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Conditionally replicating lentiviral-hybrid episomal vectors for suicide gene therapy

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ABSTRACT

Lentiviral vectors have been shown to be good candidates for gene transfer protocols; however, prevention of insertional mutagenesis remains problematic. Here we report on the design of a conditionally replicating integrase (IN)-defective lentiviral-hybrid episomal vector in which the insertion of the SV40 promoter/origin of replication provides long-term persistence of the extrachromosomal DNA in the presence of the corresponding *trans*-acting T antigen (Tag) for targeted suicide gene therapy. SV40-driven GFP expression from the IN-defective lentiviral-hybrid vector was sustained only in the Tag positive 293T cell line, while expression was transient in the parental Tag deficient cell line 293. Quantitative PCR for the 2-LTR circular forms indicated that the unintegrated forms remained stable in 293T for up to 56 days post-transduction, while they were undetectable in the cell line 293 after day 14. Transduction of 293T cells with the IN-defective lentiviral-hybrid episomal vector containing the thymidine kinase (TK) gene rendered the Tag expressing cells highly susceptible to ganciclovir (GCV) treatment, as opposed to the cells infected with the control vector or in Tag negative cells. These data suggest that conditionally replicating IN-defective lentiviral-hybrid episomal vectors could prove useful as vehicles for suicide gene therapy, in particular in cells transformed by SV40.

1. Introduction

One of the mainstays of successful cancer gene therapy is the selective delivery of a therapeutic gene to the cancerous tissue resulting in the removal of malignant cells while minimizing damage to normal tissue. In particular, targeting of the tumor with a therapeutic gene encoding metabolic enzymes able to convert an otherwise innocuous prodrug into a toxic metabolite would result in a high concentration of the toxic product in the target cells, thus avoiding the systemic toxicity often associated with conventional radio- and chemotherapy. In this setting, the employment of suicide gene therapy has been considered in various malignancies including prostate cancer, melanoma, ovarian cancer, mesothelioma, brain cancer, and leukemia (Mullen and Blaese, 1996; El-Aneed, 2004; Fulda and Debatin, 2004); some of these strategies are currently under evaluation in clinical trials in humans (Smythe, 2000; www.wiley.co.uk/genetherapy/clinical).

Despite significant improvement in vector design, vector production, transduction efficiency and transgene expression, one of the major shortcomings remains in the selective transduction and removal of the target cells (Dachs et al., 1997; Westphal and von Melchner, 2002). In this setting, the development of cell-specific targeting and/or expression systems is particularly important to avoid dilution of the vector or unnecessary removal of bystander cells.

Lentiviral vectors have unique properties that are both attractive and applicable for suicide gene therapy vectors (Chang and He, 2001; Loimas et al., 2001). They are easy to engineer, integrate into the host genome, and sustain stable transgene expression in dividing, nondividing, and terminally differentiated cell types (Naldini et al., 1996a,b; Blömer et al., 1997). Although lentiviral vectors just started to be used in clinical protocols, the potential risk associated with insertional mutagenesis and random integration was recently highlighted by the development of leukemia in individuals whose stem cells were transduced with a gammaretroviral-based vector (Hacein-Bey-Abina et al., 2002, 2003). However, while conventional gammaretroviral vector integration events show a predilection for the region of the transcriptional start site (thus favoring transcription of potentially harmful gene products), lentiviral vector integration events are more evenly distributed throughout genes with a predilection for the region at the end of the gene (thus decreasing the risk of transcription of potentially harmful gene products) (Mitchell et al., 2004; Hematti et al., 2004), suggesting that lentiviral vectors have a reduced risk for mutagenesis

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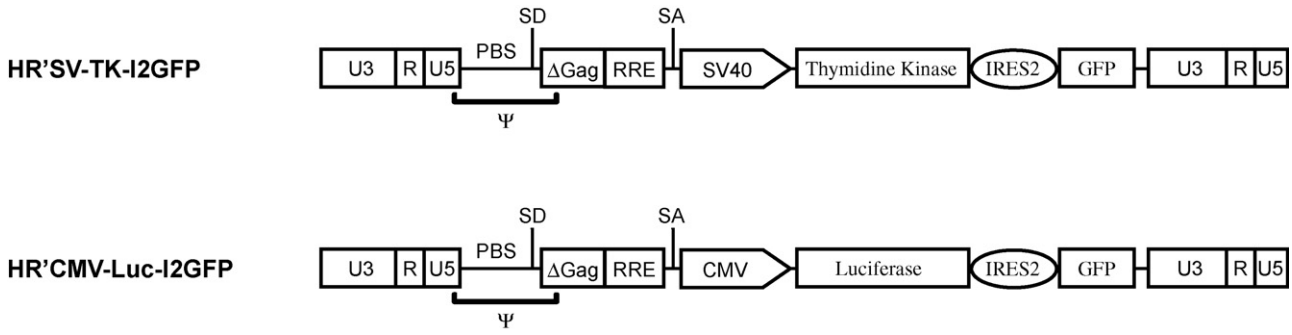

Fig. 1. Schematic representation of the HIV-based transfer vectors. For details refer to Section 2. PBS, primer binding site; (, packaging signal; SD, splice donor; SA, splice acceptor; SV40, SV40 promoter/*ori*; CMV, CMV immediate/early promoter; IRES, Encephalomyocarditis virus Internal Ribosomal Entry Site.

and a better safety profile when compared to gammaretroviral vectors. Nevertheless, gammaretroviral and lentiviral vector integration into host-cell chromosomes carries with it a finite chance of causing insertional mutagenesis. Consequently, the potential risk of insertional mutagenesis would be eliminated if using integrase (IN)-defective lentiviral vectors.

In the study presented here, we have incorporated the constituents necessary for SV40 T antigen (Tag)-driven episomal replication into the genome of an IN-defective lentiviral vector to produce a conditionally replicating IN-defective lentiviral-hybrid episomal vector. We and others have previously demonstrated that the unintegrated forms of this vector are maintained as episomes and remain transcriptionally active only in cells expressing the corresponding *trans*-acting Tag (Lu et al., 2004; Vargas et al., 2004). Here we demonstrate that the presence of the Thymidine kinase (TK) suicide gene in the unintegrated vector, results in the removal of transduced cells following GCV treatment. With appropriate modifications, IN-defective lentiviral-hybrid episomal vectors provide a novel and important tool for the delivery of therapeutic genes into cells for suicide gene therapy of diseases associated with selected episomally-replicating viruses.

2. Materials and methods

2.1. Construction of modified lentiviral vectors

The basic features of the vectors used in this work are shown in Fig. 1. Briefly, to produce HR'SV-TK-I2GFP, plasmid HR'CMV-MCS-IRES-GFPΔB (kindly provided by Dr. J. Mulloy), containing the IRES (Encephalomyocarditis virus Internal Ribosomal Entry Site)-driven GFP translation element, was digested with ClaI/BamHI, to remove the cytomegalovirus (CMV) promoter. The SV40 promoter, containing the origin of replication (*ori*) of SV40, derived from the SV40 early promoter DNA (Promega, Madison, WI) was digested with ClaI/StuI to produce a 300 bp fragment of DNA. The thymidine kinase gene from HSV-1 was derived from plasmid JB142 (a gift from Dr. J. Blaho) by amplifying a 1.13 kb fragment with primers that introduced StuI and BamHI restriction sites at the 5′ (5′TK-S: 5′-GAAAGGCCTATGGCTTCGTACCCCTGCCA-3′) and 3′ (3′TK-AS: 5′-CGCGGATCCTCAGTTAGCCTCCCCCATCT-3′) of the coding sequence, respectively. After purification, the SV40 *ori* ClaI-StuI DNA fragment, and TK gene StuI-BamHI DNA fragment were cloned into the StuI-BamHI-restricted HR'CMV-MCS-IRES-GFPΔB plasmid. To produce HR'CMV-Luc-I2GFP, plasmid pGEM-luc (Promega, Madison, WI) was restricted with BamHI/SalI to remove the luciferase gene which was inserted into the same sites of pHR'CMV-IRES2-GFPΔB. The IN competent and IN defective packaging constructs, pCMVΔR8.2 (kindly provided by Dr. I. Verma) and pC-HelpIN- (kindly provided by Dr. J. Reiser), respectively, and the envelope expression plasmid pMD.G, kindly provided by Dr. I. Verma, have been described (Vargas et al., 2004).

2.2. Virus production and cellular transduction

Lentiviral vectors were generated in human kidney 293T cells by using a non-liposomal lipid transient transfection method. Briefly, 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). For transfection experiments, 1 μg of the packaging constructs pCMVΔR8.2 or pC-HelpIN$^-$, 1 μg of the VSV-G expressing plasmid pMD.G and 2 μg of the transfer plasmid HR'-SV-TK-I2GFP or HR'CMV-Luc-I2GFP, were introduced into 293T cells using the Effectene Transfection Reagent, a non-liposomal lipid transfection method (Qiagen, Valencia, CA). Twelve hours after transfection, 10 mM sodium butyrate (Sigma Scientific, Inc., Brighton, MI) was added to increase transgene expression. After 6 h of exposure to sodium butyrate, the cells were replenished with fresh medium. Supernatants were collected every 12 h for 48 h, cleared of cellular debris by low speed centrifugation and filtered through a 0.45 μm pore size filter. Viral containing supernatants were treated with 30 U/ml RNase-free DNase-I (Invitrogen, Carlsbad, CA) for 1 h at 37 °C to remove residual plasmid DNA, ultracentrifuged at 15,000 rpm for 4 h into a pellet and resuspended in DMEM. Viral stocks were stored in aliquots at −80 °C. Recombinant viral preparations included the IN-competent HR'CMV-Luc-I2GFP/IN+ and HR'SV-TK-I2GFP/IN+ viruses and the IN-defective HR'CMV-Luc-I2GFP/IN- and HR'-SV-TK-I2GFP/IN− viruses. For transduction experiments, supernatants were normalized for p24 content by the HIV-1 p24 antigen capture assay kit (SAIC-Frederick Inc., Frederick, MD) and 1×10^5 293 or 293T cells were incubated in 6 well plates with equal amounts of p24 in the presence of 8 μg/ml polybrene. After 12–16 h, the virus-containing media was washed off, and cells were replenished with fresh media. All lentiviral vector preparations were tested for the presence of replication-competent recombinant (RCR) by infection of 293T and supernatants were assayed for p24. None of the vector preparations contained detectable RCR (data not shown).

2.3. GFP expression time course

At designated time points, 25% of the cells were harvested, fixed in 1% paraformaldehyde for 30 min at room temperature, and GFP was assessed by fluorescence-activated cell sorting (FACS), using FACS Calibur flow cytometer analysis (Beckton Dickinson, Mountain View, CA). Data acquisition and analysis were done using CellQuest software (Becton Dickinson, Mountain View, CA).

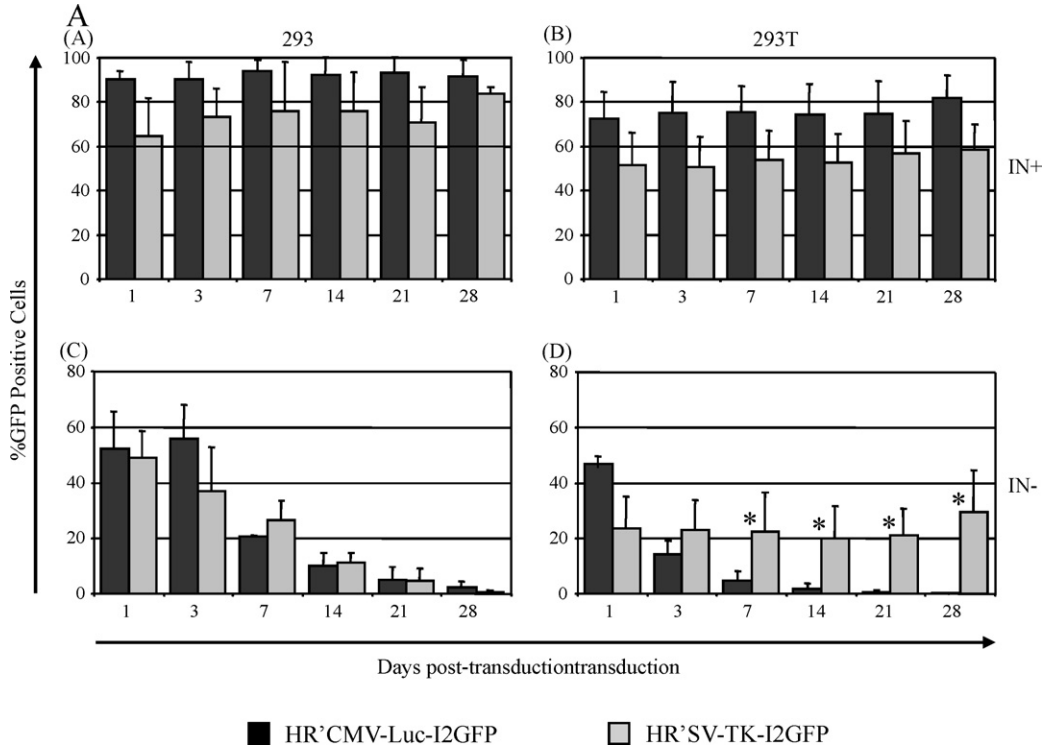

Fig. 2. Time course analysis of the percentages of GFP positive cells in 293 and 293T cells transduced with either IN-competent and IN-defective lentiviral vectors. (A) Transduction of 293 cells with IN-competent vectors; (B) transduction of 293T cells with IN-competent vectors; (C) transduction of 293 cells with IN-defective vectors; (D) transduction of 293T with IN-defective vectors. At the indicated days, transduced cells were harvested, fixed in 1% paraformaldehyde, and GFP expression was assessed by FACS analysis. Data are shown as the averages of three independent experiments, representing means ± standard deviations. $^{*}p < 0.05$.

2.4. Quantititative PCR for c2LTR

Cells were collected into pellets at various time points over the course of the experiment and frozen at −80 °C until extractions were performed. Total DNA was extracted using the QIAamp DNA Mini Kit according to the Manufacturer's procedure (Qiagen, Valencia, CA). Total DNA extracted from each sample was quantitated by optical density at 260 nm. The primers used to detect the circular 2-LTR (c2LTR) junction sequence by real-time PCR (purchased from Integrated DNA technologies) were as follows: c2LTR-S [5′-TAGGGAACCCACTGCTTA-3], c2LTR-AS [5′-TAATCAGGGAAGTAGCCTTG-3′], and c2LTR-FAM probe [5′-(FAM)-TAAAGCTTGCCTTGAGTGCTTCAAGTAGTGT-(BHQ-1)-3′]. With each experiment, a standard curve, derived from serial dilution of a plasmid containing the target sequence and ranging from 10^0 to 10^6 copies was measured in triplicate. Reaction mixtures contained 1× iTaq master mix (Bio-Rad Laboratories, Hercules, CA), 800 nM of primer c2LTR (S), 800 nM of primer c2LTR (AS), 200 nM of probe primer, and 100 ng of template DNA. After an initial incubation at 95 °C for 10 min, 40 cycles of amplification were carried out as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 47 °C, and extension for 30 s at 72 °C. Reactions were carried out and analyzed using the ICycler IQ Real Time Detection System software (Bio-Rad Laboratories, Hercules, CA).

2.5. Gancyclovir treatment of transduced cells

After transductions, cells were continually passaged until treatment with Ganciclovir (GCV). GCV surviving curves were determined 4 weeks post-transduction, by culturing the transduced cells with increasing concentrations of the drug, ranging from 0 to 800 μM, in DMEM supplemented with 10% FCS. Growth was allowed to proceed in the presence of GCV for 4 days, after which cell viability was measured by the MTS cell proliferation assay (CellTiter 96 Aqueous One Solution cell proliferation assay, Promega) according to the Manufacturer's instructions.

2.6. Statistical analysis

Analysis of variance (ANOVA) was used to make comparison between the means. Data are expressed as means ± standard deviation. All analyses were performed with Instat 2.01 (GraphPad Software, San Diego, CA).

3. Results

3.1. Transduction of the target cells by IN competent and IN-defective lentiviral-hybrid episomal vectors

Following transduction with IN-competent and IN-defective recombinant HR'SV-TK-I2GFP and HR'CMV-Luc-I2GFP vectors, percentage of GFP positive 293 and 293T cells was assessed over-time by FACS analysis (Fig. 2). Transduction of 293T with the IN− competent HR'SV-TK-I2GFP/IN+ virus, resulted in an average of 51.4% and 58.8% GFP positive cells at early (day 1) and late (day 28) timepoints, respectively (Fig. 2B); parallel transduction of 293 cells resulted in an average of 62.5% and 84.1% at early and late timepoints, respectively (Fig. 2A). This observation confirms that integration competent vectors are stable templates for sustained transgene expression. Similar results were seen following transduction of 293 and 293T cells with the IN-competent control vector, HR'CMV-Luc-I2GFP/IN+ (Fig. 2A and B), although the percentage of GFP positive cells was slightly higher, possibly due to the superior strength of the CMV promoter in driving GFP expression.

On the other hand, transduction of 293 cells with the IN-defective HR'SV-TK-I2GFP/IN− vector, resulted in a gradual

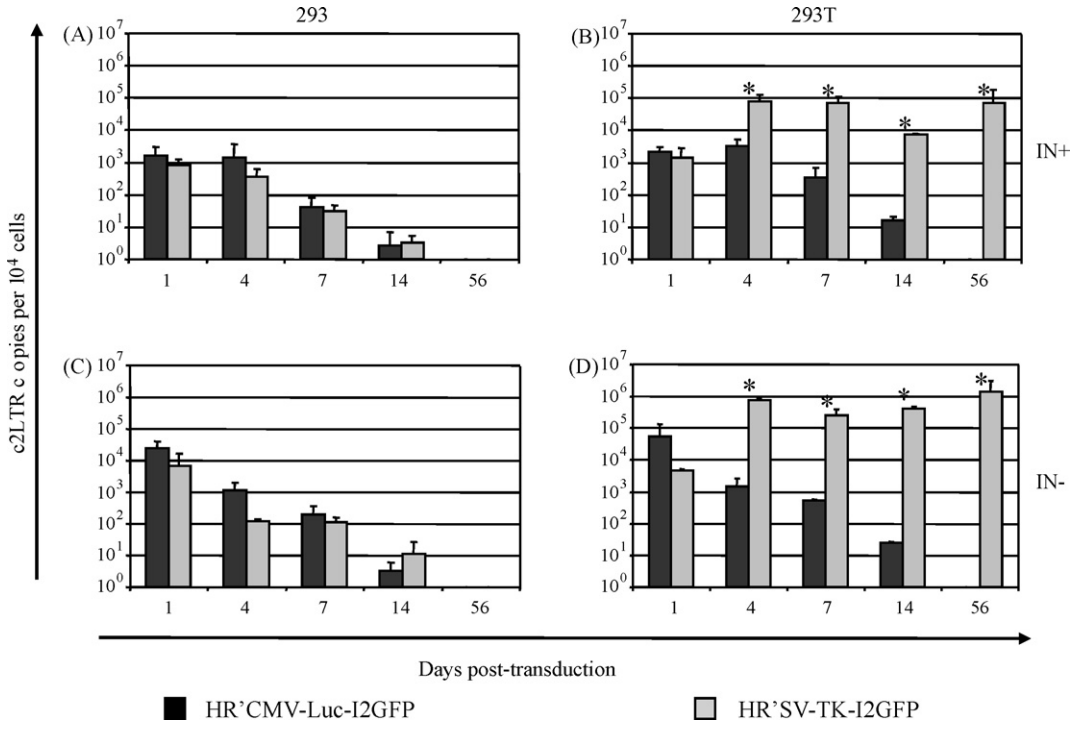

Fig. 3. Quantitative PCR (qPCR) for c2LTR episomes from DNA extracted from transduced 293 (A) and (C) and 293T (B) and (D) with IN-competent (A) and (B) and IN-defective (C) and (D) lentiviral vectors. Total DNA was extracted at the indicated days and 100 ng were used to amplify the c2LTR forms by qPCR. PCR reactions were analyzed using the ICycler IQ Real Time Detection System software as described in Section 2. Data are shown as averages of three independent experiments, representing means ± standard deviations and are expressed as copies/10^4 cells. $^*p < 0.05$.

decrease in the number of cells positive for GFP, averaging 49% at day 1 and gradually decreasing to background levels by day 28 (Fig. 2C). The transient expression of GFP was due to the transient presence of E-DNA which has been shown to be transcriptionally active in the absence of integration (Cara et al., 1995, 1996; Wiskerchen and Muesing, 1995; Wu and Marsh, 2003; Brussel and Sonigo, 2004). Although the IN− defective unintegrated vector contains the *ori* of SV40, the absence of Tag in the target cells does not allow for increased stability of the vector. As such, episomal DNA vector is diluted in the replicating cells. Similar results were obtained using the IN− defective control vector HR'CMV-Luc-I2GFP/IN−, after transducing 293 cells (Fig. 2C).

In contrast, in 293T transduced with the IN-defective HR'SV-TK-I2GFP/IN− vector, the number of GFP positive cells remained stable throughout the time-course of the experiment, averaging 24% and 29% at day 1 and day 28 post-transduction, respectively (Fig. 2D). The percentage of GFP positive cells in the IN-defective HR'SV-TK-I2GFP/IN− transduced 293T cells was statistically significant starting from day 7, and up to the end of the experiment, when compared to the control vector ($p < 0.05$). Since episomal DNA produced in the transduced cells contain the SV40 *ori*, the presence of the Tag in *trans* allowed for the increased stability of E-DNA and the consequent GFP expression throughout the time-course of the experiment. As expected, expression of GFP from 293T cells transduced with HR'CMV-Luc-I2GFP/IN− was high at day 1 (average 47%) and gradually decreased to background levels by day 28 (Fig. 2D). The absence of an *ori* in this vector resulted only in transient expression of GFP from vector E-DNA with loss of the vector as the cells divided.

3.2. Quantitative c2LTR PCR from IN competent and IN defective

In order to evaluate if expression from the IN-defective vector was ascribed to the maintenance of an episomal template, we performed quantitative PCR to measure the copy number of c2LTR unintegrated forms in 293 and 293T cells transduced with both IN-competent and IN-defective HR'SV-TK-I2GFP and HR'CMV-Luc-I2GFP vectors (Fig. 3). Real-time PCR performed on 293 and 293T cells infected with either IN-competent vector indicated that, soon after transduction (day 1), c2LTR levels ranged from 0.6×10^3 to 3×10^3 copies per 10^4 cells (Fig. 3A and B), while after infection with either IN-defective vector, c2LTR levels ranged from 6×10^3 to 6×10^4 copies per 10^4 cells (Fig. 3C and D). Subsequently, in the 293 cells lacking the Tag, the copy number for c2LTR gradually decreased over time, as the cells replicated (Fig. 3A and C); eventually, c2LTR forms become undetectable after day 14. Similar results were obtained after transduction of 293T with either the IN-competent or IN-defective HR'CMV-Luc-I2GFP vectors (Fig. 3B and D). This was expected, since the target cell contains the Tag but the transducing vector does not include the corresponding *ori*, thus preventing the episomal maintenance of the lentiviral vector.

In contrast, in the SV40 *ori* containing IN-competent and IN-defective HR'SV-TK-I2GFP vectors, the c2LTR episomal forms remained stable in Tag-expressing 293T cells. In particular, the c2LTR copy number in cells transduced with the HR'SV-TK-I2GFP/IN+ vector, ranged from an average of 1.3×10^3 copies of c2LTR at day 1 to an average 8×10^4 copies at day 56 post-transduction. Similarly, the levels of c2LTR in the 293T transduced with the HR'SV-TK-I2GFP/IN− vector, ranged from 5×10^3 at day 1 and increased to an average of 3.1×10^6 copies at day 56. The higher number of c2LTR in both cell types infected with either IN-defective vector is due to the shunting of vector DNA exclusively toward E-DNA production, while in the IN-competent infected cells a portion of the vector DNA becomes integrated. These data further confirm that the presence of the SV40 *ori* in the context of an IN-defective vector transducing 293T cells, allows for the formation and increased stability of circular E-DNA. Of note, the levels of c2LTR in the 293T cells transduced with both the IN-competent

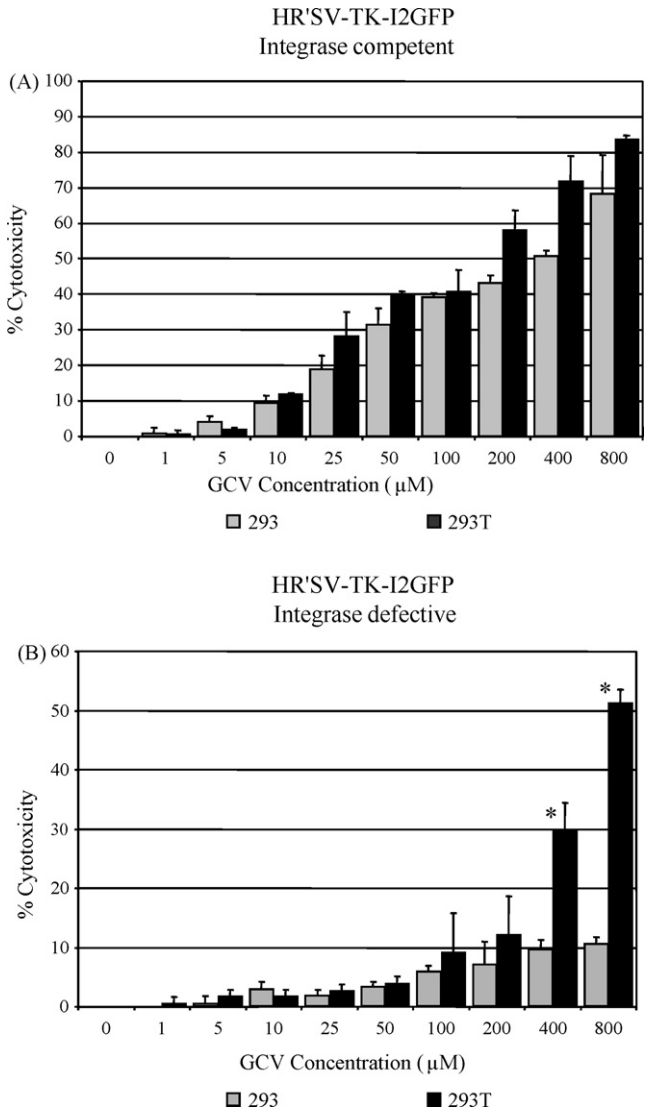

Fig. 4. Ganciclovir (GCV) activity on 293 (grey bars) and 293T (black bars) cells transduced with IN-competent (A) and IN-defective (B) HR'SV-TK-I2GFP vectors at 4 weeks post-infection. GCV sensitivity was determined by culturing the transduced cells with increasing concentrations of GCV, ranging from 0 to 800 μM. Growth was allowed to proceed in the presence of GCV for 4 days, after which cell viability was measured by MTS cell proliferation assay as described in Section 2. Data are shown as averages of three independent experiments, representing means ± standard deviations. $^{*}p < 0.002$.

and IN-defective HR'SV-TK-I2GFP vectors, were statistically significant starting from day 4 when compared to the corresponding HR'CMV-Luc-I2GFP CMV control vectors ($p < 0.05$).

3.3. GCV sensitivity of cell transduced with IN-competent and IN-defective HR'SV-TK-I2-GFP vectors

Following transduction of 293 and 293T with the IN-competent and IN-defective HR'SV-TK-I2GFP recombinant viruses, a dose–response curve for GCV sensitivity (range 0–800 μM/ml) was performed at day 28 from the infections shown in Fig. 2. As expected, 293 and 293T cells transduced with the IN-competent vectors were highly susceptible to GCV treatment. In particular, cytotoxicity in cells transduced with the IN-competent vector increased with increasing molar amounts of GCV (Fig. 4A) approaching 70% and 84% in 293 and 293T cells, respectively, at the highest amount of GCV. On the other hand, 293 cells transduced with the IN-defective recombinant vector HR'SV-TK-I2GFP showed minimal levels of toxicity (up to 10%) even at the highest concentration of GCV (Fig. 4B), which reflected the toxicity obtained in untransduced 293 and 293T cells (data not shown). This was expected, since at time of GCV treatment, day 28 from the infection, unintegrated forms of the vectors were absent (Fig. 3C) and the vector did not express the indicator gene GFP (Fig. 2C), thus leading to unresponsiveness to GCV treatment.

Interestingly, after transduction of the Tag positive 293T cells with the IN-defective HR'SV-TK-I2GFP vector, the cells were susceptible to GCV at 4 weeks post-transduction (Fig. 4B). In particular, cytotoxicity increased in a dose responsive manner with increasing molar amounts of GCV and sensitivity to GCV was statistically significant at 400 and 800 μM ($p < 0.002$) when compared to 293 cells transduced with the IN-defective vector (Fig. 4B), which were not sensitive to GCV, as described above. These results indicate that Tag expression from 293T sustained expression (Fig. 2D) and persistence (Fig. 3D) of E-DNA in target cells resulting in killing of target cells. The lower level of overall cytotoxicity noticed in the Tag positive 293T cells transduced with the IN-defective vector, as compared to the level of cytotoxicity found in 293 and 293T cells transduced with the IN-competent vector was due to the lower number of 293T cells transduced with the IN-defective vector, approaching an average of 29% GFP-positive cells at the time of analysis and GCV treatment (Fig. 2D).

4. Discussion

Currently, 67% of all gene therapy protocols have focused on cancer as a therapeutic target (Smythe, 2000; www.wiley.co.uk/genetherapy/clinical). More conventional methods of treating cancer include cytotoxic chemotherapy alone or in combination with radiotherapy and surgery. Herein, effective treatment is achieved only if a sufficiently high concentration of the cytotoxic drug is delivered to the target cells. However, the lack of selectivity of the drugs makes it problematic to selectively eliminate tumor cells while preventing toxicity to normal cells. On the other hand, cancer gene therapy requires a therapeutic gene to be delivered efficiently and selectively to the tumor cells, thus removing the malignant cells while minimizing damage to normal tissue. Consequently, targeting of the tumor with a therapeutic gene encoding metabolic enzymes that convert systemically delivered prodrugs into toxic metabolites would result in a high concentration of a toxic product in the target cells of tissue, thus avoiding the systemic toxicity often associated with conventional radio- and chemotherapy. As such, selective transduction of cancerous cells and/or selective expression of genes within these cells would achieve selective removal of tumor cells.

The system reported here takes advantage of differences between tumor and normal cells that would lead to selective persistence and expression of the delivered gene. Tumor specific markers include differentially expressed proteins that permit selective activation of a therapeutic agent in the tumor cell. To this aim, we have developed a suicide gene therapy vector based on an IN-defective lentiviral vector modified to allow conditional episomal replication and persistent expression in SV40 Tag positive cells, leading to removal of Tag expressing cells in the presence of GCV. Episomal replication and persistent expression of this lentiviral-hybrid episomal vector is dependent on two elements: (i) the presence of the *trans*-acting tumor specific marker, Tag, in the target cells and (ii) the SV40 promoter/*ori* in the lentiviral vector.

We and others have previously demonstrated that an IN-defective lentiviral vector containing the SV40 *ori* can be

continually maintained as episomes, resulting in persistence of gene expression only in the cells containing the *trans*-acting viral protein Tag (Lu et al., 2004; Vargas et al., 2004). In this report, the IN-defective lentiviral episomal vector was further modified so that the expression from the internal SV40 promoter would include the TK suicide gene along with an IRES for optimal bicistronic expression of GFP. This vector design suggests that this system could be useful to specifically express the suicide gene in naturally occurring virus-infected cells for removal of the target cells in the presence of GCV, while non-infected cells would remain healthy and unaffected.

Selective transcription and persistence of the IN-defective episomal vector was evident only in Tag expressing cells. In addition, sustained transcription from the IN-defective hybrid lentiviral vector was convincingly associated with replication of the c2LTR forms. In particular, quantitative evaluation of c2LTR established that c2LTR forms were present early after transduction, independently of the integration competency of the vector. Subsequently, the c2LTR forms exhibited a two-log increase only in the Tag expressing cells 293T cells, suggesting that the episomal vector was replicating along with the cells, and were maintained for up to 56 days. In particular, Tag expressing cells transduced with the SV40 *ori*-containing IN-defective lentiviral vector showed an average of 20–29% GFP positive cells throughout the time course of the experiments shown, representing the number of transduced cells sensitive to GCV. Indeed, following GCV treatment, an average of 52% of 293T cells were eliminated from the culture (Fig. 4B), including all cells transduced with GFP (data not shown). Conversely, transduction with IN-competent vectors, did not discriminate between Tag positive and negative cells, since 293 and 293T cells were similarly affected by the GCV treatment. This suggests that conditional replication of the IN-defective lentiviral-hybrid episomal vector, allows for the removal of Tag expressing transduced cell in a dose-responsive manner in the presence of GCV.

This vector system has been specifically designed for treatment of malignancies caused by SV40. However, the association of SV40 with human malignancies remains under debate (Klein et al., 2002; López-Ríos et al., 2004; Poulin and DeCaprio, 2006). Importantly, for malignant mesotheliomas, recent data from *in vitro* and *in vivo* studies strongly support a causal role for SV40, probably as a cofactor with asbestos (Cristaudo et al., 2005; Kroczynska et al., 2006; Comar et al., 2007). These and other previous findings led the Institute of Medicine of the National Academies to recognize that SV40 is an emergent human pathogen and conclude that the present biological evidence indicates that infections with this DNA tumor virus could lead to cancer in humans under natural conditions (Stratton et al., 2003).

With appropriate modifications, such a therapeutic approach could be used to target cells associated with the expression of other virally encoded antigens. Such modifications could be accomplished by including replicons from episomally-replicating DNA viruses that infect human cells, including, BK virus, JC virus, Human papilloma virus (HPV) and Epstein-Barr virus (EBV), for treatment of other virus associated diseases. This includes BK-associated nephropathy (PVAN) (Hirsch et al., 2006; Dall and Hariharan, 2008), JC-associated progressive multifocal leukoencephalopathy (PML) (Boren et al., 2008) and HPV-associated high-grade squamous intraepithelial lesions (HSIL) containing episomal forms of the virus (Fontaine et al., 2005; Yoshida et al., 2008). We speculate that the incorporation of an *ori* of viral origin into the backbone of IN-defective lentiviral vectors would enable the vector to remain in an episomal replicative form throughout the course of the infection, if the target cells provide in *trans* the essential viral factor to allow for the episomal replication of the hybrid vector. However, a recent report (Lu et al., 2004) showed that the incorporation of oriP of EBV into the backbone of an IN-defective HIV-1 did not allow sustained expression of the virus when *in trans* was provided the corresponding EBNA1 protein, suggesting that the vector system we described might not be suitable against diseases caused by EBV. This might be explained by the fact that, while SV40 uses the Tag to replicate and amplify the viral replicons (Cole and Conzen, 2001), EBV uses the EBNA1 protein for maintaining a steady copy number of oriP containing episomes (Kieff and Rickinson, 2001). Finally, potential applications would also include the selective expression of cytokine, immunogenic factors, and prodrug activators in order to eliminate the virus-infected cells.

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