCID mice, transplantation models.

INTRODUCTION

Recombinant vectors based on retroviruses (including both oncoretroviruses and lentiviruses) remain the only choice to efficiently transduce primary mammalian cells stably. Compared to oncoretroviral vectors (RVs), which have been the mainstay of stable gene delivery in the past 20 years, lentiviral vectors (LVs) offer several advantages. First, LVs can transduce not only mitotically active but also slowly dividing cells, including freshly isolated hematopoietic stem-progenitor cells (HSCs). Second, transgenes introduced with the LV backbone integrated into the host genome are more resistant to transcriptional silencing [1–3], commonly associated with RV-transduced cells after prolonged in vitro culture or in vivo transplanta- tion. For example, we have previously reported high-level and sustained transgene expression in engrafted human cells after transplantation of LV-transduced HSCs into nondiabetic/severe combined immunodeficient (NOD/SCID) mice [1]. In addition, LV-transduced rodent eggs or embryonic stem cells have been used to generate transgenic animals with sustained transgene expression [2,3]. Third, LVs can accommodate the use of various transcriptional promoters, either ubiquitous or cell-specific [1–4]. The self-inactivating (SIN) safety modification of LVs, which permanently disables the viral promoter within the viral long-terminal repeat (LTR) after integration, enables transgene expression in the targeted cells to be controlled solely by internal promoters [1–5]. Importantly, SIN modification of LVs does not reduce
viral titers [1,5]. We previously constructed two SIN LVs in which transgene expression (GFP) was controlled by the promoter of either the ubiquitous elongation factor 1α (EF) housekeeping gene (EF.GFP LV) or the lineage-specific human major histocompatibility HLA-DRα (DR) gene (DR.GFP LV). Using in vitro assays and transplantation into NOD/SCID mice, we found that transgene expression controlled by the selected promoter paralleled the specificity and levels of the endogenous gene expression in LV-transduced human CD34+ cells and progeny [1]. These advantages, together with other improvements, such as minimizing HIV-1 viral sequences and eliminating viral accessory proteins, make LVs the vectors of choice for stable gene transfer and expression.

Many basic and clinical gene transfer experiments require vectors that express more than one protein (e.g., a gene of interest plus a marker gene, multiple genes encoding different subunits of a complex protein, or multiple independent genes that cooperate functionally). For example, we are studying the potential use of Fas ligand (FasL)-transduced HSCs to decrease donor cell rejection in allogeneic transplantation. Since we did not have specific antibodies to monitor human FasL expression, our previous studies were accomplished by using an EF.GFP-based LV expressing a FasL–GFP fusion protein [6,7]. We found that GFP fluorescence from the GFP fusion protein in transduced target cells was several times reduced, compared to cells transduced with the parent vector containing unmodified GFP; thus, GFP in this fusion protein was an insensitive reporter. Furthermore, in other cases, use of such a protein fusion might significantly reduce or inactive functionality of one or both fusion partners.

The simplest way to transfer two genes might be to cotransduce the target cells with two separate LVs. However, it is very difficult to ensure the cotransduction of both vectors into the same cells, particularly when transduction efficiency is low and preselection is not possible. In the past 2 decades, alternative strategies of expressing two transgenes from a single RV vector have been developed [8,9], and certain of these may be applicable to LVs. In nearly all the high-titer RVs in current use, the RV LTR is retained so that it functions as a transcriptional promoter of transgene expression after integration. It has been found that, if another promoter is placed internal to the LTR, it is typically dominated by the LTR promoter [8,9]. Other strategies have included utilizing multiple alternatively spliced transcripts from the integrated (pro-viral) DNA, engineering duplication of the LTR after integration, or using an internal ribosomal entry site (IRES) sequence that mediates protein synthesis of the second gene product from an internal translational starting site within a single “bicistronic” mRNA transgene [8,9]. Currently, the most widely used strategy for two-gene RVs (or plasmid vectors) is to insert between the two transgenes an IRES sequence, such as the encephalomyocarditis virus (ECMV) IRES. IRES elements, first identified in unintegrated 5’ ends of picornaviruses, have also been identified in other viral and cellular genomes. Multiple mechanisms, not fully elucidated, may contribute to the capacity of an IRES to specify translation of the second gene (downstream of the IRES) from a bicistronic transcript [10,11]. However, in many instances, only the (first) gene transcribed upstream of the IRES is expressed strongly, from either plasmid or RV [12]. A recent study systematically examined expression of the second gene (downstream) in cells transfected by bicistronic plasmid vectors [13]. Similar to our observations with RV and plasmid vectors, this group found that the expression of the second gene (downstream) ranged from 6 to 50% of the level of expression of the first (upstream) gene.

Collectively, we have constructed 12 bicistronic LVs using the ECMV IRES in the past 2 years. In these LVs, various genes of interest were inserted as the first (upstream of the IRES) gene, and the GFP gene was the second gene (downstream of the IRES). To our surprise, more than half of these new LVs did not confer detectable levels of GFP expression in transduced cells. This may explain why nearly all (hundreds) reported LVs have contained only one transgene, and only a few papers have reported efficient coexpression of two transgenes from a single LV [14,15]. This prompted us to use different IRES elements and other strategies to express two transgenes consistently and efficiently, based on our optimized EF.GFP LV or DR.GFP SIN LV [1]. Herein, we report that LVs with dual independent internal promoters can coexpress two separate transgenes at high efficiencies in transplantable human CD34+ HSCs and other cell types.

RESULTS

Inconsistent Expression of the IRES-Dependent Second (Downstream) Gene in LV-Transduced Cells

Over the past 2 years, we have attempted to express various genes of interest utilizing LVs engineered with the gene of interest immediately upstream of an IRES and a GFP reporter gene immediately downstream. We compared GFP expression in cells transfected or transduced with these bicistronic LVs with that obtained using the “parental” monocistronic EF.GFP LV [1]. Although we had expected somewhat reduced expression of GFP when positioned as the second gene, we were surprised that GFP expression was totally undetectable by fluorescence-activated cell sorting (FACS) in cells transduced with many of these bicistronic LVs. As shown in Table 1, the insert size of the first gene was not the only factor that determined reduced expression of the second gene (GFP). For example, a vector containing a truncated version of the FasL cDNA (“delFasL,” 0.7 kb) as the first gene (upstream of the IRES) failed to confer GFP expression to transduced cells, whereas a LV containing the glucocerebrosidase or luciferase gene (1.6 and 1.7 kb, respectively) displayed a lower but detectable level of GFP. In each of these bicistronic
LVs that failed to confer detectable GFP expression in transduced cells (Table 1), the GFP transgene was present in genomic DNA from the transduced cells, and the first (upstream) transgene was efficiently expressed by various assays (data not shown).

We next examined whether varying the IRES element might enhance the expression of the second (downstream IRES-dependent) gene. A set of LVs was made with influenza hemagglutinin (HA) as the first gene, placed upstream of one of three IRES elements and GFP (Table 1). The IRES from ECMV was the control. The SP163 IRES element (163 bp) was derived from the 5' untranslated region of the human VEGF gene; SP163 has previously been shown to function as an IRES and a "translational enhancer" [16]. The Gtx IRES is a 208-bp synthetic element containing a sequence of the Gtx homeodomain gene, which has been reported to be superior to the ECMV IRES in one study [14]. Disappointingly, none of these LVs transferred detectable GFP (IRES-dependent) gene expression to transduced cells that were shown to express the HA transgene as the first (upstream) gene (Table 1).

Coexpression of Two Reporter Genes in a Single Cell by a Single LV Containing Dual Promoters

Next, we explored the possibility of expressing two transgenes by two promoters, separately but in a single LV. To avoid DNA recombination between repeated sequences in the same vector, we used two different promoters. To simplify detection of transgene expression, we used red fluorescent protein (RFP; encoded by the DsRed2 gene [17,18]) as the second reporter gene, with GFP as the first (upstream) gene. We constructed EF.GFP-CMV.RFP, a new dual-promoter LV with the EF promoter driving the GFP gene followed by the cytomegalovirus (CMV) promoter driving the RFP gene (Fig. 1B). This EF.GFP-CMV.RFP LV was produced in parallel with the EF.GFP parental vector (Fig. 1). Based on GFP expression, the EF.GFP-CMV.RFP LV had a viral titer of 10^7 transduction units (TU)/ml.

**TABLE 1: Inconsistent expression of the second gene mediated by the IRES in bicistronic LVs**

<table>
<thead>
<tr>
<th>Vector name*</th>
<th>Upstream (first) gene (size in kb)</th>
<th>Type of IRES</th>
<th>Downstream (second) gene</th>
<th>GFP expressionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF.GFP</td>
<td>GFP (0.7)</td>
<td>None</td>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>EF.GC-IRES.GFP</td>
<td>GC (1.6)</td>
<td>ECMV</td>
<td>GFP</td>
<td>++</td>
</tr>
<tr>
<td>EF.luc-IRES.GFP</td>
<td>luciferase (1.7)</td>
<td>ECMV</td>
<td>GFP</td>
<td>+</td>
</tr>
<tr>
<td>EF.hTERT-IRES.GFP</td>
<td>hTERT (3.4)</td>
<td>ECMV</td>
<td>GFP</td>
<td>–</td>
</tr>
<tr>
<td>EF.delFasL-IRES.GFP</td>
<td>delFasL (0.7)</td>
<td>ECMV</td>
<td>GFP</td>
<td>–</td>
</tr>
<tr>
<td>EF.myc-IRES.GFP</td>
<td>c-myc (1.3)</td>
<td>ECMV</td>
<td>GFP</td>
<td>–</td>
</tr>
<tr>
<td>EF.HA-IRES.GFP</td>
<td>HA (2.0)</td>
<td>ECMV</td>
<td>GFP</td>
<td>–</td>
</tr>
<tr>
<td>EF.HA-SP.GFP</td>
<td>HA</td>
<td>SP163</td>
<td>GFP</td>
<td>–</td>
</tr>
<tr>
<td>EF.HA-Gtx.GFP</td>
<td>HA</td>
<td>Gtx</td>
<td>GFP</td>
<td>–</td>
</tr>
</tbody>
</table>

*EF, elongation factor 1α gene promoter; GC, human glucocerebrosidase gene; luc, the American firefly luciferase gene; hTERT, human telomere reverse transcriptase gene; delFasL, (cleavage-site) deleted mouse Fas ligand gene; c-myc, the mouse c-myc gene; HA, influenza virus hemagglutinin gene; ECMV, encephalomyocarditis virus; SP, the 163-bp IRES element (from the human vascular endothelial growth factor gene); Gtx, the synthetic Gtx IRES element.

**METHOD**

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equivalent to that of the EF.GFP LV (10^7 TU/ml). We used the EF.GFP-CMV.RFP LV to transduce primary human iliac artery endothelial cells (HIAEC) and human 293T and TF1 cell lines. To determine if the LV allowed efficient coexpression of the two proteins within single cells, we examined transgene fluorescence in individual cells of the EF.GFP-CMV.RFP or mock-transduced cell population by fluorescence microscopy and FACS analyses (Fig. 2). Four days after a single round of transduction by the EF.GFP-CMV.RFP LV, 59% of the total individual cells (or ≥80% of transduced cells) were GFP+/RFP+ double positive by FACS analysis (10,000 cells analyzed), and 5–7% of the total cells expressed only one marker gene. Approximately 30% of cells lacked expression of either marker (fluorescence less than or equal to that of the mock-transduced control cells) and were interpreted as untransduced cells (Fig. 2E). The percentages of GFP+ and/or RFP+ cells were further confirmed by quantifying 10 microscopic fields (1333 cells counted) documented by digital camera imaging, using separate filters specific for either GFP or RFP (Figs. 2A–2E). Similar results were obtained with the 293T and TF1 cell lines (data not shown). It is not clear why these minor cell subsets expressed only one of the two markers, but the stringent FACS gating we used to define “double marker positivity” may contribute. In addition, it is known that detection of RFP is suboptimal using the 488 nm laser of our FACS instrument [17,18].

Functional and Quantitative Assays of Expression of Both Transgenes by Dual-Promoter LVs
Elsewhere, we are studying the potential use of FasL-transduced donor HSCs to decrease donor cell rejection in allogeneic transplantation models [7]. We deleted a protease-cleavage site (creating the “delFasL” deletion mutant) within the mouse FasL gene to engineer expression of FasL that would remain cell membrane bound [19,20]. Then a dual-promoter LV EF.delFasL-CMV.GFP was constructed (Fig. 1C). In contrast to our results using our previous versions of FasL-expressing LVs, a high level of GFP expression was readily seen in cells transduced with this novel LV (Fig. 3D).

To demonstrate that expression of the delFasL gene resulted in a functional protein, we tested EF.delFasL-CMV.GFP LV-transduced cells in a cytotoxicity assay against the FasL-sensitive Jurkat (human T) cell line. In the representative experiment shown in Fig. 3, 293T cells were transduced by either EF.delFasL-CMV.GFP or EF.GFP. Forty-eight hours after transduction, an aliquot of transduced 293T cells was analyzed for GFP expression. Interestingly, the GFP expression levels transferred by the EF.delFasL-CMV.GFP LV, which contains GFP as the second gene, were consistently even higher than the GFP levels transferred by the EF.GFP LV in transduced 293T cells, by microscopy (Figs. 3A and 3D), FACS (not shown), and mRNA analyses shown below. Another aliquot of transduced 293T cells was cocultured with Jurkat target cells to assess the functional effects of the delFasL transgene expressed on the surface of EF.delFasL-CMV.GFP LV-transduced cells. After overnight coculture with EF.delFasL-CMV.GFP LV-transduced 293T cells, the majority of Jurkat cells were dead or apoptotic, demonstrated by microscopy (Fig. 3E) or FACS-quantitated binding of 7-aminactinomycin D (7-AAD) and annexin V (Fig. 3F). In contrast, Jurkat cells cocultured with the control EF.GFP LV-transduced 293T cells were mostly viable (Figs. 3B and 3C). We consistently observed a low level of cell apoptosis and death of cocultured Jurkat cells after coculture with 293T cells; this may be due to a low level of FasL expression on 293T cells or the multiple-step cell harvesting and staining procedures before FACS analysis. Nonetheless, this experiment clearly showed the expression and functionality of the delFasL transgene in the EF.delFasL-CMV.GFP LV-transduced cell populations (that had a high level of GFP expression).
FIG. 4. Efficient expression of two transgenes in human CD34+ cells by dual-promoter LVs. Purified CB CD34+ cells were cultured overnight and transduced three times on days 1, 3 and 5. GFP and RFP expression in cells was analyzed 2 days after the last transduction (day 7 of culture). Representative dot plots of two-color FACS analyses of CD34+ cells transduced by (A) mock, (B) EF.GFP.CMV-RFP LV, and (C) EF.delFasL-CMV.GFP LV are shown. The values in each selected region are the percentages of gated cells analyzed. The coexpression of GFP and RFP in individual cells after the EF.GFP-CMV.RFP LV transduction was also analyzed by fluorescence microscopy (original magnification 10×), (D) under bright field (BF), (E) with filter for GFP, and (F) with filter for RFP. A merged image of (E) and (F) is shown in (G). The expression of both the delFasL and the GFP transgenes at mRNA level in the EF.delFasL-CMV.GFP LV-transduced CB CD34+ cells was analyzed by qRT-PCR (H), as described in Fig. 3G. No delFasL transgene was detected in the cells transduced with the control vector (indicated by * in H).
Since the specificity of available and commonly used FasL antibodies is in question [6], we could not determine FasL protein expression in individual cells by immunostaining. Instead, we examined the transgene expression of both the (mouse) delFasL and the GFP transgenes in transduced human cell populations by quantitative real-time RT-PCR (qRT-PCR) using specific primers and probe [21]. Expression of the delFasL transgene was not detected by qRT-PCR in the cell population transduced with the control EF.GFP vector, but was readily detected in the EF.delFasL-CMV.GFP-transduced cell population (Fig. 3G, left), whereas GFP expression was detected in cells transduced by either EF.GFP or EF.delFasL-CMV.GFP LV (right). The detected GFP transcript signal in the EF.delFasL-CMV.GFP-transduced 293T cells was approximately threefold greater than that in EF.GFP-transduced cells. To assess GFP mRNA levels in 293T cells that might be initiated by either the upstream EF promoter or the downstream CMV promoter, we performed Northern blotting using the GFP gene as the probe (Fig. 3H). As a total RNA loading control, we reprobed the blot using the β-actin housekeeping gene. Transcripts from the internal promoter (EF in the EF.GFP LV and CMV in the EF.delFasL-CMV.GFP LV) were detected as dominant bands (band a in Fig. 3H). The Northern blotting confirmed the result by qRT-PCR that the level of GFP transcript in the EF.delFasL-CMV.GFP-transduced 293T cells was approximately threefold greater than that in EF.GFP-transduced cells. The high level expression from the CMV promoter is consistent with the notion that the CMV promoter is exceedingly strong in 293T cells due to activation by the adenoviral E1A protein [22]. A longer transcript (band b) originating from the EF upstream promoter was also detected in the cell population transduced by EF.delFasL-CMV.GFP LV. It is unclear how much each transcript contributed to GFP expression at the protein level.

Coexpression of Two Genes by Dual-Promoter LVs in CD34+ HSCs

We next used the EF.GFP-CMV.RFP and EF.delFasL-CMV.GFP LVs to transduce human CD34+ HSCs from human placental/umbilical cord blood (CB). After transduction (on days 1, 3, and 5 of 7 days in culture), we analyzed transduced CB CD34+ cells for transgene expression (Fig. 4). In EF.GFP-CMV.RFP LV-transduced cells, GFP+/RFP+ double-positive cells were observed by FACS (Fig. 4B) and fluorescence microscopy (Figs. 4D–4G), although considerable numbers of single-positive GFP+ cells were also present. Next, we used the EF.delFasL-CMV.GFP LV to transduce CB CD34+ cells. Fifty-six percent of cells were GFP+ by FACS (Fig. 4C), in contrast to the low GFP expression using either the IRES or the GFP fusion strategy, as discussed above. In this case, GFP was the second (downstream) gene, whereas in the EF.GFP-CMV.RFP LV, GFP was the first gene and RFP the second gene. In cell populations transduced with the EF.delFasL-CMV.GFP LV, high levels of expression of both the GFP and the delFasL transgenes were observed by qRT-PCR (Fig. 4H). No delFasL transgene expression was present in control vector-transduced cells. The GFP expression in CD34+ cells transduced with the dual-promoter LV was even higher than in cells transduced with the EF.GFP control LV, similar to the results in transduced 293T cells (Fig. 3G). Our data showed that transduced cells expressing FasL can be prospectively identified by the coexpression of the GFP reporter gene (which was impossible in our previous studies).

Sustained Expression of Two Transgenes by a Dual-Promoter LV in Human Cells Engrafted in NOD/SCID Mice

In a separate ongoing project, we are investigating the role of the c-myc oncogene in leukemia induction using HSC transplant models. Based on the EF.GFP vector backbone, we made two vectors to express the mouse c-myc gene controlled by the EF promoter. In the first vector (EF.myc-IRES.GFP), the ECMV IRES was used to regulate GFP gene expression. In the second vector (EF.myc-PGK.GFP), the promoter of the phosphoglycerate kinase (PGK) housekeeping gene was used to mediate GFP expression (Fig. 1D). CB CD34+ cells were transduced by either the dual-promoter EF.myc-PGK.GFP or the bicistronic EF.myc-IRES.GFP LV, as described for Fig. 5. As we had observed in cell lines, GFP expression was not detectable in CD34+ cells transduced with the bicistronic EF.myc-IRES.GFP LV (Fig. 5B). In contrast, 37% of CB CD34+ cells expressed GFP after transduction with the EF.myc-PGK.GFP dual-promoter LV (Fig. 5C). Comparable levels of the mouse c-myc transgene expression were present in both transduced groups by qRT-PCR (Fig. 5D), indicating that the lack of GFP expression in cells transduced by the bicistronic EF.myc-IRES.GFP LV was not due to low transduction efficiency. Next, we transplanted the transduced cells into NOD/SCID mice (Figs. 5E–5G). Eight weeks after transplantation, we sacrificed the mice and then isolated bone marrow cells and subjected them to analysis by immunostaining/FACS and hematopoietic colony-forming cell (CFC) assays [23,24]. The presence of human leukocytes engrafted in mouse bone marrow was identified by staining with a monoclonal antibody against the human CD45 (common leukocyte antigen) (Figs. 5E and 5F). We detected similar levels of human CD45+ cells in all transplanted mice. In the mice that received CD34+ cells transduced by the dual-promoter EF.myc-PGK.GFP LV, 20% of the human CD45+ cells were GFP+. In contrast, only a background (0.6%) level of the human CD45+ cells were GFP+ in the mice that received the EF.myc-IRES.GFP LV-transduced cells (Fig. 5E). To enrich and analyze engrafted human cells further, we plated bone marrow cells from transplanted mice in either methylcellulose or liquid culture medium containing recombinant human cytokines that selectively stimulate the pro-
liferation of human cells [23,24,25]. After liquid culture for 7 days, human CD45\(^+\) cells were enriched similarly between the two groups. Forty-seven percent of the human CD45\(^+\)/H11001 cells in the EF.myc-PGK.GFP group were GFP\(^+\), whereas 0.4% of the human CD45\(^+\) cells in the EF.myc-IRES.GFP group were GFP\(^+\) (Fig. 5F). In CFC assays, 23% of the human hematopoietic cell colonies in the EF.myc-PGK.GFP group were uniformly green (GFP\(^+\)), as opposed to none in the EF.myc-IRES.GFP group (not shown). We randomly plucked individual green colonies in the EF.myc-PGK.GFP group or (any) colonies in the EF.myc-IRES.GFP group. We isolated total RNA from each plucked colony and examined it by qRT-PCR for the expression of the two transgenes (mouse \(c\)-\(myc\) and GFP, normalized to the expression of human \(\beta\)-actin as an internal control). Expression of both mouse \(c\)-\(myc\) and GFP was detected in all plucked green colonies (\(n=5\)) from the dual-promoter EF.myc-PGK.GFP LV-transduced group (Fig. 5G). The correlation of the expression levels of the two transgenes was high from these five random samples. Since all the progeny cells were visibly homogeneously green (GFP\(^+\)) and the both transgene expression levels were high in each plucked colony (by RT-PCR), many if not all of the individual cells within each colony which was derived from a single CFC must have expressed both \(c\)-\(myc\) and GFP. In the EF.myc-IRES.GFP group, the average level of the \(c\)-\(myc\) transgene expression in 6 plucked colonies was comparable to that in EF.myc-PGK.GFP group (Fig. 5G). However, GFP expression was undetectable in any of the same six colonies in the EF.myc-IRES.GFP LV-transduced group. By qRT-PCR, we estimated the GFP transcript level to be at least 50 times lower than that of the \(c\)-\(myc\) transgene in the same EF.myc-IRES.GFP LV-transduced colonies or that of the GFP transgene in the EF.myc-PGK.GFP LV (Fig. 5G). In a separate CFC assay using the CD34\(^-\) cells transduced by the EF.myc-CMV.GFP (in which the PGK.GFP expression cassette is replaced by the CMV.GFP cassette), we plucked 10 green (GFP\(^+\)) colonies as described previously. The qRT-PCR analysis revealed that both the GFP and the \(c\)-\(myc\) transgenes were highly and correlatively expressed harvested from the transplanted mice. The presence of human leukocytes was quantified by the human CD45 staining (\(x\) axis) and correlated with GFP expression (\(y\) axis). The percentages of GFP\(^+\) cells among the engrafted human (CD45\(^-\)) cells in the EF.myc-IRES.GFP and EF.myc-PGK.GFP groups are 0.6 and 20\%, respectively. The engrafted human cells within mouse bone marrow cells were further analyzed by liquid culture (F) and CFC assays (G) to favor the growth of human cells. The cells in liquid culture after 7 days were assessed again by FACS (F). The percentages of GFP\(^+\) cells among the engrafted human (CD45\(^-\)) cells in the EF.myc-IRES.GFP and EF.myc-PGK.GFP groups are 0.4 and 47\%, respectively. In the colony assay, individual GFP\(^+\) colonies (\(n=5\)) from the EF.myc-PGK.GFP-transduction group were randomly plucked for RNA isolation. Although no GFP\(^+\) colonies were present in the EF.myc-IRES.GFP-transduction group, individual colonies (\(n=6\)) were plucked and analyzed as well. The mRNA level of the \(c\)-\(myc\) and GFP transgenes from each single colony was quantified by qRT-PCR (G).
in each of the 10 colonies (data not shown). Therefore, our data strongly suggest that the dual-promoter LV mediated a high level of expression of both transgenes in individual transduced CFC and likely in all or most of their progeny. In contrast, the IRES-containing EF.myc-ires.GFP LV allowed the efficient expression of only the mouse c-myc transgene, and not the GFP transgene placed downstream of the IRES.

**Independent Transgene Expression in Cells Transduced with a Single LV Containing Dual Promoters**

The ability of SIN LVs to host two different internal promoters also provides the possibility of differentially or independently regulating expression of the two transgenes. We thus tested a dual-promoter LV containing a tissue-specific promoter and a ubiquitous promoter. We showed that the DR promoter in a single-promoter LV (DR.GFP) drove transgene expression selectively in major histocompatibility class II (MHC II⁺) cells [1]. To determine whether the DR promoter is independently regulated in MHC II⁺ cells from a dual-promoter LV, we cloned the CMV.DsRed2 reporter cassette into the DR.GFP vector to make CMV.RFP-DR.GFP (Fig. 1E). We monitored the expression of GFP (driven by the DR promoter) and RFP (driven by the CMV promoter) in transduced TF1 (MHC II⁺) cells (Fig. 6) or 293T (MHC II⁺) cells. GFP expression in 293T cells transduced by the CMV.RFP-DR.GFP LV was low (data not shown), as previously reported with the parental DR.GFP LV [1]. In CMV.RFP-DR.GFP-transduced TF1 cells, the majority of cells expressed both GFP and RFP (Fig. 6C). The percentages of GFP⁺ cells and the intensity of GFP fluorescence mediated were comparable to those obtained with the single-promoter DR.GFP vector (Fig. 6B). Therefore, the inclusion of a ubiquitous promoter (and a marker gene) did not alter the cell-specific activity of the DR promoter in a dual-promoter LV.

**Discussion**

We describe herein a new method to stably coexpress two (and possibly more) transgenes efficiently via LV-mediated gene transfer. In the past, we observed inconsistent results when an ECMV IRES element was used to mediate coexpression of both transgenes of a bicistronic LV. Although reduction of the expression level of the second gene downstream of the IRES was expected, quite often the protein encoded by the second gene was essentially undetectable, as observed in Fig. 5 with the bicistronic EF.myc-IRES.GFP LV. The exact mechanism of this phenomenon remains to be determined, but it appears to be associated with the specific sequence (and not merely the size) of first transgene. We have also analyzed the mRNA levels in cells transduced by either the bicistronic EF.myc-IRES.GFP LV or the EF.myc-PGK.GFP LV, using RT-PCR with specific primers for each transgene (Fig. 5G). Although c-myc mRNA levels were comparable between the two groups, GFP mRNA levels were at least 50 times lower in cell populations transduced with the IRES-containing LV. A similar result was observed with mRNA levels in cells transduced with the bicistronic EF.delFasL-IRES.GFP LV. The GFP (downstream gene in the bicistronic LV) mRNA level in the EF.delFasL-IRES.GFP LV-transduced cells was 80 times lower than in cells transduced with the dual-promoter EF.delFasL-CMV.GFP LV or the parental EF.GFP LV. In contrast, levels of the delFasL (upstream) mRNA were comparable in cells transduced by either EF.delFasL-IRES.GFP or EF.delFasL-CMV.GFP. Therefore, in these two cases in which we failed to detect significant GFP protein (fluorescence) in cells transduced with the IRES-containing LV, the block in GFP expression appears to occur prior to the translation initiation stage at which the IRES has been presumed to act. It is unclear whether the presence of the IRES (together with the upstream gene and possibly LV sequences) in a LV containing the EF promoter somehow either interferes with the transcription or results in a skip of the downstream GFP portion in the bicistronic transcript. More experiments are needed to explain these unexpected results further.

In contrast, when two independent ubiquitous promoters were used in the SIN LV backbone, we found that the two separate transgenes, each under the independent control of one internal promoter, were coexpressed efficiently in single cells or colonies. Using this type of dual-promoter LV, consistent and efficient coexpression of two transgenes was achieved in human CB (CD34⁺) HSCs and their progeny in vivo and in vitro and in a variety of other primary and cultured human cells. For example, the ma-
The majority of EF-GFP-CMV-RFP-transduced primary endothelial cells expressed both GFP and RFP proteins at the single-cell level (Fig. 2). In the CB CD34+ cells that were transduced by a LV expressing the murine c-myc as well as GFP, we detected the expression of both transgenes in each plucked colony derived from a single CFC after transduction and transplantation (Fig. 5). The expression levels of the two transgenes were highly correlated. The success of this approach using the LV backbone contrasts with reported failures associated with similar strategies in RVs, which resulted in severe titer reductions of transgene expression due to promoter interference [8,26,27]. One explanation may be that, with RVs, the intact LTR is required for generating high-titer viruses and is more sensitive to interference from an internal promoter [26,27]. Since the viral promoter/enhancer in the LTR has been deleted in the SIN LVs we used, the LTR limitation no longer exists. This may explain our success using this dual-promoter strategy based on SIN LVs.

We achieved favorable results in multiple cell types, using LVs containing various combinations of two genes and two promoters. However, the exact levels of transgene expression varied, likely depending upon the activity of promoters employed in a specific cell type (or specific developmental stage). Interestingly, the activity of a given promoter in the dual-promoter LVs largely paralleled what was observed in single-promoter expression vectors, either in a simple plasmid form or in the SIN LV backbone. For example, the relative strength of housekeeping gene promoters EF and PGK in the dual-promoter LVs was similar to that observed with single-promoter LVs [1], the PGK promoter was consistently weaker than the EF promoter in many cell types tested. The CMV promoter was more variable among different cell types than the EF and PGK promoters (see below). The DR promoter retained its cell specificity in a dual-promoter LV that contained a second ubiquitous promoter. Therefore, our approach is flexible, allowing choices as to which two different promoters are used in the LV to regulate the expression of two transgenes, either coordinately or independently, in the cells under investigation.

We can envision two potential problems with the dual-promoter LV design in some specific applications. In the first case in which fully independent expression of the two transgenes is desired, the expression from two adjacent transcriptional units may mutually interfere. Although we have not (yet) observed significant interference, it is possible that an enhancer element from one transcriptional unit can activate the promoter in the other unit from a distance. One way to prevent such interference might be to use insulator sequences that shield the influence from adjacent or remote DNA regulatory elements [28]. A more direct issue involves a "readthrough" transcript of the upstream promoter. With our dual-promoter LVs, two transcription units would elongate in the same direction and share the same downstream polyadenylation signal at the "R" region of the 3′ LTR.

Therefore, two transcripts would be made, which overlap in the downstream gene sequence. The presence of both transcripts was clearly demonstrated in our Northern blotting analysis (Fig. 3H). If tissue-specific transcription of a transgene is desired in cells transduced with a LV that also contains a ubiquitous promoter driving a marker gene, it may be preferable to place the tissue-specific promoter upstream of the ubiquitous transcriptional unit.

Another problem involves an application in which it is desired to coexpress two genes at precisely the same level. In theory, the same promoter/enhancer could be used twice to direct two separate genes. However, we did not use this approach to avoid the potential problem of DNA recombination (during vector construction and in transduced cells), although the exact risk level of recombination is unknown. Instead, we relied on the existence of multiple promoter elements with similar specificity (or lack of specificity) and strength. With increasing numbers of characterized promoter elements becoming available, there will be increasing choices for promoters. In the majority of gene transfer applications, it is not critical to have perfectly matched regulation expression of the two transgenes. For example, in many cases using coexpression of a reporter gene, it is sufficient if the reporter gene is efficiently and consistently expressed (regardless of its precise level relative to the first gene). Therefore, the LVs described herein will be useful in many applications. In these vectors, the EF promoter is used as the upstream gene to drive the gene of interest. The PGK or CMV promoter is used to drive the GFP or RFP reporter gene. CMV promoter-driven transgenes have been introduced by LVs into several types of cells, such as dendritic cells [29], hepatocytes [30], and neuronal cells [31], although CMV-regulated transgenes have been reported to be less active in vivo in HSC-derived progeny than the EF or PGK promoter [32–34]. Thus, our dual-promoter LVs containing the CMV promoter/enhancer driving a reporter gene will be useful for many in vitro applications and in applications in vivo with cell types permissive to the CMV promoter. If a lower but more consistent level of reporter expression is acceptable, as in our NOD/SCID mouse transplants with CD34+ HSCs (Fig. 5), then the EF and PGK dual-promoter LV is recommended.

There may be multiple potential improvements in the dual-promoter LVs described herein. For example, it has been shown that the central polypurine tract (cPPT, also called DNA flap) sequence from the HIV-1 gag gene region can enhance LV transduction [35]. It has also been reported that the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) increased gene expression levels in RVs and LVs by two- to threefold, when inserted downstream of the transgene and upstream of the 3′ LTR [36–38]. It is believed that WPRE enhances posttranscriptional processing of RNA, similar to the effect of RNA splicing. Although the LVs used in this study did not include either the cPPT or the WPRE yet still
produced satisfactory transcription efficiency and transgene expression, our dual-promoter LVs might be further improved by utilizing these elements to achieve a higher level of expression from the both transgenes. Last, insulator sequences could be added to our dual-promoter LVs, to prevent transcriptional interference between the two transcription units and/or to shield these two transcriptional units from the influence of cellular chromatin [28, 39]. Insulator elements may also reduce the potential activation of adjacent cellular genes after integration by the internal promoter/enhancer in LVs. Therefore, the addition of insulator elements to our SIN LVs, which lack the functional viral promotor/enhancer in both LTRs of integrated vector sequences, should further reduce the risk of adverse activation of cellular genes.

In conclusion, we developed SIN LVs carrying dual internal promoters that efficiently and independently mediated expression of two functional transgenes in multiple cell types, including engrafting human CD34+/HSCs. Consistent, efficient, and versatile LV transduction of multiple genes into a single cell will further enhance our ability to conduct laboratory investigations and clinical gene therapies.

METHODS AND MATERIALS

Human cell lines and primary cells. Human 293T cells were cultured in DMEM (Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA). The Jurkat human T leukemia cell line and human TF1 (CD34+/HLA-DR-) leukemia cell line were cultured in RPMI 1640 medium containing 10% FBS. GM-CSF (Peprotech, Rocky Hill, NJ) was added to TF1 cell cultures at 1 ng/ml. HI-AEC were obtained from Clonetics (BioWhittaker, Walkersville, MD) and cultured using the manufacturer’s protocol. Normal human placental/umbilical CB CD34+ cells, purchased from AllCells (San Mateo, CA), had been purified by immunomagnetic selection (Miltenyi Biotech, Auburn, CA) and were >90% CD34+ upon reanalysis by FACS. CB CD34+ cells were cultured at 2.5–5 × 10^5/ml in QBSF-60 serum-free medium (Quality Biologicals, Gaithersburg, MD) supplemented with 20 ng/ml thrombopoietin, 100 ng/ml Flt3-ligand, and 100 ng/ml stem cell factor (all recombinant human products from Peprotech) [24].

Construction of dual-promoter LVs. The EF.GFP-CMV.RFP LV was constructed by inserting, immediately downstream of EF.GFP in the EF.GFP transducing vector into 293T cells with two packaging vectors: pMD.G, a plasmid expressing the VSV-G envelope gene, and pCMVΔR8.91, a plasmid expressing the HIV-1 gag/pol, tat, and rev genes as previously described [1]. The virus supernatants were collected at 24 and 48 h after transfection. Viral titers were determined by the percentage of GFP+ 293T or TF1 cells that had been transduced with serial dilutions of neat or concentrated LV supernatants as previously described [1].

LV transductions. LV supernatants were concentrated using filtration columns (Centriprep Plus-20, molecular weight cutoff 100 kDa; Millipore, Bedford, MA) as previously described [1] and added to target cells at multiplicity of infection of 1–5 in the presence of 4–8 μg/ml Polybrene (Sigma, St. Louis, MO). Transduced cell lines were placed at a density of 5 × 10^3/ml in 2 ml and analyzed 48–72 h after one transduction. To achieve high transduction efficiencies in CB CD34+ cells, transduction was done three times (on days 1, 3, and 5), and then cells were examined on day 7. Transduced cells were analyzed by multicolor FACS analysis (see below) and by fluorescence microscopy using green and red filters (Nikon). Microscopic images were photographed using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

RNA isolation and qRT-PCR analysis. RNA isolation and qRT-PCR assays were used to determine the gene expression level with the use of gene-specific primers and fluorescently labeled TaqMan probes (ε-carboxy fluorescein as the 5’ fluorescent reporter, tetramethylrhodamine as 3’ end quencher) or SYBR green dye (Molecular Probes, Eugene, OR) in a 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The mouse delFasL primer and TaqMan probe sequences were used as follows: forward primer, 5’ CCTG-CAGAACAGACTGCG 3’; reverse primer, 5’ CCGTGAGGGGCAAACGCAGTACACC 3’. The GFP primer and probe sequences were forward primer, 5’ AGTGCTTACGG-CGCTACCC 3’; reverse primer, 5’ ATGTGTTGCGTCTTCAGG 3’; probe, 5’ AGTCGCGCATGGCGAAGGCT 3’. Mouse c-myc primer sequences were forward primer, 5’ CAGCGACGTGAAAGAGGACG 3’; reverse primer, 5’ GGCTGTCATTCCAGGCCGTC 3’. The human β-actin primer and probe sequences have been described previously [21].

Northern blotting. Ten micrograms of total RNA isolated from LV-transduced cells was denatured and separated via a 1% agarose gel. RNA was transferred to a nylon transfer membrane (Gene Screen Plus; Perkin-Elmer Life Sciences, Inc., Boston, MA) and fixed by UV irradiation. The membrane was blotted with the GFP probe first and then was stripped for second blotting using the β-actin probe. The GFP and β-actin probes were 5’P labeled by a random primer labeling kit (Prime-it II; Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Prehybridization and hybridization were carried out at 42°C using ULTRAhyb hybridization buffer (Ambion, Austin, TX).

Antibodies and flow cytometry. FACSscan and FACSsort (Becton-Dickinson, San Jose, CA) flow cytometers equipped with an argon ion laser (tuned at 488 nm) were used for FACS analysis [23,24]. Green fluorescence was detected in the FL2 channel (525 ± 20 nm), and red fluorescence from RFP was detected in the FL3 channel (578 ± 28 nm). This machine setting allows the near-maximal excitation for GFP, but only 40% of maximal RFP excitation (peaked at 583 nm). PerCP-conjugated anti-human CD45 antibody and isotype control antibodies were purchased from Becton-Dickinson and monitored by the FL3 channel. Fluorescence microscopy of GFP and RFP expression was performed with a Nikon (TE300) microscope with separate filters for either GFP or RFP (Chroma Technology Corp., Brattleboro, VT). The excitation and emission spectra for the GFP filter is 470 ± 20 and 525 ± 25 nm, respectively, and for the RFP filter is 545 ± 30 and 610 ± 75 nm, respectively.

Fas-mediated cytotoxicity assay. 293T cells (2 × 10^3 per 2 ml) were plated in six-well plates. After overnight incubation, cells were transduced using concentrated LVs containing a noncleavable delFasL coding sequence or controls. Forty-eight hours after transduction of the 293T cells, 105 Jurkat target cells were added to each well and cocultured overnight. The Jurkat cells were carefully removed for assays of cell death and apoptosis. Briefly, suspension Jurkat cells were harvested and stained with annexin V and 7-AAD (Viaprobe; Becton-Dickinson) for 15 min in the dark and immediately analyzed by multicolor FACS [7,40].
Human CB CD34+ cell engraftment of NOD/SCID mice and CFC assays. Immunodeficient NOD/SCID mice, originally obtained from The Jackson Laboratory (Bar Harbor, ME), were bred and housed in the animal facility at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins. Transduced cells were intravenously injected into sublethally irradiated (5 Gy) 6- to 8-week-old mice (10^6 input cells per mouse). Cells were plated for direct CFC assays simultaneously. Mice were sacrificed 8 weeks after transplantation for collection of BM cells. Engraftment of human cells was determined using PerCP-conjugated anti-human CD45 antibody staining. Engrafted human cells were further enriched in liquid human cell selective culture for 7 days and then analyzed by FACs. BM cells from transplanted mice were also plated for secondary CFC colonies using conditions selective for human CFUs as previously described [1,23,24]. After 14 days, green grafted human cells were further enriched in liquid human cell selective medium. Transduced cells were intravenously injected into sublethally irradiated (3 Gy) 6- to 8-week-old mice (10^5 input cells per mouse). Cells were plated for direct CFC assays simultaneously. Mice were sacrificed 8 weeks after transplantation for collection of BM cells. Engraftment of human cells was determined using PerCP-conjugated anti-human CD45 antibody staining. Engrafted human cells were further enriched in liquid human cell selective culture for 7 days and then analyzed by FACs. 

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