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Development of constitutive and inducible self-inactivating lentiviral vectors and their application in cardiovascular gene transfer

Research Article

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Abbreviations: central polypurine track, (cPPT), Dulbecco's modified Eagle's medium, (DMEM), encephalomyocarditis virus, (EMCV), green fluorescence protein, (GFP); Human Umbilical Vein Endothelial Cells, (HUVECs), internal ribosomal entry site, (IRES), mean fluorescence intensity, (MFI); multiplicity of infection, (MOI), murine leukemia virus, (MLV), recombinant adeno-associated virus, (rAAV), replication competent retrovirus, (RCR), reverse tetracycline trans-activator, (rtTA), reverse transcriptase, (RT), Secreted Alkaline Phosphatase, (SEAP), self-inactivating, (SIN), tet operators, (tetO), tetracycline repressor, (tetR), tetracycline trans-activator, (tTA), vesicular stomatitis virus envelope glycoprotein G, (VSV-G), Woodchuck Hepatitis Virus posttranscriptional regulatory element, (WPRE)

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Summary

Regulatable lentiviral vectors are among the newest gene transfer tools that are being used increasingly for basic research. They are being explored for clinical applications as well. In this study, we conducted investigations that were aimed toward improving the biosafety and the efficiency of our previously described one piece tetracycline-autoregulatable lentiviral vector (Ogueta et al, 2001). The new genetically modified regulatable vectors are now self-inactivating (SIN) minimal vectors and contain the central polypurine track (cPPT) region and the Woodchuck Hepatitis Virus posttranscriptional regulatory element (WPRE) for increased transgene expression. Newly designed lentiviral vectors were tested both *in vitro* and in an *in vivo* rat model of myocardial gene transfer. The *in vitro* results show that while all three generations of the lentiviral vectors respond to doxycycline control in both 293T and HUVEC cells, increased green fluorescence protein (GFP) expression in the presence of WPRE element is only observed in 293T cells, suggesting a tissue/cell type dependent effect of the WPRE. *In vivo* experiments in rats showed that positive LacZ/GFP gene transfer was accomplished by direct myocardial injection and that LacZ gene expression was regulatable by doxycycline. The success of direct myocardial gene transfer with one piece tetracycline-autoregulatable lentiviral vector adds an attractive gene transfer tool to the field of cardiovascular gene delivery.

I. Introduction

Lentiviral vectors have proven to be useful tools for gene transfer into a wide variety of cells and tissues both *in vitro* and *in vivo* (Vigna and Naldini, 2000). Since the initial development of the HIV based lentiviruses, several groups have worked on improving their efficiencies by adding sequences that act at the transcriptional and translational level (Zufferey et al, 1999; Follenzi et al, 2000; Zennou et al, 2000). Biosafety of the lentiviral vectors has also been improved by inactivating the U3 region of the 5' LTR and by deleting accessory protein genes from the transfer vector and from the packaging constructs (Zufferey et al, 1997, 1998; Dull et al, 1998;

Kim et al, 1998; Miyoshi et al, 1998). All these modifications have led to great improvement in the lentiviral vectors and have made them more attractive for clinical gene therapy applications. However, effective gene-based therapies will require not only efficient delivery of the therapeutic gene but in many cases, controllable gene expression in a dose-dependant and reversible manner (Agha-Mohammadi and Lotze, 2000; Koransky et al, 2002).

One of the most attractive and widely used regulatable systems in mammalian cells is the tetracycline-regulatable system. This system is particularly attractive for clinical applications because of the pharmacokinetics, cost-effectiveness and excellent safety record of tetracycline and its analog doxycycline. Moreover, the system has been engineered as either a suppressor (Tet-Off) or as an inducer (Tet-On) in regulating transgene expression using a tetracycline trans-activator (tTA) or a reverse tetracycline trans-activator (rtTA), respectively. In the latter system the tetracycline repressor (tetR) itself, rather than the tetR-mammalian cell transcription factor fusion derivative, can function as a potent trans-modulator to regulate gene expression in mammalian cells (Yao et al, 1998). This system has an advantage over the previously described rtTA system in that it avoids potential toxic effects caused by the use of a chimeric transactivator (Gossen and Boujard, 1992).

Like retroviral vectors such as murine leukemia virus (MLV), lentiviral vectors can be used for stable gene transfer due to their ability to integrate into its host genome which in turn enables long-term gene expression. One of the major advantages of the lentiviral vectors over MLV is its ability to provide gene transfer into non-dividing cells. This advantage is very important for several target organs such as the liver, heart, skeletal muscles, brain, and hematopoietic progenitor cells. Long-term transgene expression using lentiviral vectors has been demonstrated in brain, liver, muscle, retina and kidney (Naldini, et al, 1996 a and 1996b; Zufferey et al, 1997, 1998; Follenzi et al, 2002; Gusella et al, 2002). *In vitro* and *in vivo* lentivector mediated gene transfer into cardiomyocytes have been recently reported as well (Zhao et al, 2002; Bonci et al, 2003; Fleury et al, 2003).

We have previously shown the efficacy of a one piece autoregulatable lentiviral system (Ogueta et al, 2001). This vector is composed of a chimeric promoter, containing two tandem repeats of the tet operators (tetO) fused to the immediate-early cytomegalovirus enhancer promoter that drives the expression of the transgene, and tetR which is expressed on the same transcrptional unit through an internal ribosomal entry site (IRES) (Ogueta et al, 2001). The main objective of this research was to further improve the tetracycline-regulatable lentiviral vectors SIN1PiN and SIN1PcN (Ogueta et al, 2001 and **Figure 1**). In order to improve biosafety, genes encoding HIV-1 accessory proteins as well as the residual gag/pol DNA sequences were deleted from the transfer vector. The cPPT element was restored in the modified vector because of its important role in HIV-1 and lentiviral vector DNA nuclear import (Follenzi et al, 2000; Zennou et al, 2000) and its ability to increase the transduction efficiency of lentiviral vectors (reviewed by Ailles and Naldini, 2002). In order to increase gene expression in the transduced cells, the Woodchuck Hepatitis Virus posttranscriptional regulatory element (WPRE, Donello et al, 1998; Zufferey et al, 1999) was inserted into the vector as well. Experiments were performed to compare the efficacy of these newly modified lentiviral vectors in terms of overall level of gene expression as well as the regulatable gene expression in different cells. Furthermore, the newly designed lentiviral vector was tested in an *in vivo* rat model of myocardial gene transfer. The initial results showed that these vectors are very promising for further studies where both efficient and regulatable gene expression are desired. In addition, success in direct myocardial gene transfer using lentiviral vectors adds to the field of cardiovascular delivery an attractive gene transfer tool with the regulatable gene expression capability.

II. Materials and methods

A. Cell lines and reagents

Human embryonic kidney 293T cells were obtained through the NIH AIDS Reagent Program and were maintained in Dulbecco's modified Eagle's medium, DMEM, (Gibco-BRL, Rockville, MD) supplemented with 10% FBS (Sigma, St. Louis, MO) or 10% Tet System approved FBS (Clontech, Palo Alto, CA, USA), 100 I.U./mL Penicillin, 100 μg/mL Streptomycin, and 2 mM Glutamine (Gibco-BRL, Rockville, MD, USA). Human Umbilical Vein Endothelial Cells (HUVECs) were maintained in Medium 200 supplemented with LSGS (all from Cascade Biologics, Portland, OR, USA). All ligations were performed using DNA Ligation Kit from TaKaRa (PanVera, Corp., Madison, WI, USA) according to the manufacturer instructions. PCRs were performed using Expand High Fidelity PCR System (Boehringer-Mannheim, Indianapolis, IN, USA). All chemical reagents were obtained from Sigma (St. Louis, MO, USA).

B. Plasmid construction

The helper packaging plasmid, pCMVΔR8.2ΔVPR, has been described elsewhere (Naldini et al, 1996a, 1996b). The envelope-encoding plasmid, pCMV VSV-G, contains the vesicular stomatitis virus glycoprotein under regulation of a CMV promoter. Generation of the SIN1PiN and SIN1PcN lentiviral vectors was described previously (Ogueta et al, 2001).

1. Generation of SINΔtat1PiN and SINΔtat1PcN

To generate *tat* independent vectors, the SIN1PiN and SIN1PcN lentiviral vectors were utilized. Specifically, a frameshift mutation was introduced by PCR at the start codon of the *tat* open reading frame, changing the first two amino acids of the first exon from Met-Ile to Glu-STOP. The PCR primers used were: forward primer - 5' gtt act cga cag agg aga gca aga gat cta gcc agt aga tcc tag act aga gcc 3'; reverse primer - 5' ggc tct agt cta gga tct act ggc tag atc tct tgc tct cct ctg tcg agt aac 3'.

2. Generation of SINmin1PiNW and SINmin1PcNW

The Woodchuck Hepatitis B Virus Posttranscriptional

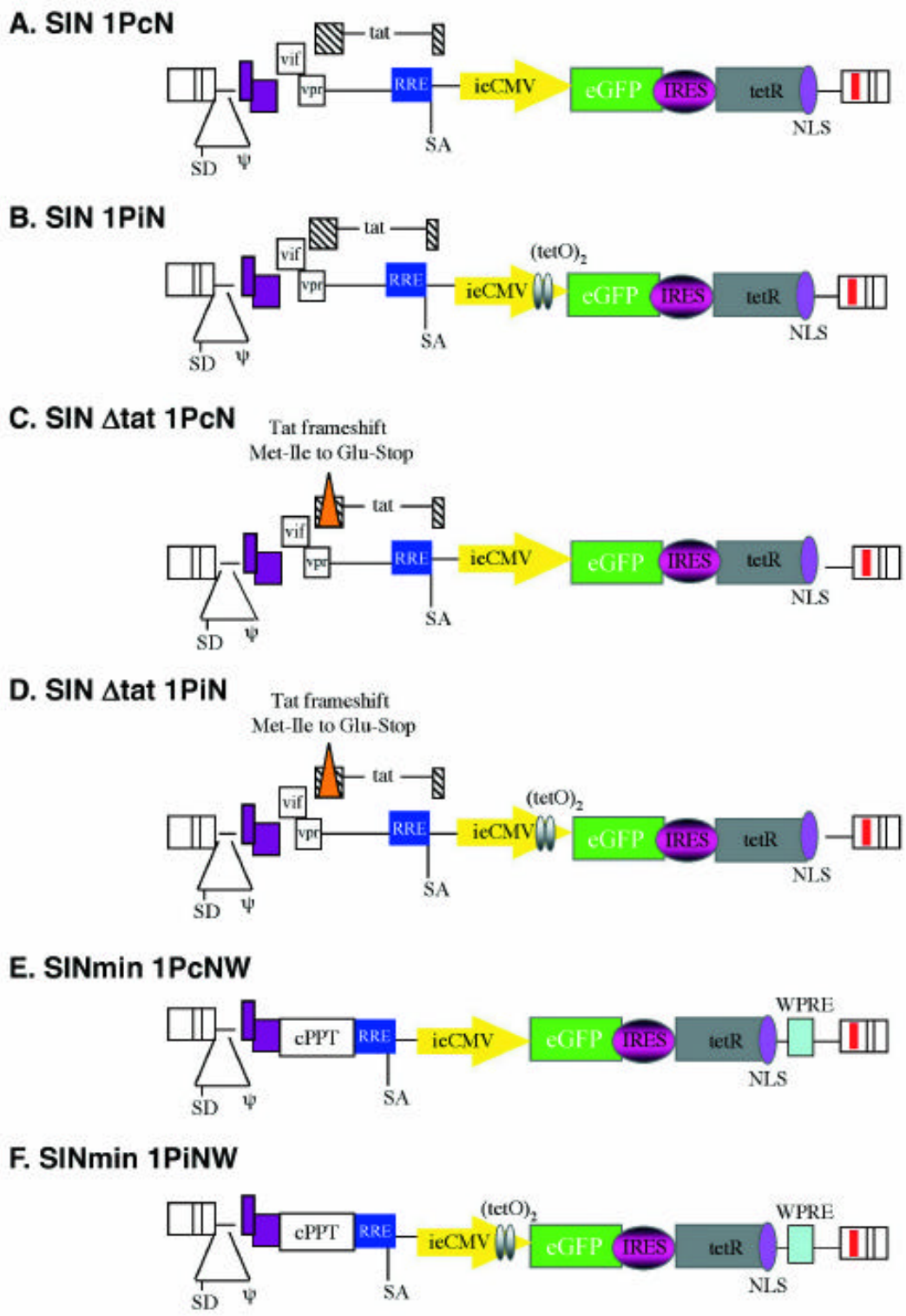

Figure 1. Schematic representation of three generations of the lentiviral transfer vectors. ***A*** and ***B*** represent the first generation of self-inactivating vectors SIN 1PcN and SIN 1PiN (Ogueta et al, 2001). ***C*** and ***D*** represent the second generation of self-inactivating vectors SINΔtat 1PcN and SINΔtat 1PiN in which the expression of the HIV *tat* gene is eliminated from the first generation vectors through a frame-shift point mutation. ***E*** and ***F*** represent the third generation of self-inactivating minimal vectors SINmin 1PcNW and SINmin 1PiNW in which a 2.2 kb *pol/gag* sequence was deleted from the second generation vectors and replaced with the cPPT sequence. The third generation vectors also contain the Woodchuck Hepatitis B Virus posttranscriptional regulatory element designated as ***W*** in the vector name. For all vectors, the ***N*** represents the nuclear localization signal (NLS) of the SV40 large T antigen fused to the C-terminus of tetR protein. ***1Pc*** denotes a single cassette containing the immediate-early CMV enhancer/promoter (ieCMV) driving constitutive expression of the trans-gene. ***1Pi*** denotes a single cassette containing two tandem repeats of the tetO inserted 10 bp downstream of the TATA box within the ieCMV driving tetracycline-regulatable expression of the trans-gene.

Regulatory Element (WPRE) was inserted into the Cla I sites of either SINΔtat1PiN or SINΔtat1PcN to obtain SINΔtat1PiNW and SINΔtat1PcNW. In order to generate the SIN minimal vector, a 2.2 Kb of the gag/pol region was deleted from SIN Δtat1PiNW and SINΔtat1PcNW by restriction enzyme digestion using ClaI and XhoI. The cPPT/CTS region was amplified from a wild type HXBc2 based HIV-1 vector, pHlibeGFP, using the following pair of primers: cPPT forward - 5' aac cca tcg atg ata aac aaa tgg cag tat tca tcc 3'; cPPT reverse. 5' aag ccg ctc gag cga cgc gtc ccc ttc acc ttt cca 3'. The 241 bp PCR fragment was digested with ClaI and XhoI and cloned back into the vector backbone. These alterations generated the SINmin1PiNW and SINmin1PcNW vectors.

3. Generation of the SINmin 1Pi(SEAP)NW

To generate an inducible plasmid with the Secreted Alkaline Phosphatase (SEAP) reporter, the SEAP reporter gene was obtained by PCR from the pSEAP2-Control plasmid (Clontech, Palo Alto, CA, USA) using the following primer pair: Forward-Age I-SEAP 5' cga ccg gtg ccc acc atg ctg ctg ct 3' and Reverse-Not I-SEAP 5'gcg gcc gct atc atg tct gct cga agc 3'. The PCR product was cloned into the SINmin1PiNW plasmid through the AgeI/NotI sites to generate the SINmin 1Pi(SEAP)NW construct.

4. Generation of the SINmin 1Pi(LacZ)NW

For cloning purposes, a Pme I site was introduced by PCR at the 5' end of the IRES in the SINmin1PiNW vector previously to create SINmin1PiNW-Pme-IRES vector. Lac Z gene was obtained from pCMVtetOlacZ (a gift from Dr. Feng Yao) and cloned via Pme I into the SINmin1PiNW-Pme-IRES lentiviral vector.

C. Production and assay of viral vectors

Virus supernatants were generated by three-plasmid co-transfection into 293T cells using the calcium phosphate method. Briefly, twenty-four hours prior to transfection, 5 x 10^6 293T cells were seeded onto a 100-mm tissue culture dish coated with poly-D-lysine and human fibronectin (Sigma, St. Louis, MO, USA). One hour prior to transfection, the media was replaced with 6 ml of fresh 293T cell growth medium. Transfection cocktail contained 15μg of transfer plasmid, 10μg pCMVΔR8.2ΔVPR, and 5μg pCMV VSV-G. After 15-16 hours of incubation, the DNA/$CaPO_4$ mixture – containing medium was removed and replaced with 5 ml of fresh medium containing 10 mM of sodium butyrate. Cells were treated with sodium butyrate for 10-12 hours, washed once with PBS, and replenished with 293T cell growth medium that contained the Tet System FBS. Viral supernatants were harvested every 17-24 hours thereafter, filtered through 0.45 μm filter, aliquoted and stored at -80°C. For the viruses used in the animal experiments, the supernatants were concentrated by ultracentrifugation at 27,000 rpm, 4°C for 2 hours. Viral pellets were resuspended in 1X PBS at 4°C for 1-2 hours, aliquoted and stored at -80°C. Alternatively, the supernatants were concentrated using Centricon-80 ultrafiltration devices (100,000 dalton MWCO) according to the manufacturer instructions (Fischer, USA).

Titers of the viral supernatants were assayed on 293T cells. Briefly, the cells were seeded 24 hours prior to infection at a density of 5 x 10^5 293T cells per well in a 6-well tissue culture plate. Cells were infected with serial dilutions of viral supernatants in the presence of 8 μg/ml protamine sulfate, grade III, herring, (Sigma, St. Louis, MO, USA) in 1 ml 293T growth medium by spin-inoculation for 2 hours at 1,600 rpm (Sorvall RT-3000B rotor), 32°C. The cells were then incubated at 37°C in the presence of 5% CO_2 overnight. Next day, the inoculums were removed and replaced with 1 μg/ml doxycycline (for Pi vectors) or without doxycycline otherwise. The GFP expression of transduced cells was measured by FACS on a FACScan (Becton Dickinson) at 48 hours post-infection. Titers of GFP-expressing lentiviral vectors were estimated by the following formula: Titer = the cell numbers at initial seeding x % GFP positive cells x the fold of dilution. For analyzing the transducing units of the SINmin1Pi(lacZ)NW vector, 48 hours post-transduction, the media were removed and the cells were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. Cells were then washed with PBS and permeabilized with 0.05% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA) in PBS containing 1.5 mM $MgCl_2$. Cells were washed twice and stained with X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.5 mg/ml X-gal in PBS) for 1 hour at 37°C. Titers of β-galactosidase-expressing lentiviral vectors were estimated by multiplying the number of blue cells (foci) per well with the folds of dilution. Levels of reverse transcriptase (RT) activity were also determined for each viral preparation.

D. *In vitro* characterization of lentiviral vectors

1. Transduction of 293T and HUVEC cells

For 293T transductions, 2 x 10^5 293T cells per well were seeded in 12-well plates coated with poly-D-lysine and human fibronectin. One day after seeding, the cells were transduced with different generations of the lentiviral vectors at MOI between 1-2.5 by spin-inoculation as described above for titration. For HUVEC transductions, 24 hours prior to transductions, 4 x 10^4 HUVECs per well were seeded in 12-well tissue culture plates. On the day of transduction, equal amount of viruses (MOI 3-6) were added to cells in the presence of 16 μg/ml of DEAE-Dextran (Sigma, St. Louis, MO, USA). Cells were spun as above and incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. Following the incubation, cells were washed and replaced with fresh Medium 200 supplemented with LGS in the presence or absence of 1μg/mL doxycycline.

2. Analysis of the reporter gene expression

The transduced 293T or HUVEC cells were treated with or without 1 μg/mL doxycycline as indicated in the text. At different time post-transduction, the cells were assayed for GFP expression by FACS analysis or β-galactosidase expression by X-gal staining as discussed above. To analyze the secreted alkaline phosphatase (SEAP) reporter expression, supernatants were harvested from the lentiviral vector transduced cell cultures at the time indicated and stored at –70°C. The SEAP activity in the conditioned medium was assayed using the BD Great EscAPe SEAP Chemiluminescence Detection Kit (Catalog number 631701) according to the manufacturer's instruction.

3. Real-time PCR for estimation of lentiviral vector copy number

Real-time PCR was performed to estimate the intracellular copy number of each lentiviral vector. The procedure was essentially as described by Sastry et al, (2002) with following modifications. DNA was isolated from mock transduced or lentiviral vector transduced 293T cells using the DNeasy Tissue Kit (Qiagen, catalog # 69506). Real-time PCR analyses were carried out in duplicate as the following: Total of 10 μl (500 ng) DNA samples was mixed with 40 μl of a PCR master mix

containing 300 nM each of the primers FPLV2 and RPLV2, 100 nM of the probe LV2 (Sastry et al, 2002), 1 X TaqMan buffer A, 3.5 mM $MgCl_2$, 200 uM of each dNTP, 0.025 units/µl of AmpliTaq Gold polymerase, and 0.01 units/µl of AmpEraseUNG. All reactions were carried out on a PE Applied Biosystems 7700 sequence detection system using one cycle of 95°C for 10 min, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. A standard curve was generated using plasmid DNA at 10^{10} to 10^3 molecules/µl diluted with untransduced 293T cell genomic DNA. The data were analyzed using Applied Biosystems' Sequence Detections Systems 1.7 software and normalized as lentivirus copy number per cellular genome.

E. *In vivo* gene delivery of lentiviral vectors

1. Direct myocardial gene transfer

Adult male Sprague-Dawley rats (270-280 grams) were purchased from Harlan, Indianapolis, IN. All animals were maintained in the BL2+ bio-containment animal facility of Dana-Farber Cancer Institute and all surgical procedures were performed with approval of the Institutional Animal Care and Use Committee. Direct myocardial gene transfer was performed following a procedure established earlier (Melo et al, 2002) and briefly described as follows. Adult male rats were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by I.P. injection and properly intubated. The chest was opened through a midline incision. The lentiviral vector solution or PBS (200-500 µl total volume per rat) was injected directly into the left ventricle at multiple injection sites (2-5 sites) using a 30-gauge needle. The chest was then closed and the recovery of each rat from its surgery was confirmed before returning to the cage.

2. Tissue processing

At various times following gene transfer with or without doxycycline treatment [1µg/ml doxycycline, 2% (w/v) sucrose in drinking water] animals were euthanized by CO_2 inhalation. The hearts were perfused with 8-10 ml of cold PBS and the left ventricle wall of the rat heart was excised from sacrificed animals. The section was thoroughly washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 30 minutes on ice (Byun et al, 2000). After fixation, the tissue was rinsed twice in PBS, cut into 2-3 mm thickness pieces, and saturated in 30% (w/v) sucrose in PBS containing 0.02% Na-Azide at 4°C overnight. For immunohistochemistry analysis of GFP gene expression, the tissue samples were embedded with the Tissue-Tek O.C.T. compound (Sakura, Torrance, CA) and frozen on dry-ice. The frozen tissues were further sectioned to 8-10 µm using a cryostat. Sections were incubated with mouse monoclonal anti-GFP antibodies at 1 µg/ml (Clontech, Cat. #8362-1) as the primary antibody followed by incubation with rat adsorbed, biotinylated horse-anti-mouse IgG(H+L) at 7.5 µg/ml (Vector Laboratories, cat. # BA-2001) as the secondary antibody. The sections were stained with Vectastain ABC reagent and Vector NovaRED substrate kit followed by counter-staining with Vector Hematoxylin QS reagent, all from Vector Laboratories. The mounted sections were observed under light microscope and photographed using a Nikon camera. For analysis of β-galactosidase expression, gross X-gal staining was performed on the tissue pieces overnight at 37°C with X-gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal, 0.02% NP40 and 0.02% Na deoxycholate in PBS). Tissues were photographed using a Nikon Cool Pix 950 camera.

III. Results

A. Generation of tat-independent and minimal lentiviral vectors expressing eGFP mediated by either constitutive or tet-regulatable CMV promoter

In order to improve their safety and possibly efficacy, previously published first generation vectors SIN1PiN and SIN1PcN transfer cassettes (Ogueta et al, 2001) (**Figure 1**, panels A and B) were first made Tat protein-independent by introducing a frame shift mutation in the first exon of the *tat* gene (SINΔtat1PcN and the tetracycline regulatable SINΔtat1PiN construct – the second generation, see **Figure 1**, panels C and D). The *tat* mutation has an added biosafety feature in that any recombination that may occur during lentivector production in mammalian cells would not result in a replication competent retrovirus (RCR) since Tat protein would not be available for transactivation of the LTR. The third generation minimal lentiviral vectors that lack all HIV accessory proteins and contain only minimal regions of *gag*/*pol* sequence were generated next with additional deletions (**Figure 1**, panels E and F). A 2.2 kb gag/pol fragment including the sequences encoding the HIV accessory genes was deleted and replaced by the cPPT/CTS region which has been shown to improve the efficiency of infection or transduction (Follenzi et al, 2000). The Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) was also inserted since WPRE has been shown to increase protein production of the transgene by 5-8 fold (Zufferey et al, 1999). These modifications generated the SINmin1PiNW and SINmin1PcNW vectors (**Figure 1**, panels E and F).

B. Evaluation of newly generated tat-independent and minimal lentiviral vectors

All three generations of the constitutive expressing lentiviral vectors, SIN1PcN, SINΔtat1PcN, and SINmin1PcNW were produced by three-plasmid co-transfection into 293T cells using the calcium phosphate method. pCMVΔR8.2 with a VPR deletion was used as the helper plasmid for packaging. The lentiviral vectors were pseudotyped with the vesicular stomatitis virus envelope glycoprotein G (VSV-G) encoded by pCMV VSV-G plasmid. There was no significant drop in viral titers with the two latest modifications. The resulting vectors were used to transduce in parallel 293T and Human Umbilical Vein Endothelial Cells (HUVECs). The experiments were performed at a multiplicity of infection (MOI) of 1-2.5 for 293T cells and 3-6 for HUVECs according to GFP titers on 293T cells.

For 293T cells, high levels of viral transduction and gene expression were achieved in all three generations of lentiviral vectors constitutively expressing eGFP over a period of 10 days (**Table 1A,** 4-day experiment, and **1B,** 10-day experiment). The lack of *tat* in the gene transfer vector SINΔtat1PcN did not confer any significant advantage/disadvantage over the parental SIN1PcN vector.

Table 1. Characterization of GFP expression with different generations of the constitutive lentiviral vectors in 293T and HUVEC cells

1A

293T Expt.1	Day 2 GFP + cells		Day 3 GFP + cells		Day 4 GFP + cells	
	%	MFI	%	MFI	%	MFI
Mock	0.7	16.1	0.5	15.3	0.4	11.2
SIN 1PcN	84.2	170.4	85.7	168.7	88.8	247.9
SINΔtat 1PcN	78.7	136.4	75.1	124.7	86.3	217.0
SINmin 1PcNW	72.9	307.7	72.8	352.3	77.8	698.1

1B

293T Expt.2	Day 3 GFP + cells		Day 7 GFP + cells		Day 10 GFP + cells	
	%	MFI	%	MFI	%	MFI
Mock	1.3	20.8	0.7	16.5	1.8	20.3
SIN 1PcN	64.5	159.2	68.0	164.7	71.9	151.9
SINΔtat 1PcN	60.0	139.2	65.3	172.6	69.0	168.3
SINmin 1PcNW	61.0	456.6	55.4	762.7	55.2	443.2

1C

293T Expt. 3 Low MOI (Day 4)	% GFP + cells	MFI GFP + cells	Lentivirus DNA (copies/cell genomes)
Mock	0.6	11.4	0
SIN 1PcN	24.1	49.7	1.6-1.9
SINΔtat 1PcN	30.9	65.3	0.9-1.1
SINmin 1PcNW	34.0	282.0	0.4-0.6

1D

Summary of HUVEC Experiments (Day 3)	Expt. 1 GFP + cells		Expt. 2 GFP + cells		Expt. 3 GFP + cells		Expt. 4 GFP + cells	
	%	MFI	%	MFI	%	MFI	%	MFI
Mock	0.5	18.0	2.0	19.6	0.7	12.8	0.2	10.7
SIN 1PcN	71.8	372.7	89.8	644.9	95.7	769.7	95.2	598.4
SINΔtat 1PcN	78.7	422.6	86.6	688.1	92.8	532.1	93.0	567.6
SINmin 1PcNW	64.1	231.8	77.0	537.2	81.7	349.2	80.8	426.6

Characterization of GFP trans-gene expression with different generations of the constitutive lentiviral vectors in 293T cells and HUVEC. 293T cells were transduced with equal amounts (an estimated 1-2.5 TU by GFP titer) of different generations of the constitutive lentiviral vectors. Following 16 hours incubation, transduced cells were washed and replaced with fresh growth medium. HUVEC were transduced with equal amounts of the lentiviruses (an estimated 3-6 TU by GFP titer on 293T). The inoculants were removed after 4-hour incubation and replaced with fresh growth media. At indicated time of post-transduction, the cells were assayed for GFP expression by FACS analysis. Data represented are an average of duplicate samples. ***Tables 1A-1C*** represent three separate experiments of 293T cell transduction. ***1A*** shows a representative experiment with the GFP expression during earlier phase of the transduction (Day 2-4 post-tranduction). ***1B*** represents a different experiment with the GFP expression during early and later phase of the transduction (Day 3, 7 and 10 post-tranduction). ***1C*** contains data from a 293T cell experiment where lower multiplicity of infection (MOI, ~0.5TU judged by GFP titer) was used. Day 4 post-transduction data for both GFP expression and the copies of lentivirus DNA per cell genome as analyzed by real-time PCR are presented in **1C**. ***Table 1D*** is a summary of four HUVEC experiments at Day 3 post-transduction.

The minimal vector with WPRE, SINmin1PcNW, produced a 2 to 4-fold increase in transgene expression as judged by the mean fluorescence intensity of the accumulated GFP as compared to the two previous generations of the vectors when transduction of similar number of cells were confirmed (**Table 1A** and **1B**), indicating a positive role of the WPRE in up-regulating gene expression in 293T cells. To further confirm the increased GFP expression is a result of the presence of the WPRE, not a difference in the number of provirus integration, a transduction at MOI of 0.5 (293T GFP titer) was conducted and real-time PCR analysis was used to confirm that similar copies of the three generation vectors could be detected within transduced cells (**Table 1C**). Note the difference in GFP and DNA titer have been previously observed and reported (Sastry et al, 2002). Although the positive effect of WPRE obtained in 293T cells are in agreement with data previously published by Zufferey et al (1999), an unexpected observation was that while high levels of GFP expression over an eighteen days period were accomplished in lentiviral vector transduced HUVECs (**Table 1D** and data not shown), the WPRE sequence in the minimal vector, SINmin1PcNW, did not exert any positive effect on GFP transgene expression when compared to SIN1PcN or SINΔtat1PcN vectors

(compare **Table 1D** with **Table 1A or 1B**), indicating the effect of WPRE may be cell type specific.

C. Evaluation of tetracycline regulated GFP expression among three generations of lentiviral vectors

Tetracycline regulated GFP expression in the context of different generations of lentiviral vectors were compared by transducing 293T cells with the inducible version (Pi) of the lentiviral vectors at similar infectious units. After transduction, cells were incubated in complete growth media in the presence or absence of 1 μg/ml of doxycycline based on previously determined dose-dependency with doxycycline (Ogueta et al, 2001). GFP expression of transduced cells were analyzed by FACS at different times post-transduction. As shown in **Table 2**, in the presence of doxycycline, GFP expression were induced in all three generations of lentiviral vectors as evidenced by the increased mean fluorescence intensity when compared to the background expression in the absence of dxycycline. The actual folds of induction are calculated using data from **Table 2** and presented in **Figure 2A**. The data indicate that the induction folds are similar for SIN1PiN and SINΔtat1PiN but slightly higher for SINmin1PiNW. **Figure 2B** represents average induction from three (293T cells) or four (HUVEC) experiments. Again, SINmin1PiNW has higher induction in 293T cells but lower induction in HUVEC when compared to the other two vectors, possibly due to the previously discussed cell type specific effect of WPRE on GFP transgene expression of this vector (**Table 1**).

It was further determined that GFP expression of the two later generations of lentiviral vectors, like the original version, could be induced either immediately after induction or at later stages after transduction (**Figures 2A** and **3**). The doxycycline-induced GFP expression could be reversed by withdrawal of the drug (**Figure 3**). It generally takes about 6-9 days for the induced GFP gene expression fully return to the basal level, presumably due to the long half-life of the GFP proteins employed in the study (Corish and Tyler-Smith, 1999). It should be noted that doxycycline had no effect on GFP expression in the cells transduced with the constitutive vectors (data not shown).

Since the slow turnover rate of GFP makes it unsuitable to assess the kinetics of gene regulation by doxycycline, the gene encoding GFP was replaced with the secreted alkaline phosphatase (SEAP) gene within the SINmin1PiNW vector. This marker has the added benefits of faster RNA turnover (Noda et al, 1987) and feasibility of assaying for secreted protein production on a daily basis. The initial results indicate that it takes about 1-2 day to fully induce or reverse SEAP production (data not shown).

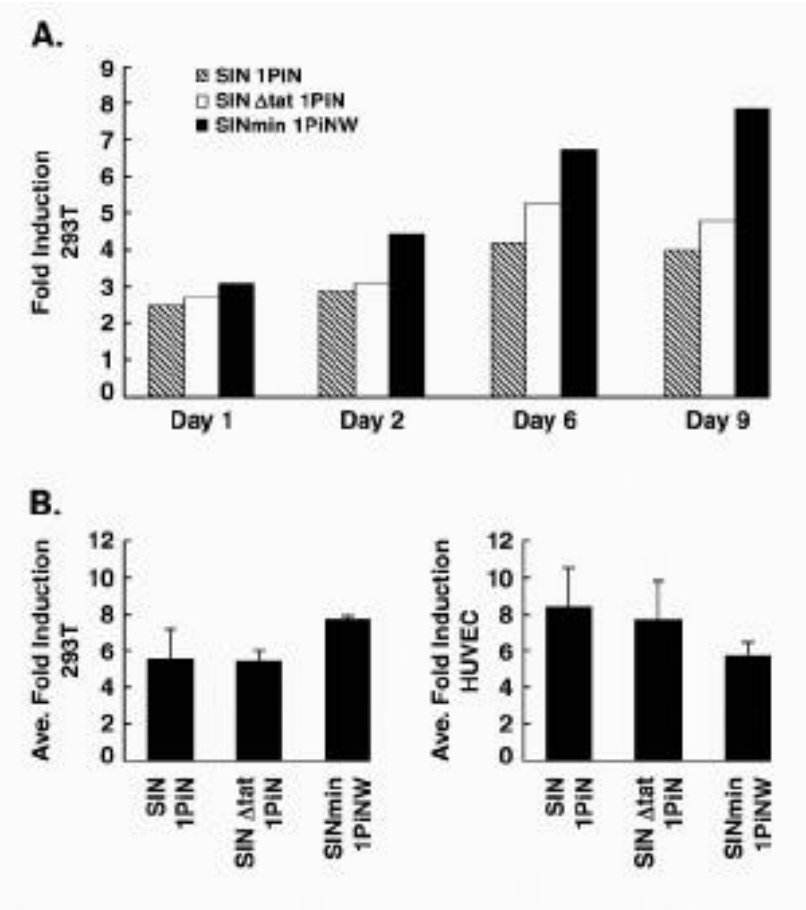

Figure 2. Fold induction of GFP transgene expression within different generations of the inducible lentiviral vectors in 293T cells and HUVEC. 293T cells were transduced and induced as described in **Table 2**. HUVEC were transduced with equal amounts of the lentiviruses (an estimated 3-6 TU by GFP titer on 293T). The inoculants were removed after 4-hour incubation and replaced with fresh growth media with or without 1μg/mL doxycycline. At indicated time of post-induction, the cells were assayed for GFP expression by FACS analysis. The induction fold is calculated by dividing the mean fluorescence intensity (MFI) of the doxycycline-induced sample with the non-induced sample after subtraction of the background MFI from mock-transduced cells. The fold induction in ***Panel A*** is calculated from a representative 293T cell experiment and the actual GFP induction data (both percentage of GFP+ cells and MFI) are presented in **Table 2**. ***Panel B*** represents an average fold induction on day 9 post-induction from three 293T cell experiments (left graph) and four HUVEC experiments (right graph).

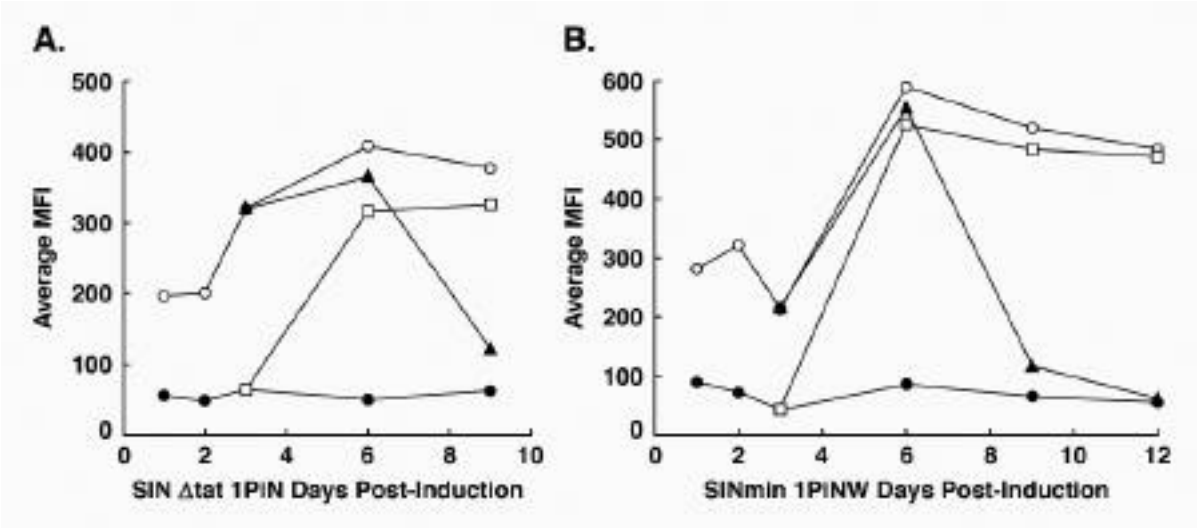

Figure 3. Mean fluorescence intensity analysis of GFP trans-gene expression upon addition or withdrawal of doxycycline 4 days post transduction. 293T cells were transduced with equal amounts (an estimated 1-2.5 TU by GFP titer) of different generations of the inducible lentiviral vectors. Following 16 hours incubation, transduced cells were washed and replaced with fresh growth medium in the presence (-○-, +/+) or absence (-●-, -/-) of 1 μg/mL doxycycline. At the Day 3 post-induction (Day 4 post-transduction), the cells were passed. A portion of the cells was maintained their original +/- doxycycline treatment. The doxycycline treatment in the other portion was switched, meaning doxycycline was withdrawn from the original drug containing samples (-▲-, +/-) or doxycycline was added into the previously drug absent samples (-□-, -/+). At indicated time of post-induction, the cells were assayed for GFP expression by FACS analysis and the data represented are an average of duplicate (***panel A***, SINΔtat 1PiN) or quadruplet (***panel B***, SINmin 1PiNW) samples.

D. *In vivo* myocardium gene transfer

For developing therapeutic approaches to treat cardiac diseases, we tested myocardial gene transfer using the SINΔtat1PcN and SINmin1PiNW lentiviral vectors. For *in vivo* myocardial gene transfer studies, Harlan Sprague Dawley male rats were first injected directly into the left ventricle at multiple sites with 1.5 x 10^5 TU of the VSV-G envelope pseudotyped SINΔtat1PcN lentivirus vectors that allow constitutive GFP expression. To test long-term gene transfer by the lentiviral vector, rats were euthanized at 8 weeks following viral transduction. Due to the low transduction units of lentiviral vectors utilized in this experiment, expression of GFP gene was below the detection threshold of direct fluorescent microscopy. GFP expression in the frozen heart tissue sections was thus analyzed by immunohistochemical staining using anti-GFP monoclonal antibodies. As shown in **Figure 4**, GFP specific staining is observed with the SINΔtat1PcN lentivirus transduced tissue sections (compare **Figure 4B** with **4A**). In addition, no GFP expression was detected with mock-transduced animals (data not shown).

For better evaluation of the inducibility of the lentiviral vectors and easy detection/direct visualization of transduced heart tissues, a SINmin1Pi(LacZ)NW lentiviral vector was constructed in which LacZ gene expression is regulated by the tetO-containing CMV promoter. The expression of the LacZ gene was first tested *in vitro* by transient transfection of 293T cells. **Figure 5A** shows that LacZ gene expression in the context of SINmin1Pi(LacZ)NW lentiviral transfer plasmid vector is regulated nicely by doxycycline in 293T cells.

For the *in vivo* myocardial gene transfer experiments, 1-2 x 10^7 TU of the VSV-G envelope protein pseudotyped SINmin1Pi(LacZ)NW lentivirus vectors were injected directly at multiple sites into the left ventricle of the rats following the same procedure as described above for the SINΔtat1PcN vector study. Animals were euthanized at 3 weeks following gene transfer and 1 μg/ml doxycycline was added to drinking water of certain animals 4 to 5 days prior to sacrifice. Expression of LacZ gene in the frozen heart tissues was analyzed by x-gal gross-staining (**Figure 5B**). As shown in **Figure 5B** and summarized in **Table 3**, LacZ gene was successfully transfered to the rat myocadium by lentiviral vectors. All rats that received doxycycline treatment were detectable for LacZ gene expression, indicating that CMVtetO promoter within the context of SINmin1Pi(LacZ) lentiviral genome is inducible *in vivo*.

IV. Discussion

Lentiviral vectors are excellent tools for *in vivo* gene transfer because of their unique ability to transduce non-dividing cells and establish long-term gene expression with minimal toxicity (review by Vigna and Naldini, 2000; Connolly, 2002; Galimi and Verma, 2002). One of the key issues for clinical applications with lentiviral vectors is biosafety. We have previously reported on the construction and activity of a one-piece tetracycline-inducible autoregulated lentiviral vector (Ogueta et al, 2001). The major goals of this study were to further modify this one-piece tetracycline-regulatable lentiviral vector to include additional safety features and elements that could enhance transgene expression, and to test the utility of these modified vectors in an *in vivo* model of myocardial gene transfer. To increase their safety, the one-piece tetracycline-regulatable lentiviral vectors were genetically modified to eliminate the Tat protein (the second generation SINΔtat1PiN and 1PcN). An additional

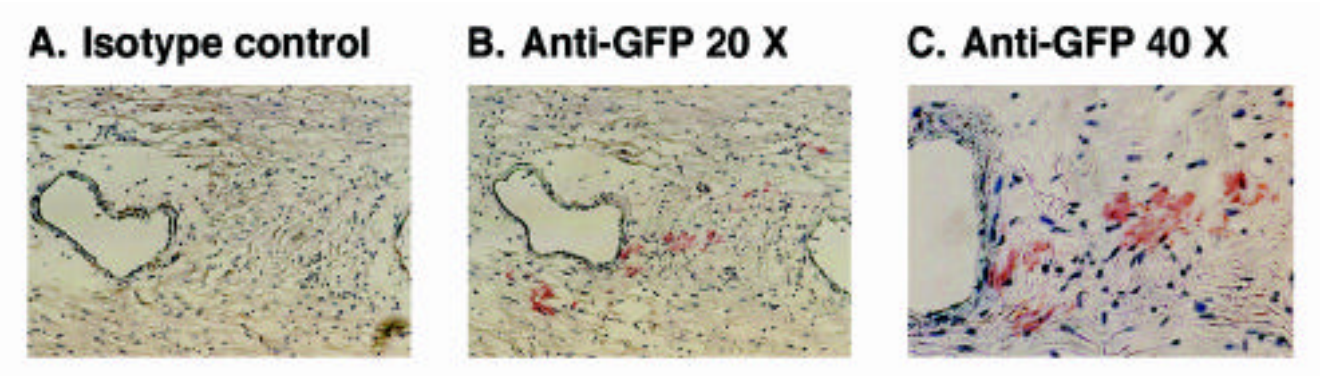

Figure 4. *In vivo* GFP gene transfer to rat myocardium by SINΔtat1PcN lentiviral vector as visualized by immunochemical staining. Eight weeks post-transduction, frozen tissue sections from SINΔtat1PcN lentiviral vector transduced heart were processed and incubated with mouse monoclonal anti-GFP antibodies (***B*** and ***C***) or an mouse IgG1 isotype control (***A***) followed by incubation with rat adsorbed, biotinylated horse-anti-mouse IgG(H+L). The sections were stained with Vectastain ABC reagent and Vector NovaRED substrate kit followed by counter-staining with Vector Hematoxylin QS reagent. The mounted sections were observed under light microscope and photographed using a Nikon camera. ***Panel A*** and ***B*** represent consecutive tissue sections. ***C*** is from an identical section of **B** and photographed with a higher power optical objective (40x vs. 20x). No GFP staining was observed in mock-transduced rat heart tissue sections.

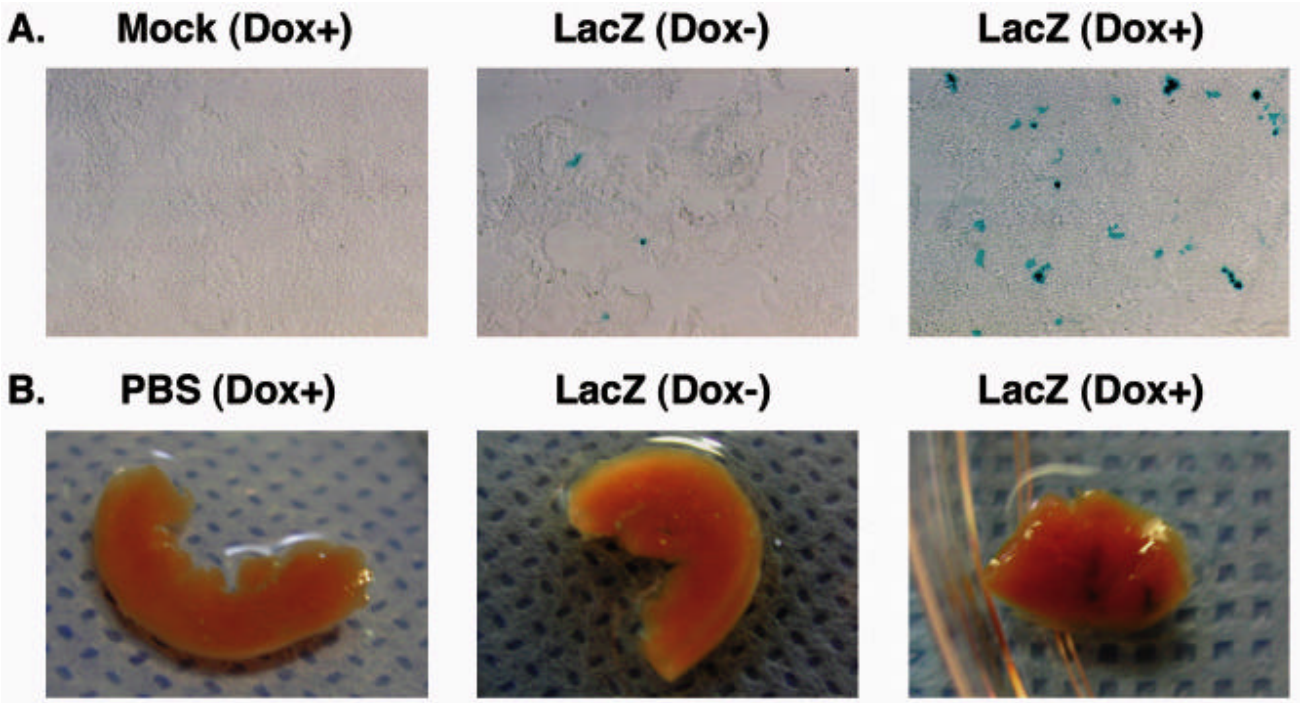

Figure 5. *In vitro* and *in vivo LacZ* gene transfer by lentiviral vector SINmin1Pi(LacZ)NW. **Panel A.** Transient transfection of 293T cells with SINmin1Pi(LacZ)NW vector. 293T cells were transfected with 1μg of SINmin1Pi(lacZ)NW transfer plasmid in the presence or absence of 1 μg/ml doxycycline. 48 hours post-transfection, the media were removed and the cells were subject to X-gal staining. Left image represents the mock-transfected cells in the presence of doxycycline. The SINmin1Pi(LacZ)NW transfected cells are presented in the middle (Dox -) or the right (Dox +) images. ***Panel B.*** *In vivo* lacZ gene transfer to rat myocardium by SINmin1Pi(LacZ)NW lentiviral vector as visualized by X-gal gross-staining of transduced tissues. Representative X-gal staining of rat heart tissues 3 weeks after SINmin1Pi(LacZ)NW transduction are shown. Left image represents a heart tissue from the rat mock-transduced with PBS in the presence of doxycycline treatment. The SINmin1Pi(LacZ)NW transduced tissue samples are presented in the middle (Dox -) or the right (Dox +) images.

~2kb of gag/pol sequence was removed from the third generation SINmin1PiNW and SINmin1PcNW to eliminate all HIV accessory proteins and minimize the potential homologous recombination during the production of the recombinant vectors. These modifications are comparable to those reported previously by others (Zufferey et al, 1997, 1998; Dull et al, 1998; Kim et al, 1998; Miyoshi et al, 1998; and reviewed by Ailles and Naldini, 2002). The third generation of our lentiviral vectors also contains the Woodchuck Hepatitis Virus posttranscriptional regulatory element (WPRE) in consideration of its ability to enhance the transgene expression (Zufferey et al, 1999) as well as the cPPT element for increasing transduction efficiency especially in human primary cells (Follenzi et al, 2000; Zennou et al, 2000). All above mentioned genetic modifications did not have a significant effect on the viral vector yield as reflected by the RT values or GFP titers and all vectors

were able to establish long-term gene expression in 293T or HUVEC cells (**Table 1** and data not shown).

One important observation of this study is that although the presence of the WPRE element in the viral vector was able to enhance the transgene GFP expression in 293T cells, no increased GFP gene expression was detected in HUVEC under similar transduction conditions. It should be noted that others have reported similar observations. In the case of an SIV vector system, Mangeot et al (2002) observed increased transgene expression in 293T cells while the presence of the WPRE was detrimental to gene transfer in dendritic cells. Ramezani et al (2000) reported that when tested in combination with a variety of viral or cellular promoters in a self-inactivating HIV-1-based vector, the effect of the WPRE on enhancing GFP gene expression is dependent upon the promoter it was paired with and the cell type used. Taken together, these results suggest that the effect of regulatory elements such as WPRE are both promoter and cell type dependent and that this specific modification to a lentiviral vector may prove to be only advantageous when utilized in certain cell/tissue types.

Lentiviral vectors that are capable of regulating transgene expression would be ideal for many clinical applications. Several tetracycline-based lentiviral vector systems have been established to date (Kafri et al, 2000, Reiser et al, 2000, Ogueta et al, 2001, Vigna et al, 2002). The tetracycline repressor or its fusion derivatives can be encoded in the same (single vector) or different (two vectors) vector backbone as the transgene it regulates. Unlike other reported tet-regulatable lentiviral vectors, our auto-regulatable tet-on single vector system uses the tetracycline repressor (tetR) itself, rather than the TetR-mammalian transcription factor fusion proteins, as the transcriptional regulator, thus avoiding the potential pleiotropic and toxic effects associated with the chimeric transcription regulators. The tetR expression is directed by the encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) as the second cistron in a bi-cistronic message whose expression is controlled by the tet-operon (tetO)-containing CMV immediate–early promotor (Ogueta et al, 2001 and **Figure 1**). The transgene expression in the two newly developed generations of inducible lentiviral vectors, like in the first generation, is modulated *in vitro* by doxycycline in both HUVEC and 293T cells (**Figure 2, 3** and **5A**, **Table 2**) and is fully reversible by withdrawal of the drug (**Figure 3**). The observed folds of induction as well as the 6-9 day timeframe required for fully reversal of induced GFP expression are similar to those reported by others (Reiser et al, 2000 and Kafri et al, 2000). The long half-life of the GFP may be the main contributor for the long reversal time and the lower fold of induction as compared to the case of EGF (Ogueta et al, 2001). It should also be noted that in our system, the initial GFP expression is not controlled by doxycycline until sufficient amount of tetR protein is accumulated. This could cause the higher basal level of GFP accumulation in the absence of doxycycline leading to an observed lower level of induction (**Table 1, Figures 2 and 3**). This may also explain the low level of LacZ transgene expression in 293T cells or some transduced rat hearts in the absence of doxycycline in **Figure 5** (middle panels). Since the degree of regulation in our system is directly associated with the level of tetR, one could increase the efficacy in achieving regulated transgene expression by elevating the tetR expression. To achieve this goal, new vectors currently under development in our laboratory include the use of codon optimized tetR, reposition of tetR into the first cistron of the bi-cistron message and/or expressing tetR under control of a separate strong promoter.

In the present study gene transfer was observed in the hearts of rats that received direct myocardial injection of the constitutive SINΔtat1PcN-GFP vectors (**Figure 4**). In a recent report, Zhao et al (2002) demonstrated gene transfer of GFP into neonatal and adult ventricular cardiac myocytes *in vitro* and by direct myocardial injection of transplanted hearts *in vivo* using an early generation constitutive lentiviral vector. Bonci et al (2003) and Fleury et al (2003) also demonstrated efficient myocardial gene transfer of GFP by direct myocardial injection *in vivo* using more advanced constitutive lentiviral vectors. Thus, all four studies demonstrate the feasibility of lentiviral gene transfer into the myocardium. However, an additional and significant technical advance was achieved in our studies with the demonstration that direct myocardial injection in rats with the one-piece tetR regulated lentiviral vector [SINmin1Pi(LacZ)NW] can lead to doxycycline-regulated LacZ transgene expression in the myocardium (**Figure 5** and **Table 3**). The data demonstrates that all inducible lentiviral vector transduced rats receiving doxycycline treatment were detectable for LacZ gene expression in their hearts. The different levels of LacZ expression observed in these rats may be caused by either difference in individual doxycycline uptake or in individual transduction efficiency. Compared with two previous *in vivo* studies using "tet-off" lentiviral vector systems for gene delivery to rat brain and transplanted tumor (Kafri et al, 2000, Vigna et al, 2002), our results not only add an additional format (i.e. "tet-on") of tetracycline-regulatable lentiviral vector for *in vivo* gene transfer in general but also expands its utility specifically to myocardial gene delivery.

In summary, our results indicate that regulatable gene expression through direct myocardial gene transfer can be achieved with the use of a "tet-on" auto-regulatable self-inactivating lentiviral vector. The tet-inducible transgene expression, in addition to other aforementioned advantages of lentiviral vectors, should prove to be a very useful tool for the field of cardiovascular delivery. For example, it has been suggested that regulatable vectors could help overcome the problems caused by continuous VEGF expression in gene therapy treatment of ischemic cardiovascular disease (Koransky et al, 2002). More efficient gene transfer than what was achieved in our present studies can also be accomplished with highly concentrated lentiviral vector preparations as has been observed using adenovirus or recombinant adeno-associated virus (rAAV) vectors (Wright et al, 2001; Melo et al, 2002) or in the case of constitutive lentiviral vectors

Table 2. Mean fluorescence intensity of the inducible lentiviral vectors in 293T cells in the presence or absence of 1 μg/ml doxycycline

Vector	Doxycycline	Day 1 GFP+ cells		Day 2 GFP+ cells		Day 6 GFP+ cells		Day 9 GFP+ cells	
		%	MFI	%	MFI	%	MFI	%	MFI
Mock	-	0.5	14.9	0.4	11.2	0.9	12.4	0.6	13.6
	+	0.7	14.4	0.5	10.6	0.7	12.6	0.7	13.4
SIN 1PiN	-	65.9	55.4	68.3	40.8	62.7	32.8	55.1	35.2
	+	67.7	138.1	71.8	119.9	71.3	136.3	70.0	139.3
SINΔtat 1PiN	-	58.0	52.4	71.5	44.7	73.9	49.0	78.6	58.0
	+	64.6	140.0	69.9	137.0	73.8	259.6	74.0	276.1
SINmin 1PiNW	-	43.1	90.2	45.5	72.9	45.7	87.1	43.9	66.1
	+	45.5	281.0	47.1	322.0	48.2	588.5	44.7	519.6

GFP Mean fluorescence intensity in 293T cells after transduction with the inducible lentiviral vectors and treatment in the presence or absence of 1 μg/ml doxycycline. 293T cells were transduced with equal amounts (an estimated 1-2.5 TU by GFP titer) of different generations of the inducible lentiviral vectors. Following 16 hours incubation, transduced cells were washed and replaced with fresh growth medium in the presence or absence of 1 μg/ml doxycycline. At indicated time of post-induction, the cells were assayed for GFP expression by FACS analysis. Table 2 shows the percent transduction and GFP mean fluorescence intensity (MFI) data from a representative experiment at Day 1, 2, 6 and 9 post-induction and represents an average of duplicate samples.

Table 3. Summary of the *in vivo* rat myocardial LacZ gene transfer results

Reagents and treatment received by the rats	Number of rats displayed the following x-gal staining patterns		
	No staining (-)	Light Blue (+)	Blue (++/+++)
PBS (dox+)	2	0	0
Lentivirus (dox-)	1	2	0
Lentivirus (dox+)	0	3	3

Note that levels of x-gal staining described in this table correlate with images presented in Figure 5B. No staining (-) is represented in the left panel PBS (Dox+) image of Figure 5B. Light Blue (+) is depicted by the middle panel "LacZ (Dox-)", and Blue (++/+++) can be seen in the right panel "LacZ(Dox+)" image.

(Fleury et al, 2003). In addition, a study of the effects of tet-inducible therapeutic transgene delivery in a model of myocardia ischemia and injury should help to more precisely define important roles of these tet-inducible lentiviral vectors in the cardiovascular field.

Acknowledgments

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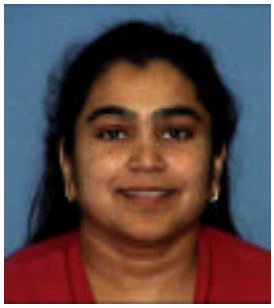

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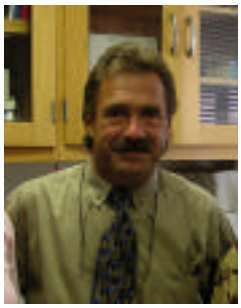

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