Use of the *piggyBac* Transposon to Create Stable Packaging Cell Lines for the Production of Clinical-Grade Self-Inactivating γ -Retroviral Vectors

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Abstract

Efforts to improve the biosafety of γ -retroviral-mediated gene therapy have resulted in a shift toward the use of self-inactivating (SIN) γ -retroviral vectors. However, scale-up and manufacturing of such vectors requires significant optimization of transient transfection-based processes or development of novel platforms for the generation of stable producer cell clones. To that end, we describe the use of the *piggybac* transposon to generate stable producer cell clones for the production of SIN γ -retroviral vectors. The *piggybac* transposon is a universal tool allowing for the stable integration of SIN γ -retroviral constructs into murine (PG13) and human 293-based Phoenix (GALV and RD114, respectively) packaging cell lines without reverse transcription. Following transposition, a high-titer clone is selected for manufacture of a master cell bank and subsequent γ -retroviral vector supernatant production. Packaging cell clones created using the *piggybac* transposon have comparable titers to non-SIN vectors generated via conventional methods. We describe herein the use of the *piggybac* transposon for the production of stable packaging cell clones for the manufacture of clinical-grade SIN γ -retroviral vectors for *ex vivo* gene therapy clinical trials.

Introduction

γ -RETROVIRAL VECTORS HAVE BEEN USED successfully in a variety of gene therapy clinical trials for the treatment of patients with immunodeficiency, infectious disease, and cancer (Blaese *et al.*, 1995; Walter *et al.*, 1995; Heslop *et al.*, 1996; Cavazzana-Calvo *et al.*, 2000; Gaspar *et al.*, 2004; Morgan *et al.*, 2006; Ott *et al.*, 2006). Most vectors used in these clinical trials have been replication-deficient, but non-self-inactivating (non-SIN). The transgene in non-SIN vectors is driven by the enhancer/promoter from the 5' long terminal repeat (LTR) following reverse transcription and integration of the provirus. Manufacture of non-SIN clinical-grade γ -retroviral vectors typically involves use of a packaging cell line that has been transduced with the vector encoding the gene of interest followed by selection of a high-titer packaging cell clone (Onodera *et al.*, 1997; Hughes *et al.*, 2005; Feldman *et al.*, 2011). A majority of the clinical trials to date have used non-SIN vectors. However, in a trial designed to treat patients with X-linked severe combined immunodeficiency (X-SCID), some patients de-

veloped leukemia following transfer of CD34⁺ hematopoietic stem cells transduced with a non-SIN γ -retroviral (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2003). This finding has raised concerns over the safety of such vectors as there may be an increased risk of insertional mutagenesis, particularly in CD34⁺-transduced cells.

In an attempt to improve vector biosafety, some in the field of gene therapy have moved to using SIN vectors. The primary focus was on SIN lentiviral vector (LVV) development, as LVV appeared to have a more random integration profile. However, the risk of insertional transformation is not completely abrogated by the use of these vectors (Wu *et al.*, 2003; Modlich *et al.*, 2009). Emerging evidence suggests that the enhancer/promoter elements driving transgene expression may play a greater role in insertional mutagenesis than the actual vector insertion pattern (Zychlinski *et al.*, 2008; Modlich *et al.*, 2009). There is evidence to suggest that the use of SIN vectors, γ -retroviral or LVV, can reduce the genotoxic potential of these vectors following integration of the provirus (Modlich *et al.*, 2006; Montini *et al.*, 2006). Therefore, γ -retroviral vectors are still a viable

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option for gene therapy clinical trials. Currently, there are no data to definitively demonstrate that SIN vectors will have a safer biosafety profile. However, if such vectors are to be used in gene therapy clinical trials, the challenge that remains is the difficulty of large-scale production of SIN γ -retroviral vectors.

Currently, most manufacturing of SIN γ -retroviral vectors involves transient transfection of human 293T cells followed by extensive downstream processing (Segura *et al.*, 2011). Manufacture of non-SIN γ -retroviral vectors has been relatively simple because of the availability of stable producer cell clones that can be easily expanded for vector production. However, such producer cell clones have been difficult to generate for SIN γ -retroviral vectors using conventional methods (Maetzig *et al.*, 2011). Recently, efforts to develop packaging cell lines for SIN γ -retroviral vector production have been forthcoming. Novel PG13-based packaging cell lines for production of clinical-grade SIN γ -retroviral vectors have been recently described (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006; Loew *et al.*, 2009). SIN γ -retroviral vectors were integrated into a predefined region of the PG13 chromatin using homologous recombination (referred to as recombinase-mediated cassette exchange [RMCE]). A stable high-titer producer clone was selected using a tagging vector encoding green fluorescent protein (GFP) that could then be exchanged via RMCE with a second γ -retroviral vector cassette encoding a novel transgene (Loew *et al.*, 2009). We describe an alternate approach to generating SIN γ -retroviral vector packaging cell clones utilizing the *piggybac* transposon system.

The *piggybac* transposon system, derived from the cabbage looper moth *Trichoplusia ni*, was discovered from a mutant baculovirus strain (Cary *et al.*, 1989), and integrates into the host cell genome by transposition into TTAA nucleotide elements (Fraser *et al.*, 1996). Transposons, *Sleeping Beauty* (SB) and *piggybac*, are becoming more common gene delivery vehicles for the genetic modification of human cells (Geurts *et al.*, 2003; Wilson *et al.*, 2007; Singh *et al.*, 2013). The advantages of *piggybac* system include more efficient gene transfer into mammalian cells and the ability to package large DNA inserts ranging from 9 to 14 kb, such as a retroviral vector cassette, without a significant loss in transposition efficiency (Wilson *et al.*, 2007). Thus, the *piggybac* platform allows for stable integration of a packageable SIN γ -retroviral vector into a variety of γ -retroviral packaging cell lines without the need for reverse transcription. High-titer stable producer cell clones can be generated within 1 month's time, which fits easily into an established production platform for the manufacture of conventional clinical-grade γ -retroviral vectors (Feldman *et al.*, 2011).

Materials and Methods

Patient peripheral blood mononuclear cells and cell lines

Peripheral blood mononuclear cells and lymphocytes used for transduction were obtained from aphereses of melanoma patients on IRB-approved surgery branch protocols and cultured in AIM-V medium (LifeTechnologies, Carlsbad, CA) supplemented with 5% human AB serum (Valley Biomedical, Winchester, VA), 0.05 mM β -mercap-

toethanol, 0.1 mM nonessential amino acids, 25 mM HEPES, and 2 mM L-glutamine (LifeTechnologies). The following packaging cell lines were used: PG13 (gibbon ape leukemia virus packaging cell line; ATCC CRL-10686); Phoenix ECO (human ecotropic packaging cell line, National Gene Vector Biorepository, and provided by Dr. Gary Nolan, Stanford University, Stanford, CA); and Phoenix-GALV and Phoenix-RD114 cell lines (kindly provided by Hans-Peter Kiem, Fred Hutchinson Cancer Research Center). All packaging cells were cultured in D10 medium consisting of high-glucose (4.5 g/l) Dulbecco's modified essential medium (LifeTechnologies) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 6 mM glutamine (final concentration; LifeTechnologies). Cells were maintained at 37°C and 5% CO₂. The following melanoma cell lines were isolated from surgically resected metastases as previously described (Topalian *et al.*, 1989): mel526 (HLA-A2⁺/NY-ESO-1⁻), mel624.38 (HLA-A2⁺/NY-ESO-1⁺), mel1300 (HLA-A2⁺/NY-ES-1⁺), and mel888 (HLA-A2⁻). The following CD19-expressing cell lines were used: bv173 (chronic lymphocytic leukemia in lymphoid blast crisis, a kind gift from Dr. A. Wiestner, National Heart, Lung and Blood Institute, Bethesda, MD) and NALM-6 (acute lymphoid leukemia from DSMZ, Braunschweig, Germany). The CD19⁺ cell line K562-CD19 (expressing CD19) and the CD19⁻ cell line K562-NGFR (chronic myeloid leukemia expressing the truncated nerve growth factor receptor) were kindly provided by Dr. James Kochenderfer (National Cancer Institute, Bethesda, MD). The cells were cultured in R10 medium consisting of RPMI 1640 (LifeTechnologies) containing 10% fetal bovine serum (Hyclone).

Construction of piggybac transposable elements encoding GFP, the NY-ESO-1 T cell receptor, and CD19 chimeric antigen receptor

Non-SIN (pMSGV1) retroviral vectors encoding GFP, NY-ESO-1 T cell receptor (TCR), and CD19 chimeric antigen receptor (CAR), respectively, were generated or described previously (Robbins *et al.*, 2008; Kochenderfer *et al.*, 2009). The SIN vector, pSERS11MPSV.GFP (SIN-GFP), was kindly provided by Drs. Baum and Schambach (Hannover Medical School, Hannover, Germany). A codon-optimized *piggybac* transposon plasmid (pPB-MCS; GenBank accession number HE654700) containing a multiple cloning site and a plasmid encoding the transposase (pcDNA3.1-piggybac-64A) were synthesized from published sequences by Dr. Richard Morgan (Bluebird Bio, Inc.; Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb). pPB-SIN-GFP was constructed as follows. Briefly, the entire retroviral vector cassette encoding the SIN-GFP was PCR-amplified using the Advantage 2 PCR kit (Clontech Laboratories, Mountain View, CA) using the indicated primers: forward, 5'-aaaaaaatcgattagtactctagcttaagacgcgtgg-3', and reverse, 5'-aaaaaaatcgataccatgattacgccaagctctcg-3', both of which include a *Clal* restriction site. The SIN-GFP retroviral cassette was then cloned into the pPB-MCS (*piggybac*) backbone using *Clal*. A clone in the forward orientation (Supplementary Fig. S2) was identified and used to generate all subsequent SIN constructs. pPB-SIN-NY-ESO-1 TCR and pPB-SIN-CD19 CAR were generated by enzymatic digest of their respective pMSGV1

plasmids with *NcoI* and *NotI*. pPB-SIN-GFP was digested with *NcoI* and *NotI* and the GFP coding segment was replaced with the NY-ESO-1 TCR or CD19-CAR digest fragment, respectively.

Generation of γ -retroviral packaging clones

For a given transgene (GFP, NY-ESO-1 TCR, or CD19 CAR), a PG13 retroviral packaging cell clone was generated as described previously with the following changes (Hughes *et al.*, 2005). Phoenix ECO cells were transfected with 9 μ g of plasmid DNA (pMSGV1-GFP, pMSGV1-NY-ESO-1 TCR, or pMSGV1-CD19 CAR) using the Lipofectamine 2000 transfection reagent (Life Technologies). After 48 hr, supernatant was harvested and used to transduce the retroviral packaging cell line PG13. Phoenix-GALV packaging clones were generated in a similar manner except that the transient vector for transduction of the packaging cells was produced in 293GP cells along with the VSV-g envelope. For the transduction, non-tissue-culture-treated 6-well plates were coated with 10 μ g/ml recombinant fibronectin fragment as described by the manufacturer (RetroNectin; Takara, Otsu, Japan). Retroviral vector supernatant (4 ml) was added to each well followed by centrifugation ($2000 \times g$) at 32°C. After 2 hr, supernatant was removed and 5×10^5 PG13 cells were added to the well and centrifuged ($1000 \times g$) for 10 min at 32°C. Two rounds of transduction were performed, and then PG13 or Phoenix packaging clones were generated by limiting dilution cloning. Because of lack of a selectable marker, high-titer clones were identified by RNA dot blot as described previously (Onodera *et al.*, 1997; Hughes *et al.*, 2005). γ -retroviral vector from the six highest titer packaging clones was generated. Briefly, 175 cm² tissue culture flasks (Nunc; Cole-Parmer, Vernon Hills, IL) were seeded at 4×10^4 cells/cm², followed by a medium exchange (30 ml) on day 3. Supernatant was harvested 24 hr later, aliquoted, and stored at -80°C until further use. Supernatant from each clone was evaluated for the ability to efficiently transduce human peripheral blood lymphocytes (PBL) and elicit IFN γ in a cytokine release assay. The clone conferring the highest transduction effi-

ciency and IFN γ release following PBL transduction was selected for this study.

PBL transductions

For PBL transductions and determination of virus titer, PBL (2×10^6 /ml) were stimulated with IL-2 (300 IU/ml; Chiron Corp., Emeryville, CA) and OKT3 (50 ng/ml; Ortho, Bridgewater, NJ) on day 0. Non-tissue-culture-treated six-well plates were coated with Retronectin (Takara Bio, Otsu, Japan) at 10 μ g/ml, 2 ml/well on day 1, and stored overnight at 4°C. Because our vectors lack a selectable marker and the variability associated with γ -retroviral vector transduction and receptor expression in different patient PBL, we used the following transduction scheme for determination of transduction efficiency and titer. Briefly, vector supernatants were produced as described above and serially diluted in D10 (4 ml/well) and applied to plates on day 2 followed by centrifugation at $2000 \times g$ for 2 hr at 32°C. Half the volume was aspirated and PBL were applied (0.25×10^6 /ml, 4 ml/well), centrifuged for 10 min at $1000 \times g$, and followed by incubation at 37°C and 5% CO₂. PBL from three patients were transduced for each assessment and vector titers were calculated as follows: (Percentage GFP⁺, tetramer⁺, or CAR⁺ cells $\times$ total cells $\times$ dilution factor)/supernatant volume.

Fluorescence-activated cell sorting analysis

Analysis of the expression of cell surface markers was carried out using FITC- or PE-conjugated antibodies directed against CD3 (BD Biosciences, San Jose, CA). GFP⁺ cells were gated on FITC. Detection of the NY-ESO-1 TCR was carried out using fluorescent peptide (NY-ESO-1₁₅₇₋₁₆₅)/HLA-A*02 tetramers produced by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Detection of the CD19 CAR was carried out using a biotin-labeled goat anti-mouse F(ab)₂ antibody (anti-Fab; Jackson Immuno-research, West Grove, PA) and streptavidin-PE (BD Pharmingen, San Diego, CA). The relative log fluorescence of live cells was determined using a FACScanto flow cytometer (BD Biosciences). Analysis was performed using Flowjo software (Treestar Inc., Ashland, OR).

FIG. 1. Diagram of gammaretroviral (non-SIN and SIN) and *piggybac* transposable SIN gammaretroviral vector constructs. The SIN gammaretroviral vector (pSERS11.MPSV.GFP) was cloned into the pPB-MCS (*piggybac*) backbone using the *Clal* restriction site to generate the *piggybac* SIN γ -retroviral vector constructs encoding GFP, NY-ESO-1 TCR, or CD19 CAR. The pMSGV1 (non-SIN) and the *piggybac* SIN γ -retroviral vectors were used to generate stable packaging cell lines as described in the Materials and Methods. GFP, green fluorescent protein; IR/DR, indirect/direct repeat; LTR, long terminal repeat; MPSV, myeloproliferative sarcoma virus promoter; R, repeat region; SIN, self-inactivating; TCR, T cell receptor; Transgene, GFP, NY-ESO-1 TCR, or CD19 CAR; U3, untranslated region 3'; U5, untranslated region 5'; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element.

FIG. 2. Comparison of murine and human γ -retroviral packaging cell lines. Stable packaging cell lines were generated using the non-self-inactivating plasmid, pMSGV1-GFP, for the production of PG13-GFP. For generation of the SIN packaging lines, Phoenix-GALV (clone 7) and Phoenix-RD114 (clone 5), pSERS11MPSV.GFP was cloned into the *piggybac* transposon as described in the Materials and Methods. (A) PBMC were transduced once with γ -retroviral vector produced from PG13, G7, and R5. Representative fluorescence-activated cell sorting histograms show that γ -retroviral vectors encoding GFP from each of the packaging cell lines were able to efficiently transduce PBMC (range 74.2–85.9%). (B) Vector from each PG13, G7, and R5 γ -retroviral packaging cell line was serially diluted before transduction of PBMC. Viral titers were determined and there was no significant difference in vector titers (PG13, G7, and R5 titers, $2.7 \times 10^6 \pm 0.1$, $2.7 \times 10^6 \pm 0.1$, and $3.0 \times 10^6 \pm 0.1$, respectively). PBMC, peripheral blood mononuclear cells.

Cytokine release assays

Cytokine release was measured following the incubation of 10^5 transduced T lymphocytes with 10^5 tumor target cells in 200 μ l of R10 medium for 18 hr at 37°C. Dilutions of culture supernatant were then tested for IFN γ by enzyme-linked immunosorbent assay (ELISA; Pierce).

Statistical analysis

Fluorescence-activated cell sorting data were analyzed using Flowjo software (Treestar Inc.). Where appropriate, results were compared using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison analysis between groups.

Results and Discussion

We used the *piggybac* transposon to stably integrate a SIN γ -retroviral cassette encoding GFP, pSERS11MPSV.GFP (SIN-GFP) (Schambach *et al.*, 2007), into the 293-based Phoenix-GALV and RD114 packaging cell lines without reverse transcription (Fig. 1). The pPB-SIN-GFP plasmid was constructed by subcloning the entire SIN-GFP retroviral cassette within the *piggybac* IR/DR domains. pPB-SIN-GFP was then cotransfected along with transposase mRNA into the Phoenix packaging cells. The use of transposase mRNA allows for stable transposition as the mRNA is rapidly degraded following transfection. High-titer γ -retroviral producer cell clones were selected by RNA dot blot (Onodera *et al.*, 1997). We generated pPB-SIN-GFP producer cell clones in both the Phoenix-GALV (clone 7) and Phoenix-RD114 (clone 5) packaging cell lines. Supernatant generated from these clones was compared with a non-SIN PG13 packaging clone (pMSGV1-GFP; Fig. 1) prepared via conventional methods (Onodera *et al.*, 1997; Hughes *et al.*, 2005). Following transduction of PBL with either the SIN or non-SIN vector supernatant, no significant difference in transduction efficiency (Fig. 2A) or titer (Fig. 2B and Table 1) was observed as measured by GFP $^+$ PBL for any of the clones tested. In addition, we demonstrated that orientation of the retroviral vector cassette (Supplementary Fig. S2A) within the transposon did not significantly affect vector production or transduction efficiency of PBL (Supplementary Fig. S2B).

In order to evaluate SIN γ -retroviral vectors encoding clinically relevant targeting molecules, such as a TCR or CAR, we inserted the human NY-ESO-1 TCR $_{157-165}$ (pPB-SIN-NY-ESO-1 TCR) and a CAR directed against CD19 (pPB-SIN-CD19 CAR; Fig. 1) into the *piggybac* backbone (Robbins *et al.*, 2008; Kochenderfer *et al.*, 2009, 2010; Robbins *et al.*, 2011). Since both Phoenix packaging lines (GALV and RD114) appear to be functionally equivalent and comparable to the conventional PG13 packaging clones based on the previous data (Fig. 2 and Table 1), we focused on a comparison between the GALV-pseudotyped packaging cell lines, PG13 and Phoenix-GALV. A human TCR

TABLE 1. COMPARISON OF PHOENIX AND PG13-BASED PACKAGING CELL LINES FOR PRODUCTION OF A GFP-ENCODING GAMMARETROVIRUS

Packaging line	Parental line ^a	Env ^b	SIN	Transgene	Titer ^c (Tu/ml, $\times 10^6$)
Phoenix-GALV (clone 7)	HEK293T	GALV	+	GFP	2.7 ± 0.1
Phoenix-RD114 (clone 5)	HEK293T	RD114	+	GFP	3.0 ± 0.1
PG13	NIH3T3	GALV	–	GFP	2.7 ± 0.1

GFP, green fluorescence protein; PBL, peripheral blood lymphocytes; SIN, self-inactivating.

^aPhoenix, human; PG13, murine.

^bEnvelopes: GALV, gibbon ape leukemia virus; RD114, feline endogenous virus.

^cAverage titer $\pm$ standard error of the mean for 3 separate patient PBL. TU/ml, transducing units per milliliter = [(% GFP $^+$)(total cells)/supernatant volume]/(dilution factor).

FIG. 3. *Piggybac* transposon-mediated integration of a self-inactivating γ -retroviral vector cassette encoding a TCR without reverse transcription. A SIN γ -retroviral vector cassette encoding the human NY-ESO-1 TCR was prepared and inserted into the *piggybac* transposon, and a stable producer cell line was generated as described in the Materials and Methods. Vector generated from this produce line was compared with our clinical, non-SIN PG13-produced clone generated using pMSGV1-NY-ESO-1 TCR plasmid for both transduction efficiency (**A**) and function (**B**) as measured by IFN γ release following coculture with antigen-positive tumor cell lines. (**A**) PBMC from three separate patients were transduced once with a SIN γ -retroviral vector produced from a Phoenix-GALV packaging clone or a non-SIN PG13-derived vector both encoding the human NY-ESO-1 TCR. The cells were stained using an anti-CD3 antibody and NY-ESO-1_{157–165} tetramer. No significant difference in percentage of cells transduced was observed for either the SIN (Phoenix-GALV, 32.5% $\pm$ 4.4%) or non-SIN (PG13, 34.3% $\pm$ 3.6%). (**B**) Transduced PBMC were cocultured with HLA-A2⁺/NY-ESO-1⁺ (mel1300 and mel624.38) or HLA-A2⁺/NY-ESO-1[–] (mel526) and HLA-A2[–] (mel888) tumor cell lines. IFN γ released was measured, and both SIN (open bars) and non-SIN (closed bars) showed specific recognition of HLA-matched antigen-positive tumor cell lines. PBMC transduced with the SIN vector released significantly more IFN γ when cocultured with the mel624.38 cell line ($p < 0.001$).

TABLE 2. COMPARISON OF TITERS FROM PHOENIX-GALV PACKAGING CELL LINES VERSUS PG13 cGMP MASTER CELL BANKS FOR THE PRODUCTION OF γ -RETROVIRAL VECTORS ENCODING CLINICALLY RELEVANT TARGETING MOLECULES

Antigen specificity	Targeting molecule	Packaging line ^a	Parental line	SIN	Titer ^b (TU/ml, $\times 10^6$)
NY-ESO-1	TCR	Phoenix-GALV	HEK293T	+	1.0 $\pm$ 0.3
		PG13	NIH3T3	–	1.1 $\pm$ 0.3
CD19	CAR	Phoenix-GALV	HEK293T	+	1.5 $\pm$ 0.3
		PG13	NIH3T3	–	1.9 $\pm$ 0.05

CAR, chimeric antigen receptor; TCR, T cell receptor.

^aPhoenix, human; PG13, murine.

^bAverage titer $\pm$ standard error of the mean for 3 separate patient PBL. TU/ml, transducing units per milliliter = [(% positive cells)(total cells)/supernatant volume](dilution factor).

directed against the NY-ESO-1_{157–165} described previously (Robbins *et al.*, 2008) was cloned into a non-SIN γ -retroviral vector and used to generate a PG13 packaging cell line (Feldman *et al.*, 2011; Robbins *et al.*, 2011). In parallel, this TCR was synthesized and cloned into the pSERS11MPSV.GFP (Schambach *et al.*, 2007), whereby the GFP was replaced with the NY-ESO-1 TCR. This construct was then subcloned into the *piggybac* transposon in the forward orientation and used to generate a stable Phoenix-GALV packaging cell line as described (Figs. 1 and 3). After a single transduction, there was no significant difference in the percentage of CD3⁺/NY-ESO-1 TCR⁺ PBL using PG13-derived retroviral vector ($30.7\% \pm 4.1\%$) as compared with the SIN vector ($24.3\% \pm 1.4\%$) (Fig. 3A). The vector titers were nearly identical for both packaging cell clones ($1.1 \pm 0.3 \times 10^6$ and $1.0 \pm 0.3 \times 10^6$ TU/ml, respectively; Table 2). Interestingly, PBL transduced with the SIN vector released higher levels of IFN γ following coculture with both antigen-specific tumor cell lines; however, only the IFN γ level after coculture with mel624.38 was significant ($p < 0.001$; Fig. 3B).

Similar to the NY-ESO-1 TCR, we previously manufactured a PG13 packaging cell line used for the clinical production of a γ -retroviral vector encoding the CD19 CAR (Kochenderfer *et al.*, 2009, 2010, 2011, 2012). We cloned the CD19 CAR into the SIN retroviral cassette within the *piggybac* backbone to generate pPB-SIN-CD19 CAR. Retroviral vector produced from both a PG13 and Phoenix-GALV packaging clone was able to efficiently transduce PBL (Fig. 4A). While the transduction efficiency was slightly higher from Phoenix-GALV-derived vector supernatant ($57.1\% \pm 3.3\%$ and $49.3\% \pm 1.6\%$, respectively), the difference was not significant. In addition, there was no significant difference in the titer between the two vectors (Table 2). Interestingly, and in contrast to the NY-ESO-1 TCR supernatants, PBL transduced with the PG13-derived CD19 CAR vector supernatant showed higher levels of IFN γ release following coculture with CD19⁺ B cell lines; however, only coculture with K562-CD19 cells was significant ($p < 0.001$; Fig. 4B). Differences in IFN γ release between PBL transduced with vector supernatants from the Phoenix and PG13 packaging clones cannot be attributed to increased levels of receptor expression, as there were no significant differences in receptor MFI between the NY-ESO-1 TCR and CD19 CAR packaging cell lines, respectively (data not shown).

Currently, a majority of clinical trials using gene-modified T cells for cancer immunotherapy utilize γ -retroviral-mediated gene delivery. In addition to the high transduction efficiencies associated with such vectors, use of non-SIN γ -retroviral vectors is amenable to large-scale production through the use of packaging cell lines that constitutively release vector into the culture medium. Development of γ -retroviral packaging cell lines for SIN vector production is complicated by the fact that the cell lines cannot be easily generated without reverse transcription of the viral genome. To that end, we have developed a *piggybac* transposon-based platform for the generation of stable SIN γ -retroviral packaging cell lines. These cell lines are comparable to packaging cell lines for our non-SIN vectors in terms of both transduction efficiency and function as measured by IFN γ secretion. Thus, by cloning a SIN vector within the IR/DR repeats of the *piggybac* transposon, we can efficiently gen-

FIG. 4. *Piggybac* transposon-mediated integration of a self-inactivating gammaretroviral vector cassette encoding an anti-CD19 CAR without reverse transcription. A SIN γ -retroviral vector cassette encoding the murine CD19 CAR was prepared and inserted into the *piggybac* transposon, and a stable producer cell line was generated as described in the Materials and Methods. Vector generated from this produce line was compared with our clinical, non-SIN PG13-produced clone generated using pMSGV1-CD19 CAR plasmid for both transduction efficiency (A) and function (B) as measured by IFN γ release following coculture with antigen-positive tumor cell lines. (A) PBMC from three separate patients were transduced once with a SIN γ -retroviral vector produced from a Phoenix-GALV packaging clone (H8) or a non-SIN PG13-derived vector both encoding the murine anti-CD19 CAR. The cells were stained using an anti-CD3 antibody and biotinylated anti-(Fab)₂ and streptavidin-PE. No significant difference in percentage of cells transduced was observed for either the SIN (Phoenix-GALV, $57.1\% \pm 3.2\%$) or non-SIN (PG13, $49.3\% \pm 1.6\%$). (B) Transduced PBMC were cocultured with CD19⁺ (K562-CD19, Bv1673, and NALM6) and CD19⁻ (K562-NGFR) tumor cell lines. IFN γ released was measured, and both SIN (open bars) and non-SIN (closed bars) showed specific recognition of CD19 antigen-positive tumor cell lines. PBMC transduced with the non-SIN vector released significantly more IFN γ when cocultured with the K562-NGFR cell line ($p < 0.001$).

erate stable packaging cell clones in less than 1 month's time that transfer easily into a current GMP γ -retroviral production platform.

Acknowledgments

The authors would like to thank Arnold Mixon and Shawn Farid for assistance with flow cytometry. The authors would also like to thank the National Gene Vector Biorepository

for providing the Phoenix-ECO and PG13 cells used in portions of this study. The authors would like to thank Drs. Baum and Schambach (Hannover Medical School, Hannover, Germany) for kindly providing the pSERS11MPSV.GFP plasmid.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication June 24, 2014;
accepted after revision July 26, 2014.

Published online: July 29, 2014.