Generation of Transgenic Mice Using Lentiviral Vectors: 
A Novel Preclinical Assessment of Lentiviral Vectors for Gene Therapy

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Lentiviral vectors have become attractive delivery vehicles for gene therapy investigators. Specifically, the ability of lentiviral vectors to integrate into nondividing cells and provide stable and long-term gene expression in vivo is a desirable attribute of gene therapy approaches. We report here a simple method for generating transgenic mice using lentiviral vectors, which could be useful models for gene therapy. After removal of the zona pellucida, fertilized eggs were co-incubated with oncoretroviral or lentiviral vectors. The resulting blastocysts were transferred into uteri of pseudo-pregnant females. In both cases, around 60–70% of founder pups were transgenic as determined by PCR analysis. Southern blot analysis revealed that the transgenes were integrated at different genetic loci and transmitted through the germ line. Most of the transgenes delivered by lentiviral vectors were expressed in transgenic mice, although those delivered by oncoretroviral vectors were completely silenced. When the upstream sequences of the rhodopsin gene and the red pigment gene were used as tissue-specific promoters, consistent enhanced green fluorescent protein (EGFP) expression was observed in rod and cone photoreceptor cells, respectively, in retina. However, mice generated with the corneal epithelium-specific keratin-12 promoter displayed EGFP expression not only in cornea but also in other tissues of the mouse. We conclude that the generation of transgenic mice using lentiviral vectors is a simple and robust method to evaluate the promoter specificity in lentiviral vectors in vivo prior to undertaking a gene therapy strategy.

Key Words: lentivirus, oncoretrovirus, transgenesis, gene silencing, ubiquitous, tissue specific, promoter, rhodopsin, red pigment, keratin-12

INTRODUCTION

Oncoretroviral vectors have the potential of stable gene expression by integrating a transgene(s) into the host genome. They are the most commonly used gene delivery tool for clinical and preclinical gene therapy investigations; however, gene silencing has been an obstacle to achieving long-term expression for therapeutic purposes [1,2]. Two major mechanisms of oncoretrovirus silencing have been identified [3]. One involves trans-acting factors that bind to the viral promoters in the long terminal repeats (LTRs), and the other is methylation of the integrated oncoretroviral genome and flanking host DNA sequences. Since the mechanisms are not fully understood, it is difficult to develop a modified oncoretroviral vector that can readily escape gene silencing. Lentiviral vectors are complex retroviruses with the distinguishing property of being able to transduce both dividing and nondividing cells [4]. Moreover the transgenes delivered by lentiviral vectors are capable of escaping gene silencing and expressing stably in vivo [5,6].

In gene therapy experiments, generation of transgenic mice can provide information about safety, efficacy, and tissue specificity of transgenes. Although pronuclear injection is a commonly used method, it is not suitable to evaluate the retroviral vectors since the mechanism of integration is different. Each transgene delivered by retroviral vectors usually integrates as a single provirus into
multiple loci, while pronuclear injection often results in an integration of multiple copies into a single locus [7]. As an alternative method, infection of preimplantation embryos using an oncoretroviral vector is available, but the hurdle of gene silencing has not been overcome [1]. Recently we and others developed a method to generate transgenic mice using lentiviral vectors and showed the germ-line transmission of transgenes and the lack of gene silencing [8,9]. However, there was no direct comparison of transgene expression or integrations in oncoretrovirus- and lentivirus-mediated transgenic animals.

In the present study, we generated transgenic mice using oncoretroviral and lentiviral vectors carrying the same enhanced green fluorescent protein (EGFP) expression cassette. We generated Moloney murine leukemia virus-based oncoretroviral vectors (RVs) and human immunodeficiency virus type 1 (HIV-1)-based lentiviral vectors (LVs). In both cases, virally delivered transgenes efficiently integrated into the mouse genome and were inherited through the germ line. However, consistent with previous studies [8,9], stable and long-term expressions were observed only in transgenic mice generated by lentiviral vectors. We next evaluated the tissue specificity of various promoters in vivo. Appropriate expression of EGFP was observed when tissue-specific promoters such as rhodopsin gene and red pigment gene promoters were used. Surprisingly we observed leakage of the transgene expression in transgenic mice generated with lentiviral vector carrying the keratin-12 gene promoter, which was expected to be corneal epithelium-specific. We propose that lentiviral vectors are excellent tools for generating transgenic animals and that it is also possible to evaluate the nature of tissue-specific promoters before applying those promoters to the gene therapy strategy.

RESULTS

Preparation of Lentiviral and Oncoretroviral Vectors
Lentiviral vectors employed in this study are based on a self-inactivating vector (SIN vector) characterized by transcriptional inactivation of the integrated virus because of 3’LTR deletion mutations [10] (Fig. 1A). Lentiviral vectors carry the central polypurine tract (cPPT) of the pol gene of HIV-1, which has been shown to enhance nuclear translocation of HIV-1 DNA [11,12]. Addition of the cPPT in oncoretroviral vectors has apparently no influence on their inability to infect nondividing cells ([11,12], unpublished data). For ubiquitous transgene expression, we used CAG and cytomegalovirus (CMV) gene promoter sequences. The CAG promoter is an artificially modified chicken β-actin gene promoter [13] that shows ubiquitous EGFP expression in transgenic mice generated by pronuclear microinjection [14]. In both retroviral vectors, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was placed after the EGFP sequence to enhance the transgene expression [15]. When we transduced 293T cells by oncoretroviral and lentiviral vectors at an m.o.i. of 1, almost all cells expressed EGFP 2 days after infection (Fig. 1B). For tissue-specific expression, we employed the rhodopsin (Rho) gene [16,17], red pigment (RG) gene [18], and keratin-12 (K12) gene [19] promoters to express EGFP in rod photoreceptor cells, cone photoreceptor cells, and corneal epithelium, respectively.

Generation of Transgenic Mice Using Lentiviral and Oncoretroviral Vectors
We generated transgenic mice using viral vectors carrying EGFP under the control of the CAG and CMV promoters.
no EGFP protein was observed (Fig. 2B). Protein was loaded for RV-CMV-EGFP transgenic founders, but we detected the expression of EGFP only in lentiviral-mediated transgenic pups and not in the oncoretroviral-mediated transgenic pups (Fig. 2A). All the F0 pups (F0-1 to F0-11) show multiple integrations of proviral DNA, but we detected the expression of EGFP only in LV-CMV-EGFP transgenic mice (Fig. 2B). Four of six LV-CMV-EGFP transgenic founder mice expressed different levels of EGFP, which appeared to correlate with copy number. In contrast, although 10-fold greater total protein was loaded (Fig. 2D). We examined at least two independent transgenic founder mice that carried multiple copies of transgene (2.4 kb) [18]. With the bovine rhodopsin gene promoter, EGFP was expressed only in photoreceptor cells, which consist of rod and cone photoreceptor cells, in LV-Rho-EGFP transgenic mice. Analysis of frozen sections of the eye showed EGFP expression throughout the retina (Fig. 3A). Only the cells located at the outer nuclear layer (ONL), where the photoreceptor cells exist, expressed EGFP in the eye (Fig. 3B). These results are compatible with the phenotype of transgenic mice generated by pronuclear injection of a transgene carrying the same promoter sequence (2.4 kb) [17]. Most photoreceptor cells that express EGFP are rod photoreceptor cells because around 97% of photoreceptor cells are rod photoreceptor cells, although we cannot totally exclude the possibility that cone photoreceptor cells also express EGFP. We next obtained transgenic mice using the human red pigment gene promoter to express EGFP in a small part of the photoreceptor cells. The photoreceptor cells that express EGFP will be cone photoreceptor cells based on the following three facts. First, the cells confined to the outer half of the ONL, where the cone photoreceptor cells exist [20], expressed EGFP (Fig. 3C). Second, cone photoreceptor cells are also distinguishable from rod photoreceptor cells by their morphological differences [20]. Third, transgenic mice carrying the same promoter sequence (2.1 kb) [18] show cone

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter</th>
<th>Blastocysts transferred</th>
<th>Pups born (%)</th>
<th>Pups weaned</th>
<th>Transgenic pups (%)</th>
</tr>
</thead>
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<tr>
<td>RV</td>
<td>CAG</td>
<td>52</td>
<td>29 (56)</td>
<td>29</td>
<td>17 (59)</td>
</tr>
<tr>
<td>RV</td>
<td>CMV</td>
<td>64</td>
<td>22 (34)</td>
<td>21</td>
<td>13 (62)</td>
</tr>
<tr>
<td>LV</td>
<td>CAG</td>
<td>110</td>
<td>28 (25)</td>
<td>23</td>
<td>15 (65)</td>
</tr>
<tr>
<td>LV</td>
<td>CMV</td>
<td>40</td>
<td>7 (18)</td>
<td>7</td>
<td>7 (100)</td>
</tr>
</tbody>
</table>

Both transgenic mice expressed EGFP in all tissues that we examined (Fig. 2D). As far as we examined, there was no obvious difference in copy number between founder mice or in inheritance pattern of transgenes between transgenic mice carrying CMV and CAG promoters (data not shown). We could also detect EGFP expression ubiquitously in LV-CMV-EGFP transgenic mice, similar to LV-CAG-EGFP transgenic mice (data not shown).

**Tissue-Specific Expression in Transgenic Mice Generated Using Lentiviral Vectors**

We generated transgenic mice using lentiviral vectors that express EGFP in a tissue-specific manner. We employed the rhodopsin, red pigment, and keratin-12 gene promoters for rod photoreceptor cell-, cone photoreceptor cell-, and corneal epithelium-specific expression, respectively. As shown in Table 2, efficiency of transgenesis did not depend on the promoter. Approximately 80% of founder pups (28/34) generated by each lentiviral vector carried a transgene(s) detected by PCR and/or Southern blot analyses and were comparable to the data obtained with the CMV and CAG ubiquitous promoters (Table 1).
FIG. 2. Generation of transgenic mice using oncoretroviral and lentiviral vectors. (A) Transgenic pups derived from LV-CAG-EGFP- (upper row) or RV-CAG-EGFP- (lower row) treated embryo. Green fluorescence was observed under a fluorescence stereomicroscope (right of each row) and photos were taken under similar conditions. The whole body of the LV-CAG-EGFP transgenic pup is also shown (bottom). (B) The copy numbers of transgenic founder mice generated by LV-CMV-EGFP or RV-CMV-EGFP treatment were determined (top) by Southern blot analysis. Each specific integration band is indicated (white arrowhead). Genomic DNA from each mouse was digested with BamHI and hybridized with an EGFP probe. Lane 1, 10 pg of GFP-LV; lane 2, genomic DNA from wild-type mouse. EGFP protein expression in muscle tissue of those mice was also examined (bottom) by Western blot analysis. Lane 1, positive control, 10 μg of protein derived from muscle tissue of LV-CAG-EGFP transgenic mouse; lane 2, negative control, 10 μg of protein derived from muscle tissue of wild-type mouse. (C) The inheritance of transgenes to the F1 progeny was confirmed in the transgenic founder generated by LV-CMV-EGFP (top). Each specific integration band indicated by each small letter in lane 3 (F0-1) was also detected in the F1 progeny. EGFP protein expression in muscle tissue of those mice was also examined (bottom) by Western blot analysis. Lanes 1 and 2, see (B). (D) EGFP expression in each tissue of wild-type mouse (top), transgenic mice generated by LV-CAG-EGFP (second row) and RV-CAG-EGFP (third row) treatment, and transgenic mouse generated by pronuclear injection of plasmid carrying EGFP cassette driven by CAG promoter (TgN(beta-act-EGFP)) (bottom) was examined by Western blot analysis. Ten or 1 μg of protein derived from each tissue of wild-type and RV-CAG-EGFP transgenic mouse or LV-CAG-EGFP transgenic mouse and TgN(beta-act-EGFP) was loaded into each well. Proteins transferred to the membrane were probed by anti-EGFP antibody. One and 10 μg of protein derived from muscle tissue of transgenic mouse generated by LV-CAG-EGFP were used as positive controls (P1 and P10, respectively).
photorceptor cell-specific reporter gene expression that is similar to our results. Since EGFP fluorescence in LV-RG-EGFP transgenic mouse retina was so weak, autofluorescence derived from the interphotorceptor matrix was also captured due to long exposure (Fig. 3C). However, we never observed EGFP fluorescence in the ONL region of wild-type mice (data not shown). To confirm tissue-specific expression, we extracted proteins from various tissues and subjected them to Western blot analysis. In both LV-Rho-EGFP and LV-RG-EGFP transgenic mice, EGFP was detected mostly in the retinal tissues (Fig. 3D). Since the number of cone photoreceptor cells is smaller, the amount of EGFP detected is considerably lower.

Finally we generated transgenic mice expressing EGFP in a corneal epithelium-specific manner using the mouse keratin-12 gene promoter. Although we observed autofluorescence at the retinal region in both wild-type mouse and LV-K12-EGFP transgenic mouse as discussed above, we detected EGFP fluorescence in the corneal region only in the LV-K12-EGFP transgenic mouse (Fig. 4A). At higher magnification we could identify EGFP expression in the corneal epithelial cells (Fig. 4B). Again we examined EGFP expression in various tissues in LV-K12-EGFP transgenic mice to determine their tissue-specific gene expression. Surprisingly, not only the corneal tissue but also other tissues, including brain, skin, heart, and kidney, expressed EGFP in the different lines of LV-K12-EGFP transgenic mice, even if endogenous keratin-12 expression was detected only in cornea (Fig. 4C).

**DISCUSSION**

In this study, transgenic mice were efficiently generated by infecting fertilized eggs with vesicular stomatitis virus envelope protein (VSV-G)-pseudotyped oncoretroviral and lentiviral vectors. By using the same EGFP expression cassettes, we could compare directly the results with lentiviral and oncoretroviral vectors. EGFP expression was observed by oncoretroviral-mediated transgenesis while transgenes delivered by oncoretroviral vectors were completely silenced. Previous studies have also shown the lack of gene silencing in lentiviral-vector-mediated transgenic mice and rats [8,9]. With SIN viral vectors, the EGFP reporter gene was driven by the internal promoter inserted between the LTRs. The restricted tissue expression confirms that in SIN vectors, little or no expression is initiated in the LTR [10]. Since the procedure to generate transgenic animals is relatively easy and cost effective and the expression pattern of the transgene is similar between LV-CAG-EGFP transgenic mice and TgN(beta-act-EGFP) mice, this method could be an alternative to conventional pronuclear injection. Our approach requires neither an expensive machine such as a micromanipulator nor fine techniques to inject DNA into the pronucleus. High efficiency of transgenesis and no need to visualize the pronucleus may also be advantages when manipulating large live-stock animals or nonhuman primates [21] into which the introduction of exogenous genes is difficult and inefficient. Moreover, the multiple proviral insertions and germ-line transmission (Fig. 2) provide a prospective genetic approach for large-scale gene disruption projects that have previously been performed in a variety of species, including fly, zebrafish, and mouse [22 – 24]. Infection of fertilized eggs instead of embryonic stem cells could circumvent the expensive and time-consuming procedure of making chimeric mice prior to obtaining F1 generation. Thus, lentiviral vectors could be very useful for gene-trap experiments in vivo. However, it should be remembered that denuded embryos were delayed in their development in vivo with respect to their untreated counterparts. We cannot exclude the possibility that removal of the zona pellucida might affect each developmental stage.

Understanding the nature of a viral vector in vivo is a prerequisite for clinical gene therapy strategies. In addition to the problem of random integration, two potential problems that can occur with the lentiviral vector during gene delivery include toxicity of the virus partially due to presence of the VSV envelope protein and unexpected transgene expression in tissues other than the target site. The former problem could be lessened by replacement of the virus envelope (e.g., Ross River virus (RRV) glycoproteins or amphotrophic glycoprotein) [25]. It has been reported that RRV glycoproteins causes less cytocotoxicity [25]. The problem of inadvertent expression can be avoided by testing the reporter gene expression in transgenic mice generated by lentiviral vectors harboring appropriate promoters as shown in this study. Although multiple proviral insertions make it difficult to establish pure-breeding transgenic lines, this could be an advantage for evaluating the nature of lentiviral vectors in vivo since the situation is more similar to the clinical gene therapy.

**TABLE 2: Generation of lentiviral-mediated transgenic mice with tissue-specific promoters**

<table>
<thead>
<tr>
<th>Promoter</th>
<th>m.o.i.</th>
<th>Blastocysts transferred</th>
<th>Pups born (%)</th>
<th>Pups weaned</th>
<th>Transgenic pups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>$5 \times 10^5$/egg</td>
<td>40</td>
<td>2 (5)</td>
<td>2</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Rho</td>
<td>$5 \times 10^5$/egg</td>
<td>20</td>
<td>11 (55)</td>
<td>9</td>
<td>7 (78)</td>
</tr>
<tr>
<td>RG</td>
<td>$3 \times 10^5$/egg</td>
<td>60</td>
<td>18 (30)</td>
<td>18</td>
<td>14 (78)</td>
</tr>
<tr>
<td>K12</td>
<td>$6 \times 10^5$/egg</td>
<td>60</td>
<td>5 (8)</td>
<td>5</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>36 (20)</td>
<td>34</td>
<td>28 (82)</td>
<td></td>
</tr>
</tbody>
</table>
Our data suggest that genes driven by the rhodopsin gene and red pigment gene promoters (Fig. 3) can be used for correcting rod photoreceptor cell diseases such as retinitis pigmentosa [16] and cone photoreceptor cell diseases such as cone dystrophy. Shiraishi et al. reported that the keratin-12 gene promoter sequence (2.7 kb) contained a corneal epithelial cell-specific regulatory cis-DNA element and that reporter gene expression was observed in corneal, but not conjunctival or epidermal, epithelial cells after plasmid transfection into rabbit cornea, conjunctiva, and skin by particle-mediated gene transfer [19]. We employed the exact same region to generate transgenic mice that express EGFP in a corneal epithelium-specific manner. Surprisingly, as shown in Fig. 4C, we observed EGFP...
expression not only in the corneal tissue, but also other tissues including brain, heart, and kidney in the different lines of LV-K12-EGFP transgenic mice. The expression in noncorneal tissues might be caused by leakage of the transgene(s), since we demonstrated that endogenous keratin-12 expression is detected only in cornea and a previous report showed that keratin-12 is a corneal epithelium-specific cytokeratin [26]. The leakage of transgene expression occurred in LV-K12-EGFP transgenic mice unexpectedly. This means that more selectivity of the regulatory element will be required to develop a reliable vector for transgene delivery to the corneal epithelium.

From this study, we conclude that the lentiviral vector could be useful for generating transgenic mice for both ubiquitous and tissue-specific transgene expression. Moreover, the approach described here could be a novel method to evaluate the nature of lentiviral vectors in vivo prior to the gene therapy strategy.

MATERIALS AND METHODS

Preparation of viral plasmids and viral vector production. We constructed pRV-CMV-EGFP and pRV-CAG-EGFP in the oncoretroviral vector pCLNCX [27] by replacing a fragment containing the neom cassette and the
CMV promoter with CMV-EGFP-WPRE or CAG-EGFP-WPRE, respectively. We constructed plV-CMV-EGFP and plV-CAG-EGFP plasmids in the HIV-based lentiviral vector pRRLsin-hPGK-EGFP [12] by replacing the hPGK fragment with the CMV or CAG promoter fragment. For tissue-specific gene expression, the bovine rhodopsin gene, human red pigment gene, and mouse keratin-12 gene promoters were obtained from pHIV-Rho-GFP [16], pR2.1 LacZ [18], and 2.SKZ [19], respectively. These promoter sequences were subcloned into pUbiascuit II SK (+) (Strategene, La Jolla, CA), resulting in pBS-SK-Rho, pBS-SK-RG, and pBS-SK-K12. Lentivirus vectors plV-Rho-EGFP, plV-RG-EGFP, and plV-K12-EGFP were constructed by replacing the CMV promoter fragment in GFP-LV [28] with the Cial-Xbal fragments of pBS-SK-Rho, pBS-SK-RG, and pBS-SK-K12. All constructs were confirmed by direct sequencing. VSV-G protein-pseudotyped oncortival or lentiviral vectors were generated as described [12]. In brief, 293gp cells or 293T cells were transfected with the vector, packaging plasmids, and a plasmid coding for the VSV-G envelope protein by the calcium phosphate method. Virus was harvested over the following 3 days and concentrated by ultracentrifugation (68,000 g). The multiplicity of infection (m.o.i.) of virus carrying CMV and CAG promoter sequence was determined by infecting 293T cells, followed by flow cytometric quantification of EGFP-positive cells. The titer of lentiviral vectors was further determined by measuring the amount of HIV p24 gag antigen by ELISA (NEN Life Science Products, Boston, MA). The m.o.i. of other lentiviral vectors was estimated using the following equation: 1 ng of p24 = 10^5 infectious units. Treatment of embryos and generation of transgenic mice. B6D2F1 females were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later and then mated with B6D2F1 males. Two-cell-stage embryos were collected from the oviducts of the copulated female 36 h after injection of human chorionic gonadotropin. To remove zona pellucida, the embryos were placed in acidic Tyrode's solution [29] for 30 s to 1 min. After a dissociation of zona pellucidae was confirmed, embryos were washed three times with KSOM [30] and incubated at 37°C for 2.5 days in a 5%-CO2 drop of KSOM containing viral vectors at an m.o.i. of ~10^4. Blastocysts developed from infected embryos were transferred into 2.5-dase-pregnant females.

PCR and Southern blot analysis. DNA extracted from the tail tips of WEHI was amplified with primers EGFPr (5'-GGCAACATGGTAGACAAAGGGCCGAG-3') and EGFPrev (5'-CTTTCTCGTTCCTCT-3') to check the existence of the EGFP transgene(s). For Southern blot analysis, genomic DNA (20 µg) was digested with BamHI, separated by electrophoresis in a 0.8% agarose gel, and blotted onto a nylon membrane before hybridization with the 32P-random-prime-labeled 754-bp XbaI-SalI fragment of GFP-LV.

Western blot analysis. Lysates from each tissue were subjected to SDS-PAGE under reducing conditions. Proteins transferred to a nitrocellulose membrane were probed with a rabbit anti-EGFP antibody (Clontech, Palo Alto, CA) and rabbit anti-actin antibody (Sigma, St. Louis, MO). The immunoreactive bands were visualized with an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Histological analysis. Eyes were fixed with 4% paraformaldehyde and cryoprotected with 30% sucrose prior to being frozen in OCT compound (Sakura Finetek U.S.A., Torrance, CA) and then sectioned on a cryostat. The cell nuclei were counterstained with DAPI. Each section was observed under a fluorescence microscope and the images were recorded with the Axio Vision imaging system (Zeiss).

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