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RESEARCH ARTICLE

Human CMV immediate-early enhancer: a useful tool to enhance cell-type-specific expression from lentiviral vectors

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Abstract

Background Lentiviral vectors are attractive delivery tools for gene therapy, especially in terminally differentiated target cells. While restriction of gene expression to specific cell populations is of particular importance, highly efficient cell-type-specific gene expression after viral gene transfer so far has been hampered by low levels of transgene expression.

Methods Addressing this problem, we have integrated the human cytomegalovirus (CMV) immediate-early enhancer into an 'advanced' generation lentiviral vector. Expression cassettes with the reporter gene green fluorescent protein (GFP), combined with the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) under control of a ubiquitous phosphoglycerate kinase (mouse PGK), cardiomyocyte- (human atrial natriuretic factor (ANF), human ventricular myosin light chain (MLC2v)), or type II alveolar epithelial cell (AT-2)-specific human surfactant protein C (SP-C) promoter, were introduced. As insertion of an enhancing element can interfere with the promoter's specificity, expression levels conferred by our enhancer/promoter constructs were evaluated in target and non-target cells.

Results Transduction of target cells with human CMV enhancer containing lentiviral vectors resulted in a multiple-log increase in GFP expression compared to corresponding vectors lacking the human CMV enhancer. In the case of the ANF, the MLC2v, and the SP-C promoters, tissue-specific reporter gene expression in cardiomyocytes and in lung AT-2 cells was maintained, as expression in non-target cells increased only up to 7-fold.

Conclusions The results of this study indicate that lentiviral vectors with the human CMV enhancer conferring efficient cell-type-specific gene expression may be useful tools for gene therapy purposes or cell tracing, e.g. to analyze stem cell differentiation in transplantation and co-culture settings.

Keywords lentiviral vector; gene transfer; tissue-specific expression; CMV enhancer

Introduction

Lentiviral vectors are attractive delivery tools for gene therapy, as they have shown highly efficient stable transgene expression in terminally differentiated cells [1,2] without significant levels of silencing [3].

Expression levels seem to be relatively independent of the integration site [4] and even constructs larger than 10 kb can be packaged [5].

For local gene therapy addressing specific sites in the organism, the possibility of restricting gene expression to specific cell populations is of particular importance [6]. In principle, targeted tissue-specific expression of therapeutic or reporter genes from viral vectors can be achieved using different wild-type or engineered surface proteins, or, alternatively, by introduction of cell-type-specific promoters [6]. The use of cell-type-specific promoters, which keep the expression of the therapeutic gene in non-target cells at a minimum, can improve efficacy and safety, even in the setting of a systemic vector administration.

Although some reports demonstrated cell-type-specific expression after lentiviral transduction [7,8], the majority of cell-type-specific promoters do not result in detectable expression levels without further enhancing elements [9]. During the generation of transgenic cell lines or animals, low expression levels of cell-type-specific promoters can be overcome by selection of clones that display high-expression levels because of external genomic enhancement at the particular integration site. However, detectable cell-type-specific expression from lentiviral vectors, widely independent from the site of integration, requires increased expression levels exclusively dependent on internal enhancing elements.

In our hands, the introduction of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [10] does not sufficiently improve transgene expression levels of cell-type-specific promoters, including cardiomyocyte- (human atrial natriuretic factor (ANF), human ventricular myosin light chain (MLC2v)) and type II alveolar epithelial cell (AT-2) human surfactant protein C (SP-C)-specific promoters. Therefore, we searched for other possibilities to increase expression levels of those promoters without decreasing cell-type-specificity.

In a variety of plasmid vectors viral enhancers have been used to augment transgene expression on a transcriptional level. Among other viral enhancers from herpes virus, Rous sarcoma virus, and hepatitis B virus, the enhancer from the human cytomegalovirus (CMV) has the highest activity not only in primate cells, but also in cell lines from other species [11]. It was the aim of this study to investigate the usefulness of this CMV enhancer fragment of the human CMV immediate-early enhancer/promoter as a general tool for improvement of tissue-specific transgene expression in the context of 'advanced' generation lentiviral vectors.

Materials and methods

Lentiviral vector design

A second-generation, three-plasmid packaging system was used for virus production [12]. Self-inactivating transfer

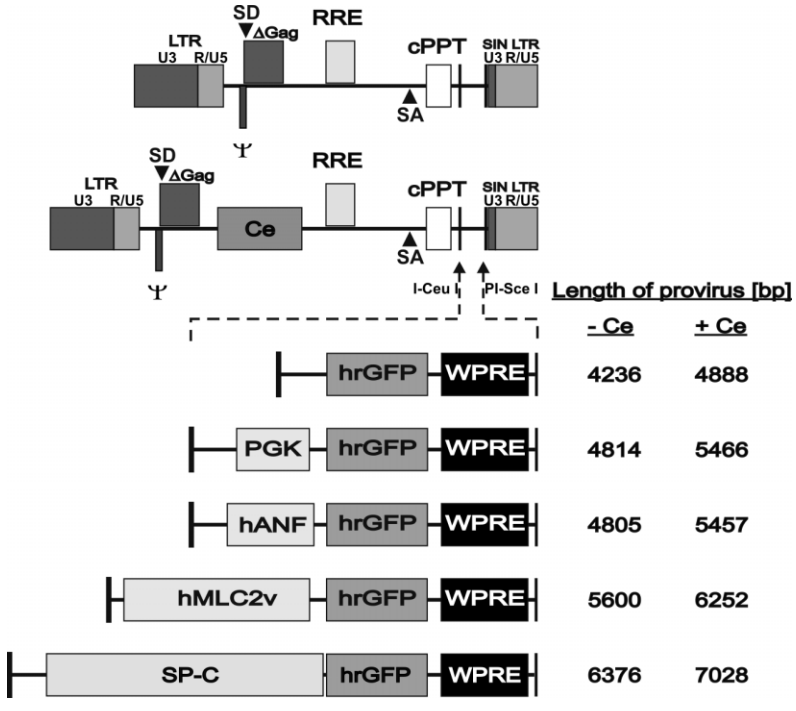

Figure 1. Overview of lentiviral vector constructs used in this study. The human CMV immediate-early enhancer was introduced upstream of the RRE in the pLentiShuttle vector backbone. Promoters included: PGK, mouse phosphoglycerate kinase; hANF, human atrial natriuretic factor; hMLC2v, human ventricular myosin light chain; SP-C, human surfactant protein C. On the right, the length of provirus derived from these vectors without (−) and with (+) the CMV immediate-early enhancer (Ce) is given. Further abbreviations: cPPT, central polypurine tract; gpt, guanine phosphoribosyl transferase gene; hrGFP, humanized *Renilla reniformis* green fluorescent protein; LTR, long terminal repeat; Ψ, packaging signal; RRE, *rev* responsive element; SA, splice acceptor site; SD, splice donor site; SIN LTR, self-inactivating LTR; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element

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plasmids were generated with the lentiviral cloning system 'LentiShuttle' [13], derived from pHR'SINcPPT CEW [14]. The human CMV immediate-early enhancer (positions −598 to −68 of the enhancer/promoter region of the major IE gene of HCMV; corresponding to positions 235–765 in pcDNA3Zeo, NCBI Accession **X90639**) was amplified by polymerase chain reaction (PCR) from the plasmid pcDNA3 using primers sense and reverse antisense introducing Eag I sites. The PCR product was inserted into the respective site in the pLentiShuttle vector backbone, placing the CMV enhancer (Ce) upstream of the *rev* responsive element (RRE) (Figure 1).

Expression cassettes contain the reporter gene hrGFP (from plasmid phrGFP-1, Stratagene, La Jolla, USA) and the regulatory element WPRE (from pHR'SINcPPT-CEW) [14] under the control of the ubiquitous murine phosphoglycerate kinase (PGK) promoter or different promoters conferring tissue-specific expression. For cardiomyocyte-specific expression, regulatory sequences of the human atrial natriuretic factor (ANF, −473 bp to +91 bp of exon 1; NCBI Accession **K02043**; 564 bp from position 1) or the human ventricular myosin light chain 2 (MLC2v, −1213 bp to +54 bp of exon 1; NCBI Accession **L01652**; 1,3 kb from position 3) were introduced. For lung tissue-specific expression, regulatory elements of the human pulmonary surfactant protein C (SP-C) gene were included. In this study, a truncated 2 kb fragment of the SP-C promoter (ΔAva SPC, −3686 bp to −1910 bp plus −215 bp to +21 bp of exon 1) was used [15].

Cell culture

Human kidney 293T cells for virus production and HeLa229 cells for titration were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Karlsruhe, Germany), 2 mmol/l L-glutamine (Invitrogen Life Technologies), penicillin (100 U/ml, PAA, Linz, Austria) and streptomycin (100 mg/ml, PAA) (complete DMEM).

Neonatal rat cardiomyocytes (NRCMs) were isolated from 1-day-old Wistar rats as previously described [16]. NRCMs were cultured in complete DMEM. HL-1 cells, a cardiac muscle cell line from the AT-1 mouse atrial cardiomyocyte tumor lineage, were maintained in Claycomb medium (SAFC Biosciences, Andover, UK) supplemented with 10% fetal bovine serum (FBS, SAFC Biosciences), 100 μM norepinephrine (Sigma-Aldrich, Seelze, Germany), 4 mM L-glutamine (Invitrogen Life Technologies), penicillin (100 U/ml, PAA) and streptomycin (100 mg/ml, PAA) [17]. In culture, both NRCMs and HL-1 cells contracted spontaneously and cardiac phenotype was further confirmed with sarcomeric α-actinin immunostaining (NRCM, see Figure 6; HL-1 data not shown).

The mouse lung epithelial cell line MLE-15 was maintained in HITES medium (medium RPMI 1640 (Invitrogen), supplemented with hydrocortisone, insulin, transferrin, estradiol and sodium selenite (all from Sigma-Aldrich), and 2% FBS (Invitrogen)) [18]. Human dermal fibroblasts were maintained in DMEM : F12 + Glutamax I (Invitrogen); human smooth muscle cells (SMCs, Promocell, Heidelberg, Germany) were maintained in human smooth muscle cell medium (Promocell); human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins [19] and maintained in endothelial cell growth medium (Promocell). Fibroblast and endothelial phenotype was confirmed by immunostaining for CD90 and CD31, respectively; the MLE-15 cells were immunostained for the alveolar type II cell marker (pro-)SP-C (data not shown). All cells were maintained at 5% CO_2 and 37 °C.

Detection of cell-type-specific mRNA expression by PCR

Total RNA was prepared from mouse heart and lung tissue as well as from NRCMs, HL-1, MLE-15, human dermal fibroblasts, HUVECs, and SMCs, using TriZol (Sigma-Aldrich). Contaminating DNA was digested by DNAse I (Stratagene, La Jolla, CA, USA) for 15 min at 37 °C followed by phenol/chloroform extraction. After ethanol precipitation, 0.75 μg RNA was used for random-primed cDNA synthesis with SuperScript™ II reverse transcriptase (Invitrogen, Karlsruhe, Germany). cDNA (1 μl) was amplified with PCR using 1.25 U REDTaq DNA-Polymerase (Sigma-Aldrich) in REDTaq 10x reaction buffer, using 0.4 μM of each primer and 0.4 μM dNTPs in a 25 μl reaction. PCR conditions included denaturation at 94 °C for 1 min, annealing at T_A for 1 min, and polymerization at 72 °C for 1 min; after 35 cycles, an extension step of 10 min at 72 °C was added. Sequences and specifications of primers can be found in Table 1. Reactions without reverse transcriptase were performed in parallel to control for any remaining contaminations of genomic DNA.

Preparation of lentiviral vector stocks

For virus production, a second-generation three-plasmid packaging system was used, including the packaging plasmid psPAX2 and the envelope-coding plasmid pMD2.G for pseudotyping the lentiviral particles with VSV-G. The lentiviral transfer construct (30 μg), psPAX2 (19.5 μg) and pMD2.G (10.5 μg) were co-transfected into 9×10^6 HEK293T cells using the calcium phosphate DNA precipitation method [1]; the medium was collected after 48 h, filtered through a 0.45 μm pore size filter and concentrated by 16 h centrifugation at 18 000 *g*. The virus pellet was resuspended in DMEM.

Titration by means of quantitative real-time PCR

The active biological titers of our vector preparations were determined by quantitative real-time PCR. HeLa229 cells

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Table 1. Sequences of oligonucleotides used, including corresponding gene information and primer position as well as PCR conditions

Oligonucleotide	Sequence 5′ – 3′	Target[a]	Position	T_A[b]	Product size [bp]	
					cDNA	Genomic DNA
GAPDH sense	ggccaaggtcatccatga	NM002046	582	55 °C	353	353
GAPDH ras	tcagtgtagcccaggatg	NM002046	935	55 °C		
ANF sense	atacagtgcggtgtccaaca	M27498	145	55 °C	268	372
ANF ras	agccctcagtttgcttttca	M27498	412	55 °C		
MLC2v sense	cttcactatcatggaccagaacag	NM010861	135	60 °C	221	2171
MLC2v ras	acactttgaatgcgttgagaatggt	NM010861	355	60 °C		
SP-C sense	gattgtaggggctctgctcat	NM011359	174	66 °C	219	769
SP-C ras	tagcagtaggttcctggagctg	NM011359	392	66 °C		

[a]NCBI accession number.
[b]T_A: Annealing temperature.

were transduced with the vectors; after two passages, cells were washed twice with phosphate-buffered saline (PBS), 1×10^6 detached cells were lysed in 100 µl of 200 µg/ml proteinase K in PCR buffer for 3 h at 56 °C, followed by 10 min inactivation at 95 °C. These crude extracts were diluted 1 : 10 and 5 µl (corresponding to 5000 cells) served as template for the PCR, detecting viral long terminal repeat (LTR) sequences within the genomic DNA of the target cells (genome titer), with primers forward 5′-AGCTTGCCTTGAGTGCTTCA-3′, reverse 5′-TGACTAAAAGGGTCTGAGGGA-3′ and probe 5′-FAM-TGCCCGTCTGTTGTGTGACTCTG-TAMRA-3′. Amplification reactions were performed using HotGoldStar polymerase (qPCR Core kit No ROX, Eurogentec, Seraing, Belgium) according to the manufacturer's instructions using an iCycler real-time PCR detection system (Bio-Rad Laboratories, Munich, Germany). For each viral preparation two transduction experiments were performed; for each experiment, the amount of proviral DNA was calculated from threshold cycle (Ct) numbers measured in triplicate. To this aim, a standard curve was generated by amplification of serial dilutions of the transfer vector pLentiShuttle CMV hrGFP WPRE, using the same PCR conditions. The quantitation is linear between 10^2 and 10^7 copies of plasmid per PCR.

Lentiviral transduction of target cells

For determination of expression levels, cells were transduced for 4 h in complete medium at 37 °C and 5% CO_2 in the presence of protamine sulfate (4 µg/ml, for HL-1 cells) or polybrene (4 µg/ml, for all other cell types) (both Sigma-Aldrich).

Immunohistological determination of cell-type-specific expression

Five days after transduction, the cells were fixed for immunocytochemistry in 4% paraformaldehyde for 1 h at 4 °C. The following primary antibodies were used: a mouse IgG1 monoclonal antibody EA-53 anti-sarcomeric α-actinin (diluted 1 : 800, Sigma-Aldrich), a rabbit polyclonal antibody targeting (pro-)SP-C (diluted 1 : 5000, Chemicon, Temecula, CA, USA), a mouse IgG1 monoclonal antibody 5E10 to CD90 (diluted 1 : 20, BD Biosciences, Heidelberg, Germany), and a mouse IgG1 monoclonal antibody JC70A to CD31 (diluted 1 : 150, Dako, Hamburg, Germany). Respective isotype controls (Dako) were used as negative controls. After incubation with an appropriate secondary antibody, Cy™3 conjugated donkey anti-mouse IgG (diluted 1 : 600, Dianova, Hamburg, Germany), or Cy™3 conjugated donkey anti-rabbit IgG (diluted 1 : 600, Dianova), cells were labeled with DAPI (Sigma-Aldrich) and images collected with a TE300 fluorescence microscope (Nikon, Tokyo, Japan).

Determination of expression levels by fluorescence-activated cell sorting (FACS)

Five days after transduction, GFP expression levels in MLE-15 cells, HL-1 cells, HUVECs, human fibroblasts, and human smooth muscle cells (from transduction experiments in triplicate) were analyzed by flow cytometry using a FACSCalibur cell analyzer (BD Biosciences). The percentage of GFP^{pos} cells and means of fluorescence intensity (MFI) of GFP-positive cells were determined using WinMDI 2.9 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 3.02 for Windows, GraphPad Software, San Diego, CA, USA); means and standard deviations are given. For comparison of viral titers, the data was analyzed by one-way analysis of variance (ANOVA) with the Bonferroni multiple comparison test for comparison of any two groups; probability values <0.05 were considered significant. For FACS analyses, the total increase in fluorescence intensity was calculated by multiplying values for mean fluorescence intensity of GFP-positive cells by the percentage of GFP-positive cells, and

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subtraction of background fluorescence of non-transduced control cells. Standard rules for error propagation were applied to determine the final composite standard deviation.

Results

Introduction of the human CMV immediate-early enhancer into lentiviral vectors does not influence packaging efficiency of infectious particles

The human CMV immediate-early enhancer (Ce) was inserted upstream of the RRE into the backbone of pLentiShuttle, a recently developed lentiviral vector system which simplifies the insertion of transgenes [13]. Several expression cassettes were introduced into pLentiShuttle with and without the CMV enhancer, respectively, to generate transfer vectors mediating the expression of GFP under control of the ubiquitous mouse PGK or cardiac human ANF- and human MLC2v-, as well as lung cell-specific human SP-C- promoters. A promoterless expression cassette was used to assess the transcriptional activity of the CMV enhancer alone. All constructs included the regulatory element WPRE downstream of the transgene and are depicted in Figure 1. All vectors were packaged in HEK293T cells.

First, we analyzed potential effects of the introduced fragment of the CMV enhancer on the packaging efficiency. On one hand, the introduction of new elements could impair the packaging process, due to a larger genome size or interference with the packaging signal [5]; on the other, the CMV enhancer theoretically could also influence the transcription from the lentiviral transfer plasmid and thereby the amount of proviral RNA available for packaging [20]. Titers, measured by quantitative real-time PCR as integrated proviral DNA within the genomic DNA of transduced HeLa229 cells (genome titer), are given as infectious units per ml (IU/ml) of concentrated cell culture supernatants (Figure 2). Values ranged from $4.6 \times 10^7 \pm 4.4 \times 10^6$ IU/ml for LentiShuttle Ce-SP-C hrGFP WPRE to $8.3 \times 10^9 \pm 5.1 \times 10^9$ IU/ml for LentiShuttle PGK hrGFP WPRE; however, direct comparison of titers of vectors with the same expression cassette revealed no significant differences for the respective vectors with and without CMV enhancer.

Functionality of the cloned fragment of the CMV enhancer was tested in combination with the mouse PGK promoter. Compared to the very strong CMV promoter, the PGK promoter confers rather moderate ubiquitous transgene expression in various cell types. Combination of the PGK promoter with the CMV enhancer resulted in enhanced GFP expression in HeLa229 cells after transduction at a multiplicity of infection (MOI) of 1, 5 and 20, as determined by flow cytometry. The CMV enhancer alone conferred no significant GFP expression at MOIs 1 and 5; however, low GFP expression was

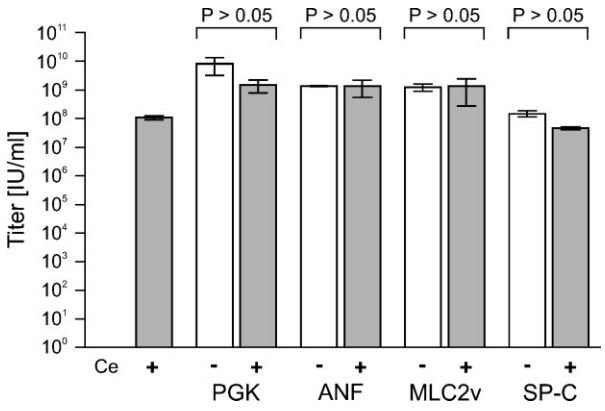

Figure 2. No significant influence of the CMV enhancer on titers of 'LentiShuttle' constructs. For lentiviral vectors used in this study, titers were determined by quantitative real-time PCR, targeting integrated proviral copies in HeLa229 cells transduced with the vectors. Results of two experiments performed in triplicate as described in Materials and Methods are shown, titers are given as infectious units per ml (IU/ml) of concentrated cell culture supernatants, error bars represent standard deviation. No significant differences in titers for vectors with and without the CMV enhancer (Ce) were observed for the respective promoters

observed at an MOI of 20 (Figure 3). In addition, all cell types used in this study (HL-1, MLE-15, human dermal fibroblasts, HUVECs and SMCs) were analyzed 5 days after transduction. In HL-1 cells, a cell line that shows a very weak expression from the PGK promoter, the insertion of the CMV enhancer only led to a 1.4-fold increase in expression level compared to the corresponding LentiShuttle vector without the CMV enhancer. Compared to the PGK promoter without CMV enhancer, the increase in expression level was up to 6-fold in human dermal fibroblasts transduced at an MOI of 5. In combination with the PGK promoter, the CMV enhancer was functional in all cell types analyzed; on average, a 3-fold increase in expression level after insertion of the CMV enhancer was observed (data not shown).

The CMV enhancer leads to increased but cell-type-specific transgene expression from the cardiac ANF-promoter

The cell-type specificity of lentiviral vector mediated transgene expression is of major significance for future gene therapy applications. To evaluate the ANF promoter with and without the CMV enhancer, the murine atrial cardiomyocyte cell line HL-1 [17] was used as a pure target cell population; human fibroblasts, endothelial cells (HUVECs) and smooth muscle cells served as non-target negative controls. First, the endogenous expression pattern of ANF in these cell types was analyzed by RT-PCR. As expected, and in contrast to HL-1 cells, no ANF expression was observed in fibroblasts, HUVECs and SMCs (Figure 4). Then, target and non-target cells were transduced with suitable MOIs dependent on the

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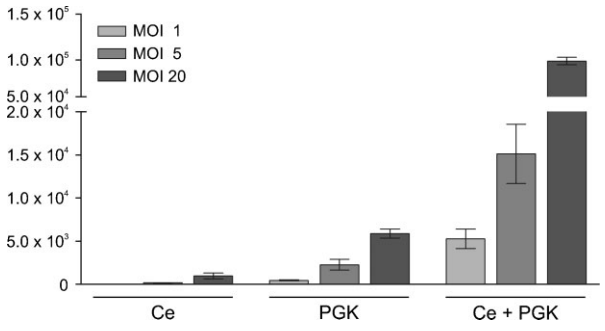

Figure 3. Increased transgene expression from the PGK promoter combined with the CMV enhancer. Lentiviral hrGFP expression from the CMV enhancer alone, and the mouse PGK promoter with and without CMV enhancer, was analyzed by flow cytometry. HeLa229 cells were transduced at MOIs of 1, 5 and 20. Columns represent the total increase in fluorescence intensity compared to non-transduced cells (negative control) from three-fold experiments measured in triplicate; error bars indicate standard deviation. The CMV enhancer alone conferred no significant GFP expression at MOIs 1 and 5; low GFP expression was observed at an MOI of 20. A significant increase in GFP expression was observed for the PGK promoter after insertion of the CMV enhancer

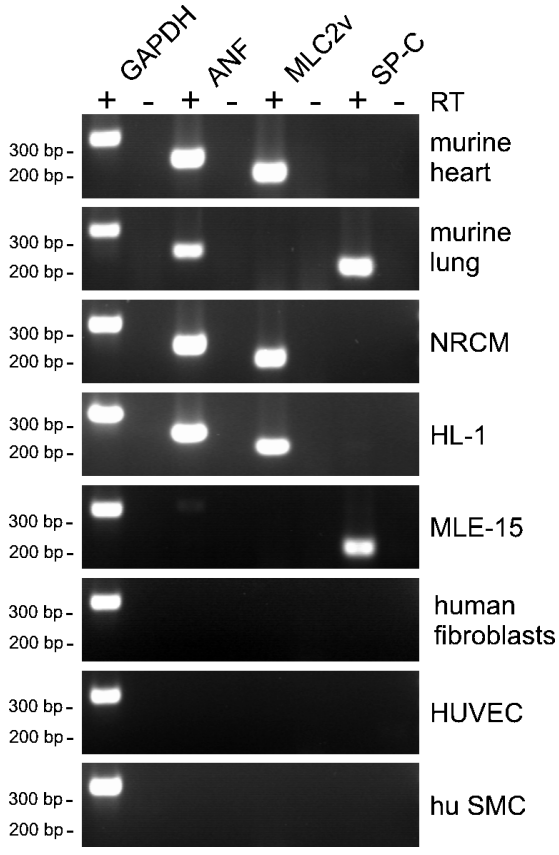

Figure 4. Cell-type-specific mRNA expression of cardiac and pulmonary cell lines. Expression of cell-type-specific transcripts was analyzed by RT-PCR. To avoid contaminations with genomic DNA, RNA was treated with DNAse I before reverse transcription. Reactions without reverse transcriptase (RT) were performed in parallel to control for remaining contaminations of genomic DNA. In addition, primer pairs for tissue-specific genes were designed to enclose intron sequences, thereby resulting in different product sizes for genomic and cDNA templates (Table 1). Integrity of RNA was confirmed by RT-PCR with primers specific for the housekeeping gene GAPDH

cell type (HL-1: MOI 50; fibroblasts: MOI 5; others: MOI 1), to yield approximately one integrated viral copy per cell. Integration of proviral sequences was assessed by real-time PCR according to the protocol described for titration of lentiviral vectors. For LentiShuttle ANF hrGFP WPRE, the following integrated copy numbers per cell were determined: HL-1 cells 0.82 ± 0.02; fibroblasts 0.11 ± 0.06; HUVECs 0.49 ± 0.22; SMCs 1.20 ± 0.70. Transduction with LentiShuttle Ce-ANF hrGFP WPRE yielded similar results of integrated copy numbers per cell: HL-1 cells 0.73 ± 0.23; fibroblasts 0.19 ± 0.18; HUVECs 0.41 ± 0.21; SMCs 0.72 ± 0.04. Positive control transduction with LentiShuttle Ce-PGK hrGFP WPRE using the same MOIs resulted in 0.58 ± 0.11 copies per cell for HL-1 cells (fibroblasts 0.19 ± 0.05; HUVECs 0.57 ± 0.68; SMCs 1.58 ± 1.21). Five days after transduction cells were analyzed by flow cytometry.

For the HL-1 cell line, insertion of the CMV enhancer led to a significant increase in the ANF promoter driven GFP expression level compared to the corresponding vector without the CMV enhancer, resulting in $18.8 \pm 1.8\%$ GFP^{pos} cells after transduction (Figure 5). Control transduction with LentiShuttle Ce-PGK hrGFP WPRE resulted in a similar efficiency of $11.2 \pm 0.2\%$ GFP^{pos} cells with an expression level lower than for LentiShuttle Ce-ANF hrGFP WPRE, thus suggesting a relatively low transcriptional activity of HL-1 cells (Figure 5). In HL-1 cells, GFP expression from the ANF promoter without CMV enhancer was hardly detectable. No GFP expression from LentiShuttle Ce-ANF hrGFP WPRE was detected in non-target cells HUVECs and SMCs, while, in this setting, weak non-specific expression occurred in a low percentage of fibroblasts. For the vector LentiShuttle Ce-ANF hrGFP WPRE, cell-type specificity of transgene expression was dependent on the number of integrated lentiviruses; application of higher MOIs led to an increase in non-specific expression. This effect was most evident in fibroblasts, but, at an MOI of 100, also HUVECs and SMCs showed considerable non-specific GFP expression (see Supplementary Material).

As expected, the atrial cell line HL-1 was unsuitable for the evaluation of LentiShuttle constructs with the ventricular MLC2v promoter. Despite the fact that these cells contain at least a low level of MLC2v mRNA, as detected by RT-PCR (Figure 4), no GFP expression was observed after transduction with LentiShuttle Ce-MLC2v hrGFP (data not shown).

Increased cell-type-specific transgene expression in primary cardiomyocytes from cardiac promoters combined with the CMV enhancer

We also evaluated the influence of the human CMV immediate-early enhancer on transgene expression from heart-specific promoters in primary cardiac target cells.

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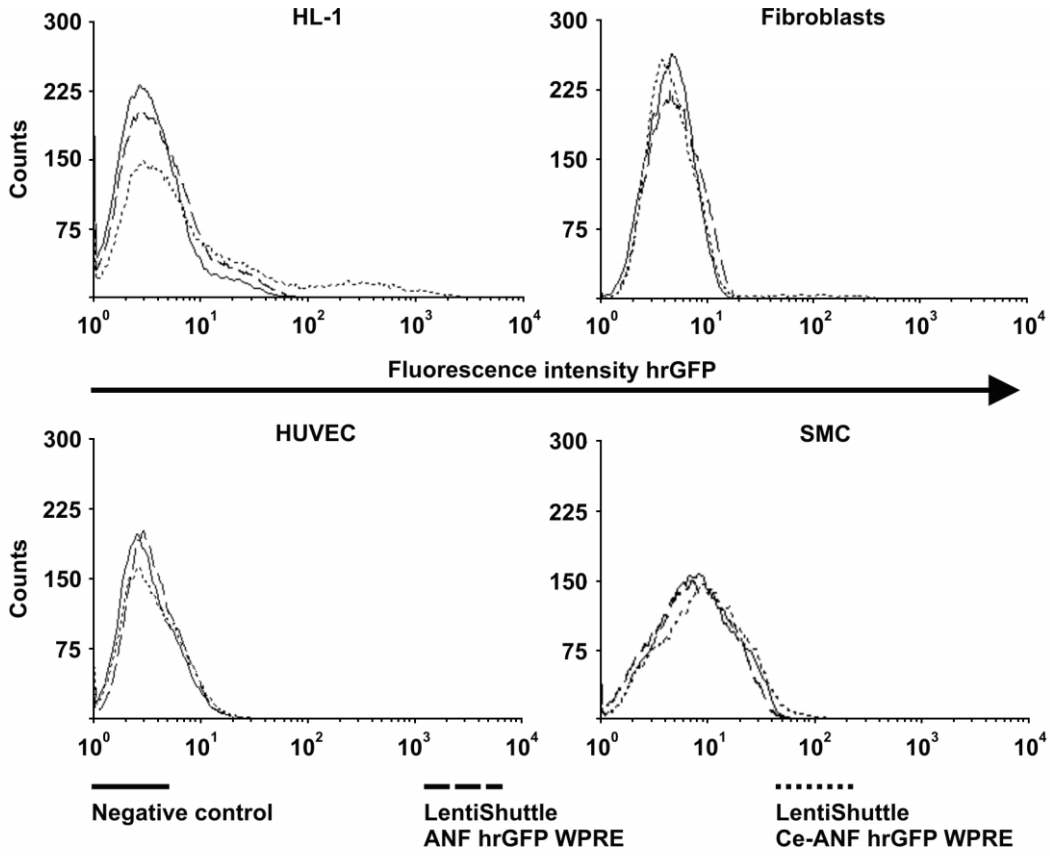

	HL-1	Fibroblasts	HUVEC	SMC
Positive control Ce-PGK	$6.6x10^2 \pm 2.1x10^1$	$8.9x10^2 \pm 6.6x10^1$	$2.4x10^3 \pm 3.7x10^1$	$6.5x10^3 \pm 6.7x10^2$
ANF	$6.2x10^1 \pm 1.8x10^1$	$4.2x10^1 \pm 7.0x10^1$	Not detected	Not detected
Ce-ANF	$2.8x10^3 \pm 6.0x10^1$	$3.1x10^2 \pm 1.2x10^2$	Not detected	$1.8x10^2 \pm 8.9x10^1$

Figure 5. Enhanced transgene expression remains cell-type-specific for the cardiac ANF promoter. HL-1 cells and non-target control cells were transduced with the ANF promoter constructs with and without the CMV enhancer to yield ~1 copy per cell. Lentiviral hrGFP expression was analyzed 5 days after transduction by flow cytometry. Background fluorescence was determined for non-transduced cells (negative control). As a positive control, cells were transduced with LentiShuttle Ce-PGK hrGFP WPRE. Fluorescence intensities for each cell type are given as representative histograms, data including standard deviation from analysis in triplicate are summarized in a table, given as the total increase in fluorescence intensity compared to the negative control. For HL-1 cells, 45-fold GFP expression was observed from the vector LentiShuttle Ce-ANF hrGFP WPRE compared to LentiShuttle ANF hrGFP WPRE. Except for a 7-fold increase in GFP expression level from LentiShuttle Ce-ANF hrGFP WPRE in fibroblasts, no significant expression was detected in non-target control cells

To this aim, cardiomyocytes were isolated from newborn rat hearts, including atria and ventricles. In contrast to the atrial cardiac cell line HL-1, the neonatal rat cardiomyocytes (NRCMs) allowed for the evaluation of constructs both with the promoter for human ANF and with the promoter for human MLC2v. Cells were transduced with lentiviral vector stocks at an MOI of 5. After 5 days, cardiomyocytes were identified within the cultures by immunostaining for sarcomeric α-actinin; GFP expression and cell morphology were analyzed by fluorescence microscopy. The LentiShuttle Ce-PGK hrGFP WPRE was used as a positive control vector. For constructs with human ANF and human MLC2v promoters, a significant increase in GFP expression levels was observed for both vectors with the CMV enhancer (Figures 6c and 6e).

In this setting, contaminating cells within the cardiomyocyte preparations, mainly consisting of fibroblasts, allowed for an evaluation of cell-type specificity of the GFP expression from the different enhancer and promoter constructs. Whereas LentiShuttle Ce-PGK hrGFP WPRE conferred GFP expression both in sarcomeric α-actinin^{pos} cardiomyocytes and in sarcomeric α-actinin^{neg} fibroblasts (Figure 6a), expression from the promoters ANF and MLC2v was restricted to the sarcomeric α-actinin^{pos} cardiomyocyte population. For the vector LentiShuttle Ce-ANF hrGFP WPRE weak unspecific GFP expression in fibroblasts was observed in a few cells (Figure 6c, see arrowheads).

Although not directly comparable, data obtained from experiments with another lentiviral vector system suggest that the effect of the CMV enhancer strongly

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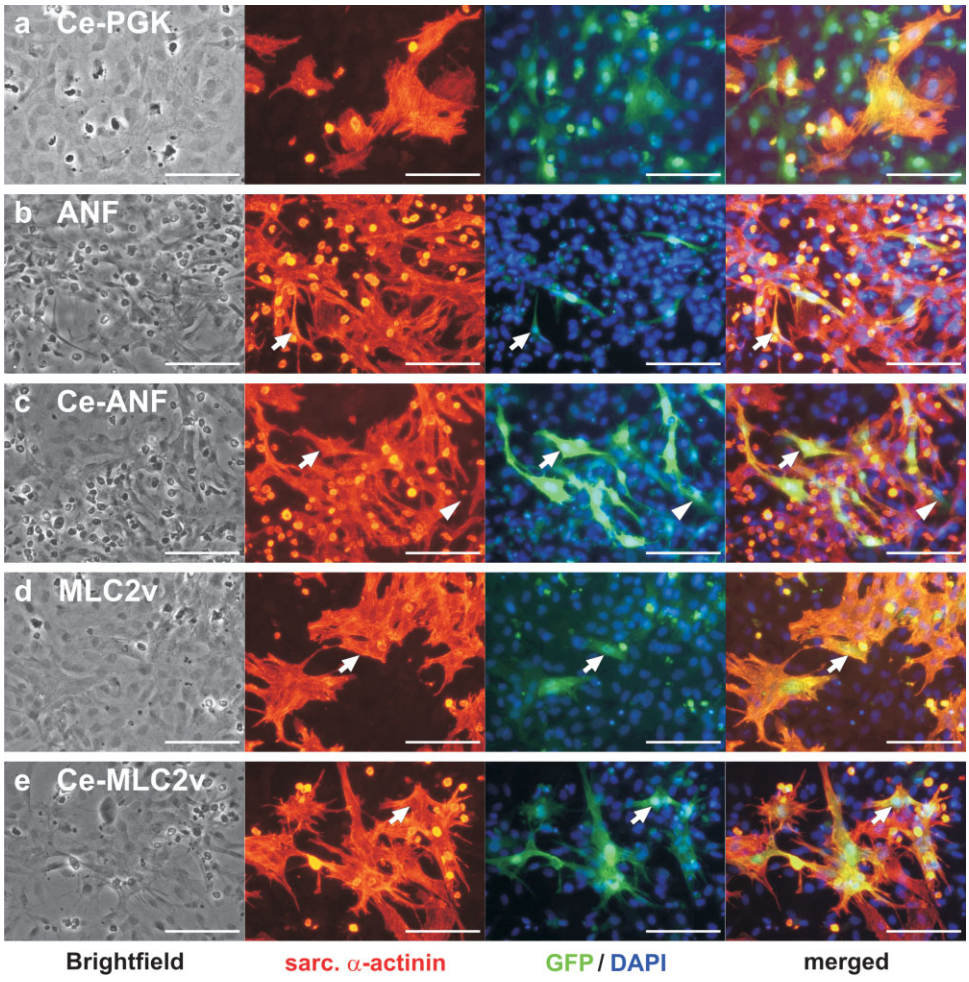

Figure 6. Increased transgene expression in primary cardiomyocytes from heart-specific promoters combined with the CMV enhancer. Lentiviral vector mediated hrGFP expression (green) in neonatal rat cardiomyocytes from enhancer/promoters Ce-PGK (a), ANF (b), Ce-ANF (c), MLC2v (d), and Ce-MLC2v (e) is shown. NRCMs were analyzed by fluorescence microscopy 5 days after transduction, following cardiomyocyte-specific staining for sarcomeric α-actinin (red). Nuclei were stained with DAPI (blue). Scale bars: 100 μm. Cell-type specificity of GFP expression can be evaluated in the merged images; while the PGK promoter confers ubiquitous expression also in contaminating cells, mainly consisting of fibroblasts, ANF and MLC2v promoter driven expression is restricted almost exclusively to cardiomyocytes (white arrows). For Ce-ANF, few non-myocytes show a weak non-specific GFP expression (white arrowheads)

depends on the promoter used. The analogous insertion of the CMV enhancer sequence into an SIVmac-derived lentiviral vector with GFP under the control of a human α-myosin heavy chain (α-MHC) promoter (see Supplementary Material) resulted in a loss of tissue specificity: GFP expression was not only observed in sarcomeric α-actinin^{pos} NRCMs, but also in contaminating non-cardiomyocytes (see Supplementary Material) as well as in transduced HeLa229 cells (data not shown).

The CMV enhancer leads to increased but cell-type-specific transgene expression from the lung cell-specific SP-C promoter

First, cell-type specificity of LentiShuttle SP-C hrGFP WPRE with and without the CMV enhancer was evaluated by comparing expression levels in alveolar epithelial cells and non-target control cells at an MOI of 1. Due to the limited availability of primary lung type II alveolar epithelial cells, the murine alveolar type II epithelial cell line MLE-15 was used.

Compared to the non-transduced control, hardly any increase in fluorescence intensity was observed for MLE-15 cells transduced with LentiShuttle SP-C hrGFP WPRE, whereas LentiShuttle Ce-SP-C hrGFP WPRE conferred GFP expression levels with a mean fluorescence intensity (MFI) of 178 ± 5. At the same time, expression levels in non-target cells were essentially the same as for non-transduced controls (data not shown).

Second, for the vector LentiShuttle Ce-SP-C hrGFP WPRE an MOI of 20 was used in order to evaluate whether higher MOIs result in expression levels above background in non-target cells. However, this MOI led to a robust GFP expression in MLE-15 cells (MFI 686 ± 161),

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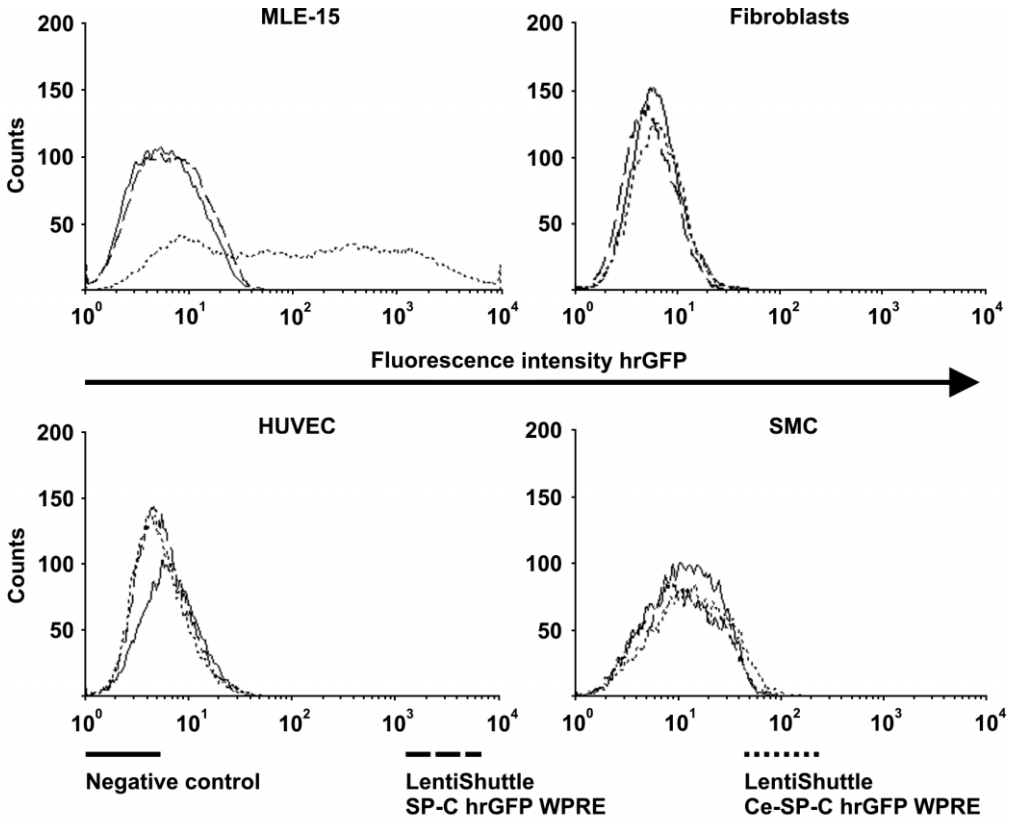

	MLE-15	Fibroblasts	HUVEC	SMC
Positive control Ce-PGK	$7.2x10^4 \pm 4.0x10^4$	$5.2x10^4 \pm 7.1x10^2$	$1.2x10^5 \pm 1.9x10^4$	$2.6x10^5 \pm 3.9x10^3$
SP-C	$1.7x10^1 \pm 7.2x10^0$	Not detected	Not detected	$3.9x10^1 \pm 2.4x10^0$
Ce-SP-C	$3.2x10^4 \pm 8.8x10^3$	$1.7x10^1 \pm 7.4x10^0$	Not detected	$1.6x10^2 \pm 2.8x10^1$

Figure 7. Enhanced transgene expression remains cell-type-specific for the lung-specific SP-C promoter. MLE-15 cells and non-target control cells were transduced at an MOI of 20 with the SP-C promoter constructs with and without CMV enhancer. Lentiviral hrGFP expression was analyzed 5 days after transduction by flow cytometry. Background fluorescence was determined for non-transduced cells (negative control). As a positive control, cells were transduced with LentiShuttle Ce-PGK hrGFP WPRE. Fluorescence intensities for each cell-type are given as representative histograms, data including standard deviation from analysis in duplicate are summarized in a table, given as the total increase in fluorescence intensity compared to the negative control. For MLE-15 cells, high-level GFP expression was observed from the vector LentiShuttle Ce-SP-C hrGFP WPRE while no significant expression was detected in non-target control cells

without significant non-specific expression in fibroblasts, HUVECs or SMCs (Figure 7). Although after transduction with LentiShuttle Ce-SP-C hrGFP WPRE a slight non-specific increase in total fluorescence intensity compared to the negative control was observed in fibroblasts and SMCs, this increase was at least 200-fold lower than in the target cell line MLE-15. Again, integration of lentiviral proviral sequences was assessed by real-time PCR to exclude false negative results in control cells due to inefficient transduction (integrated copy numbers per cell: MLE-15 cells 22.93 ± 5.11; fibroblasts 7.00 ± 3.74; HUVECs 31.73 ± 5.68; SMCs 9.85 ± 3.49). At the same time, positive control transduction with LentiShuttle Ce-PGK hrGFP WPRE at an MOI of 20 conferred efficient GFP expression in all cell types analyzed (Figure 7) and resulted in 13.71 ± 8.41 copies per cell for MLE-15 cells (fibroblasts 3.93 ± 3.62; HUVECs 9.53 ± 2.34; SMCs 7.40 ± 1.70).

Discussion

We have previously described 'LentiShuttle', a novel and efficient cloning system for lentiviral vectors [13]. In this study, we used this system for the generation of vectors for stable and specific transgene expression in two different tissues.

The therapeutic value of a viral gene transfer vector depends not only on efficacy, but also on its safety. In this view, the restriction of transgene expression to specific target cell populations is highly desirable for preventing effects in other cell types. Tissue-specific promoters can be used for controlled expression of therapeutic genes or for introducing suicidal genes into tissue-specific tumors. For this purpose, tissue or even cell-type specificity of promoter activity is absolutely crucial and has to be carefully evaluated for all vectors. For instance, Gotoh *et al.* [21] presented a prostate-specific antigen

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(PSA) promoter designated for use in tissue-specific toxic gene therapy for prostate cancer, whereas Li *et al*. [22] demonstrated high levels of transgene expression from this promoter not only in the prostate, but also in the lungs of nude mice. However, in an enhanced PSA promoter construct tissue specificity was restored [8,23].

In this study, we focused on the development of vectors for tissue-specific expression in cardiac and pulmonary cells. One of the best-characterized promoters for cardiac expression is the ventricular myosin light chain (MLC2v) promoter [24–26]. In contrast, few reports have demonstrated heart tissue-specific expression driven by the promoter for atrial natriuretic factor (ANF) from non-viral vectors [24,27], although this promoter might provide a powerful tool for specific targeting of atrial cardiomyocytes and of cells of the cardiac conduction system [28]. The pulmonary type II alveolar epithelial (AT-2) cell-specific human surfactant protein C (SP-C) promoter so far has been mainly used for the generation of transgenic mice [15,29].

A major drawback of most tissue-specific promoters is the relatively weak transcriptional activity, which limits their widespread use in gene therapy applications. For enhancing the transcriptional activity of endothelial cell-specific and gastrointestinal-specific promoters in non-viral gene transfer, a positive feedback loop provided by a chimeric transcription factor has been applied [9]. Because this rather complex system includes the introduction of several genetic elements (the transcription factor binding site and the coding sequence for the transcription factor, either with a second tissue-specific promoter or expressed in combination with a reporter gene by introduction of an IRES site), we chose the enhancer element of the human CMV immediate-early promoter/enhancer as a more simple enhancing mechanism, better suitable for the application in a lentiviral vector.

This enhancer element had been shown to be a useful tool to enhance transgene transcription in non-viral plasmid vectors. Besides its use for enhancing ubiquitous expression [30], it has been combined recently with tissue-specific promoters such as the platelet-derived growth factor B-chain (PDGF-β) promoter, conferring neuron-specific expression [31].

In this study, we investigated the usefulness of the CMV enhancer element as a tool for improvement of tissue-specific transgene expression in cardiac or pulmonary type II alveolar epithelial cells in the context of 'advanced' generation lentiviral vectors. To this aim, several lentiviral transfer plasmids with or without the CMV enhancer element, combined with tissue-specific promoters ANF, MLC2v, or SP-C, were generated as depicted in Figure 1.

Although the aim of this study was not to use the CMV enhancer to achieve improved packaging of our lentiviral vectors, it was possible that inclusion of the CMV enhancer led to increased transcription of proviral RNA from the lentiviral transfer plasmid in the 293T packaging cells. As the amount of vector RNA available for packaging in producer cells is a rate-limiting step in the production of infectious vectors [20], we compared packaging efficiencies of comparable vectors with and without the CMV enhancer; however, we did not observe significant differences in viral titers (Figure 2). Although expression of proviral RNAs in the packaging cell line has not been compared on a quantitative level, the CMV enhancer element seems to modify only the downstream promoter driven RNA transcription without altering transcription driven by the LTR upstream of the enhancer. General functionality of the CMV enhancer fragment used in our study was confirmed in combination with a PGK promoter for ubiquitous expression. An increase in expression level was observed for various cell types (data for HeLa229 cells is given in Figure 3).

To evaluate transgene expression levels from the cardiac- and lung-specific promoters together with their cell-type specificity, target and non-target cells were transduced. For a quantitative analysis of GFP expression levels mediated by the cardiac ANF promoter, the cardiac cell line HL-1 was used, which is derived from mouse atrial cardiomyocytes [17]. Although we were able to demonstrate endogenous expression of MLC2v, a ventricular marker gene, in this atrial myocyte line, no GFP expression was observed after transduction of HL-1 cells with high MOIs of LentiShuttle Ce-MLC2v hrGFP WPRE. As functionality of this vector has been proven by transduction of NRCMs, we presume that our semiquantitative RT-PCR led to an overestimation of endogenous MLC2v expression. Because HL-1 cells were unsuitable for the evaluation of LentiShuttle constructs with the ventricular MLC2v promoter, primary neonatal rat cardiomyocytes were used for these studies.

To date, two murine alveolar type II epithelial cell lines, MLE-12 and MLE-15 that exhibit sufficient expression of surfactant protein C, are available [32]. One of these lines, MLE-15, was used as a model for primary pulmonary type II alveolar epithelial cells, the designated target for our SP-C promoter constructs. We chose fibroblasts, endothelial cells and smooth muscle cells as non-target control cells, because, apart from cardiomyocytes and respiratory epithelial cells, they are the predominant cell types present in heart and lung, respectively.

Cell-type-specific promoters with the CMV enhancer conferred efficient GFP expression in our target cell lines. After transduction with vectors containing promoters ANF and MLC2v, GFP expression was restricted to cardiomyocytes (Figures 5 and 6). Only negligible levels of GFP expression in human dermal fibroblasts and contaminating rat fibroblasts were observed for vectors including the enhancer/promoter combination Ce-ANF. Despite a massive increase in fluorescence level compared to the corresponding vector without Ce, there was no significant non-specific GFP expression after transduction of control cells with the lung-specific Ce-SP-C constructs at an MOI of 1 (data not shown) and even at an MOI of 20 (Figure 7). In contrast, for the Ce-ANF the MOI used for transduction seems to have a substantial influence on cell-type specificity. We speculate that the ANF promoter may thus be subject to influences from genomic DNA

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elements flanking the integration site; if so, higher MOIs would increase the probability for integration of proviral DNA at genomic sites containing such elements.

Importantly, maintenance of cell-type specificity after insertion of the CMV enhancer seems to depend on the promoter used: data from similar experiments using simian immunodeficiency virus (SIV)-derived lentiviral vectors indicate that cell-type specificity of GFP expression from a heart-specific human α-MHC promoter is severely compromised in combination with the CMV enhancer fragment (see Supplementary Material). However, it should be noted that, although these SIV-derived vectors have a very similar design as the HIV-derived vectors used in our study (see Supplementary Material), resulting data do not allow for direct comparison.

In summary, we analyzed lentiviral vectors containing the cardiac promoters ANF and MLC2v, as well as a vector with the AT-2 lung cell-specific SP-C promoter, with and without inclusion of the CMV immediate-early enhancer. Constructs containing the CMV enhancer exhibited high expression levels in the appropriate target cells and very low expression in other cell types. While combination of the CMV enhancer with other promoters such as the human α-MHC promoter may lead to unspecific transgene expression expression, this study indicates that lentiviral vectors containing the human CMV enhancer in conjunction with many tissue-specific promoters may be useful tools for tissue-targeted gene therapy purposes. In addition, they could be used for cell tracing, e.g. to analyze stem cell differentiation in transplantation and co-culture settings. Our novel lentiviral vectors with cardiac-specific promoters may allow for selection of different cardiomyocyte sub-types (including atrial and ventricular cells) in the case of stem-cell-derived myocytes, vectors with the pneumocyte-specific SP-C promoter may enable selection of stem-cell-derived AT-2 cells. The resulting highly purified cells could then be used for more accurate molecular characterizations as well as for potential cell therapies.

Supplementary Material

The supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1099-498X/suppmat/.

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