Design and *in vivo* characterization of self-inactivating human and non-human lentiviral expression vectors engineered for streptogramin-adjustable transgene expression

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ABSTRACT

Adjustable transgene expression is considered key for next-generation molecular interventions in gene therapy scenarios, therapeutic reprogramming of clinical cell phenotypes for tissue engineering and sophisticated gene-function analyses in the post-genomic era. We have designed a portfolio of latest generation self-inactivating human (HIV-derived) and non-human (EIAV-based) lentiviral expression vectors engineered for streptogramin-adjustable expression of reporter (AmyS$_{\Delta S}$, EYFP, SAMY, SEAP), differentiation-modulating (human C/EBP-α) and therapeutic (human VEGF) transgenes in a variety of rodent (CHO-K1, C2C12) and human cell lines (HT-1080, K-562), human and mouse primary cells (NHDF, PBMC, CD4$^+$) as well as chicken embryos. Lentiviral design concepts include (i) binary systems harboring constitutive streptogramin-dependent transactivator (PIT) and PIT-responsive transgene expression units on separate lentivectors; (ii) streptogramin-responsive promoters (P$_{PIR8}$) placed 5$'$ of desired transgenes; (iii) within modified enhancer-free 3$'$-long terminal repeats; and (iv) bidirectional autoregulated configurations providing streptogramin-responsive transgene expression in a lentiviral one-vector format. Rigorous quantitative analysis revealed HIV-based direct P$_{PIR}$-transgene configurations to provide optimal regulation performance for (i) adjustable expression of intracellular and secreted product proteins, (ii) regulated differential differentiation of muscle precursor cell lines into adipocytes or osteoblasts and (iii) conditional vascularization fine-tuning in chicken embryos. Similar performance could be achieved by engineering streptogramin-responsive transgene expression into an auto-regulated one-vector format. Powerful transduction systems equipped with adjustable transcription modulation options are expected to greatly advance sophisticated molecular interventions in clinically and/or biotechnologically relevant primary cells and cell lines.

INTRODUCTION

Based on their ability to transduce a wide variety of difficult-to-transfect quiescent as well as mitotically active cell types and target tissues without eliciting significant humoral immune responses, transgenic lentiviral particles are currently at the forefront of clinical engineering initiatives (1–7). Although stripped of some 60% of their genomes including all accessory genes and produced by reassembly using multiply attenuated split-genome packaging systems, safety concerns about the clinical use of human immunodeficiency virus (HIV)-derived transduction systems are ongoing. In their latest generation, HIV-1-based lentiviral transduction systems consist of (i) helper plasmid(s) encoding *gag* (encoding major structural proteins), *pol* (coding for lentivirus-specific enzymes) and *rev* (a regulator of *gag/pol* expression and nuclear export of virus RNA); (ii) a *vsv-g* expression vector promoting pantropic transduction of pseudotyped lentiviral particles; and (iii) the actual transgene(s)-encoding lentivector which remains the only genetic material transferred to desired target cells (8).

The lentivector typically contains the transgene expression cassette flanked by *cis*-acting elements required for encapsidation, reverse transcription and integration: the extended packaging signal (ψ^+), the polypurine tracts (PPT, cPPT), 5$'$- and 3$'$-long terminal repeats (5$'$- and 3$'$-LTRs) as well as *env*-derived sequences encompassing the Rev response element (RRE) (8–12). To prevent transcriptional interference with transgene expression and provirus-flanking chromosomal cistrons, most lentivectors are self-inactivating (SIN), characterized by a 3$'$-LTR-located enhancer deletion (3$'$LTR$_{\Delta U3}$) resulting in the elimination of 5$'$-LTR-based transcriptional activities following reverse transcription and integration (13–16). Following the generic design principle of pioneering HIV-based lentiviral transduction systems, a variety of different non-human/non-primate lentivirus derivatives have recently been reported to transduce mammalian cells at similar

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efficiencies and tropism while successfully addressing HIV-associated safety concerns (17–22). Whatever lentiviral transduction system will eventually prevail in the clinics it will have to be compatible with transcription control modalities to titrate protein pharmaceuticals into their therapeutic window and/or adjust their levels to daily changing dosing regimes (23–25).

We have recently developed a human-compatible transcription control system (PIP) responsive to clinically licensed antibiotics of the streptogramin class (e.g. pristinamycin) (26). A streptogramin-dependent transactivator (PIT), designed by fusing the *Streptomyces coelicolor* pristinamycin-induced protein to the Herpes simplex-derived VP16 transactivation domain (PIP-VP16), binds and activates chimeric promoters (P$_{PIR8}$), assembled by cloning tandem PIT-specific operator modules 5′ of a minimal eukaryotic promoter, in a pristinamycin-adjustable manner (26–28).

Capitalizing on recent advances in lentivector design (29) we have engineered a variety of isogenic lentiviral particles for streptogramin-responsive transgene expression and validated their performance in different human primary cells, cell lines as well as in chicken embryos.

MATERIALS AND METHODS

Vector design

All plasmids and oligonucleotides used in this study are listed in Tables 1 and 2, respectively. Detailed information on plasmid construction is also provided in Table 1.

Cell culture

Human embryonic kidney cells transgenic for simian virus 40 (SV40) large T antigen [HEK293-T; (29)], human fibrosarcoma cells (HT-1080; ATCC CCL-121), human peripheral blood mononuclear cells (PBMC; kindly provided by Claudia Ferrara), mouse CD4$^+$ cells (kindly provided by Ivo Sonderegger) and C2C12$_{PIT/tTA}$, a mouse myoblast cell line (C2C12; CRL-1772) transgenic for constitutive expression of streptogramin (PIT)- and tetracycline (tTA)-dependent transactivators (30), were cultivated in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAN Biotech GmbH, Aidenbach, Germany; Cat. No. 3302-P231902), 100 U penicillin and 100 µg/ml streptomycin (Sigma Chemicals, St. Louis, MO), 800 µg/ml G418 (Invitrogen) (C2C12$_{PIT/tTA}$ only) and kept at 37°C in a 5% CO$_2$-containing humidified atmosphere. Chinese hamster ovary cells (CHO-K1; ATCC CCL-61) and their derivative CHO-TWIN1$_{108}$ (31) were cultivated in 10% FCS-containing FMX-8 medium (Cell Culture Technologies GmbH, Zurich, Switzerland) supplemented with 400 µg/ml G418. Normal human dermal fibroblasts (NHDFs; PromoCell GmbH, Heidelberg, Germany; Cat. No. C-12300) were cultivated in fibroblast basal medium (PromoCell GmbH; Cat. No. C-23210). Human chronic myelogenous leukemia cell line K-562 (ATCC-243) was cultivated in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% FCS.

Lentivirus production and infection

For production of replication-incompetent, SIN HIV-based lentiviral particles, a mixture containing 94 µl DMEM, 6 µl FuGENE6 (Roche Diagnostics AG, Rotkreuz, Switzerland), 25 µM chloroquine, 1 µg pLTR-G [encoding the pseudotyping envelope protein VSV-G of the vesicular stomatitis virus (11)], 1 µg pCD/NL-BH* (helper construct) (32) and 1 µg of the desired transgene-encoding lentiviral expression vector was co-transfected into HEK293-T cells. Likewise, the human HIV-1-derived vectors were replaced by non-primate equine infectious anaemia virus (EIAV) counterparts: 1 µg of envelope-encoding pRV67 (19), 2 µg pONY3.1 (helper construct) (19) and 2 µg of the engineered EIAV-derived lentivector. The medium of HEK293-T cultures was replaced 6 h post-transfection and lentiviral particles were produced for another 48 h prior to collection from the supernatant by filtration through a 0.45 µm filter (Schleicher & Schuell GmbH, Dassel, Germany; FP 030/2), which typically yielded titers of 2×10^7 cfu/ml following titration on CHO-K1 cells. To prevent cross-contamination of secreted proteins from production supernatants and increase overall transduction/production efficiency, lentiviral particles were concentrated by ultracentrifugation at 25 000 r.p.m. for 2 h at 4°C (Beckman Quick-Seal centrifuge tubes; Beckman Instruments Inc., CA; Cat. No. 342413). The pellets were resuspended in FCS-containing DMEM (see above) to adjust viral concentrations to desired levels. Lentiviral particle titers were quantified by titration on CHO-K1 cells [enhanced yellow fluorescent protein (EYFP)-encoding lentivectors] and/or using standard p24-specific enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (NEK-050; PerkinElmer, Boston, MA). Unless stated otherwise, standard transduction experiments included infection of 24 000 target cells seeded per well of a 12-well plate with 2×10^6 cfu lentiviral particles (10 ng of p24).

Quantification of reporter protein production

Product proteins were quantified in cell culture supernatants/lysates three days post-transduction. AmyS$_{\Delta S}$, SAMY and secreted alkaline phosphatase (SEAP) titers were assessed following protocols by Schlatter *et al.* (33) using the blue starch Phadebas$^®$ (AmyS$_{\Delta S}$, SAMY; Pharmacia Upjohn, Peapack, NJ) or a chemiluminescence-based assay (SEAP; Roche Diagnostics AG). Vascular endothelial growth factor (VEGF) production was quantified using the human VEGF-specific DuoSet ELISA System (R&D Systems, Minneapolis, MO) according to the manufacturer's protocol.

Characterization of adipogenic and osteogenic cell phenotypes

To visualize adipogenic cell phenotypes following transduction of C2C12$_{PIT/tTA}$ cells with pBM103-derived lentiviral particles, transduced cells were cultured to 80% confluence in FCS-supplemented DMEM (see above) and then transferred to the same medium also containing 1 µM dexamethasone, 50 µM ETYA and 5 µg/ml insulin [Dex/ETYA/Insulin, DEI; (34)]. After DEI induction for 14 days, cells were fixed for 1 h in 37% aqueous formaldehyde solution. Excess formaldehyde was removed by rinsing three times with ddH$_2$O for 30 s prior to immersion of cells in a Oil Red O working solution for 30 min [12 ml Oil Red O stock solution in 8 ml ddH$_2$O; Oil Red O stock solution: 500 mg Oil Red O (Fluka Chemie, Buchs SG, Switzerland) dissolved in 100 ml 60% triethyl-phosphate (Fluka Chemie]. Subsequently, cells were washed

Table 1. Plasmids used and designed in this study

Plasmids	Description and cloning strategy	Reference or source
pBiRex4	Streptogramin-responsive bidirectional expression vector (pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT-pA)	(27)
pBP33	Streptogramin-responsive SEAP expression vector (P$_{PIR8}$-SEAP-IRES-pA)	(28)
pCD/NL-BH*[a]	HIV-1-derived gag/pol-encoding helper plasmid	(32)
pCFl	Dual-regulated expression vector encoding c/ebp-α	Unpublished data
pCF19	Plasmid containing SEAP cassette	(30)
pCF77	Vector containing a pA$_I$-MCS-pA$_{II}$ cassette	Unpublished data
pJK16	HIV-1-derived lentiviral expression vector encoding a P$_{hCMV}$-driven EYFP expression unit (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{hCMV}$-EYFP-3′LTR$_{ΔU3}$)	Unpublished data
pLentiModule4[a]	Modular lentiviral expression vector encoding P$_{hCMV}$-driven EYFP expression unit (5′LTR-ψ$^+$-cPPT-RRE-P$_{hCMV}$-EYFP-3′LTR$_{ΔU3}$)	(29)
pLentiModule8[a]	Modular lentiviral expression vector encoding P$_{hEF1α}$-driven SEAP expression unit (5′LTR-ψ$^+$-cPPT-RRE-P$_{hEF1α}$-SEAP-3′LTR$_{ΔU3}$)	(29)
pLTR-G[a]	Expression vector encoding VSV-G (5′LTR-VSV-G-pA)	(11)
pMF156	Expression vector encoding the streptogramin-dependent transactivator (PIT) (P$_{hCMV}$-PIT-pA)	(26)
pMF351[a]	Lentiviral expression vector (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-MCSI-P$_{hCMV}$-EYFP-MCSII-3′LTR$_{ΔU3}$)	(29)
pMF356[a]	Lentiviral expression vector (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-MCS-3′LTR$_{ΔU3}$)	(29)
pMF359[a]	pMF356-derived lentiviral expression vector	(29)
pMF364[a]	Lentiviral expression vector (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE -MCSI-P$_{hEF1α}$-SAMY-MCSII-3′LTR$_{ΔU3}$)	Unpublished data
pMF392[a]	Lentiviral expression unit encoding the streptogramin-dependent transactivator (PIT). PIT was excised from pMF156 by EcoRI/XbaI and ligated into the corresponding sites (EcoRI/SpeI) of pMF359. (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{hEF1α}$-PIT-3′LTR$_{ΔU3}$)	This work
pONY3.1[b]	EIAV-derived helper plasmid (P$_{hCMV}$-tat-gag-pol-tat-S2-ΔEnv-rev3′LTR$_{ΔU3}$)	(17)
pONY8.0G[b]	EIAV-derived lentiviral expression vector (E$_{CMV}$-R-U5-MCS-ΔEnv-3′LTR$_{ΔU3}$)	(19)
pRV67	Expression vector encoding VSV-G (P$_{hCMV}$-VSV-G-pA)	(17)
pSS 185	Expression vector encoding SAMY (P$_{hCMV}$-SAMY-pA)	(33)
pSS 189	Expression vector encoding amyS$_{ΔS}$ (P$_{hCMV}$–amyS$_{ΔS}$-pA)	(33)
pBM43[a]	Lentiviral expression vector encoding a P$_{hEF1α}$-driven VEGF$_{121}$ expression cassette (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{hEFIα}$-VEGF$_{121}$-3′LTR$_{ΔU3}$)	(29)
pBM57[a]	P$_{hCMV}$ was eliminated from pJK16 by XmaI/AgeI-mediated deletion (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-EYFP-3′LTR$_{ΔU3}$)	This work
pBM60[a]	P$_{PIR8}$ was PCR-amplified from pBP33 using OBM27/OBM28, PmeI-restricted and cloned into the corresponding site (PmeI) of pBM57 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-EYFP-3′LTR$_{ΔU3I}$-P$_{PIR8}$-3′LTR$_{ΔU3II}$)	This work
pBM76[b]	P$_{hCMV}$-EYFP was excised from pMF351 by XbaI/KpnI and cloned into the corresponding sites (XbaI/KpnI) of pONY8.0G (E$_{CMV}$-R-U5-P$_{hCMV}$-EYFP-ΔEnv-3′LTR$_{ΔU3}$)	This work
pBM80[b]	P$_{hCMV}$-EYFP was eliminated from pBM76 by NotI-mediated deletion (E$_{CMV}$-R-U5-MCS-Δ Env-3′LTR$_{ΔU3}$)	This work
pBM84[a]	AmyS$_{ΔS}$ was PCR-amplified from pSS189 using OBM37/OBM38, AscI/SwaI-restricted and cloned into the corresponding sites (AscI/SwaI) of pBM60 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-amyS$_{ΔS}$-3′LTR$_{ΔU3I}$-P$_{PIR8}$-3′LTR$_{ΔU3II}$)	This work
pBM85[a]	SAMY was PCR-amplified from pSS185 using OBM37/OBM38, *AscI/SwaI*-restricted and cloned into the corresponding sites (AscI/SwaI) of pBM60 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-SAMY-3′LTR$_{ΔU3I}$-P$_{PIR8}$-3′LTR$_{ΔU3II}$)	This work
pBM90[a]	P$_{PIR8}$ was eliminated from pBM84 by PmeI-mediated deletion (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-amyS$_{ΔS}$-3′LTR$_{ΔU3}$)	This work
pBM91[a]	P$_{PIR8}$ was eliminated from pBM85 by PmeI-mediated deletion (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-SAMY-3′LTR$_{ΔU3}$)	This work
pBM92[a]	c/ebp-α was excised from pCFl by BamHI/ClaI and cloned into the corresponding sites (ClaI/BamHI) of pCF77 (pA$_I$-c/ebp-α-pA$_{II}$) in antisense orientation	This work
pBM93[a]	pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT-pA$_{II}$ was excised from pBiRex4 by EcoRV/XbaI and cloned into the compatible sites (SmaI/SpeI) of pMF356 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-EYFP← P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT-pA$_{II}$ -3′LTR$_{ΔU3}$)	This work
pBM94[a]	P$_{PIR8}$ was PCR-amplified from pBP33 using OBM39/OBM40, AscI/MluI-restricted and cloned into the compatible AscI site of pBM57 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-EYFP-3′LTR$_{ΔU3}$)	This work
pBM95[a]	c/ebp-α was excised from pBM92 by ClaI/MluI and cloned in sense orientation into the compatible sites (AscI/BstBI) of pBM60 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-c/ebp-α-3′ LTR$_{ΔU3I}$-P$_{PIR8}$-3′LTR$_{ΔU3II}$)	This work
pBM96[a]	P$_{PIR8}$ was PCR-amplified from pBP33 using OBM39/OBM40, AscI/MluI restricted and cloned into the compatible AscI site of pBM91 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-SAMY-3′LTR$_{ΔU3}$)	This work
pBM97[a]	P$_{PIR8}$ was PCR-amplified from pBP33 using OBM39/OBM40, AscI/MluI-restricted and cloned into the compatible AscI site of pBM90 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-amyS$_{ΔS}$-3′LTR$_{ΔU3}$)	This work
pBM98[a]	pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT was excised from pBiRex4 by EcoRV/PsiI and cloned in sense orientation into the compatible sites (HpaI/SwaI) of pMF356 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PTR-3′LTR$_{ΔU3}$)	This work
pBM99[b]	P$_{PIR8}$-EYFP was excised from pBM94 by AscI/PacI and cloned into the corresponding sites (AscI/PacI) of pBM80 (E$_{CMV}$-R-U5-P$_{PIR8}$-EYFP-ΔEnv-3′LTR$_{ΔU3}$)	This work
pBM101[a]	P$_{PIR8}$ was eliminated from pBM95 by Pmel-mediated deletion (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-c/ebp-α-3′LTR$_{ΔU3}$)	This work
pBM103[a]	c/ebp-α was excised from pBM101 by EcoRI/NheI and cloned into the corresponding sites (EcoRI/NheI) of pBM94 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-c/ebp-α-3′LTR$_{ΔU3}$)	This work
pBM104[a]	VEGF$_{121}$ was excised from pBM43 by EcoRI/MluI and cloned into the corresponding sites (EcoRI/MluI) of pBM94 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-VEGF$_{121}$-3′LTR$_{ΔU3}$)	This work

Table 1. *Continued*

Plasmids	Description and cloning strategy	Reference or source
pBM105[a]	SEAP was excised from pLentiModule8 by EcoRI/NheI and cloned into the corresponding sites (EcoRI/NheI) of pBM94(5′LTR-ψ⁺-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-SEAP-3′LTR$_{ΔU3}$)	This work
pBM106[a]	EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT was excised from pBiRex4 by ClaI/PsiI and cloned in sense orientation into the compatible sites (ClaI/SwaI) of pBM356 (5′LTR-ψ⁺-ori$_{SV40}$-cPPT-RRE-EYFP←P$_{hCMVmm}$-PTR-P$_{hsp70min}$→PIT-3′LTR$_{ΔU3}$)	
pBM126[a]	pA$_I$-SEAP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT-pA$_{11}$ was excised from pCF19 by PmeI/ClaI and cloned in antisense orientation into the compatible sites (ClaI/NruI) of pBM93 (pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT-pA$_{11}$)	
pBM127[a]	pA$_1$-SEAP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→ PIT was excised from pCF19 by PmeI/ClaI and cloned in antisense orientation into the compatible sites (ClaI/NruI) of pBM98 (pA$_I$-EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT)	
pBM128[a]	SEAP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT was excised from pCF19 by PmeI/ClaI and cloned in antisense orientation into the compatible sites (ClaI/NruI) of pBM106 (EYFP←P$_{hCMVmin}$-PTR-P$_{hsp70min}$→PIT)	

[a]Human immunodeficiency virus (HIV)-1-derived lentiviral expression vectors.
[b]Equine infectious anemia virus (EIAV)-derived lentiviral expression vectors. Please consult Table 2 for indicated oligonucleotides.
Abbreviations: 3′-LTR, 3′-long terminal repeat; 3′-LTR$_{ΔU3}$, 3′-LTR devoid of the enhancer; 3′-LTR$_{ΔU3I}$/3′-LTR$_{ΔU3II}$ 3′-LTR$_{ΔU3}$ subunits; 5′-LTR, 5′-LTR; amyS, *B. stearothermophilus* α-amylase; amyS$_{ΔS}$, *B. stearothermophilus* α-amylase devoid of its signal sequence; c/ebp-α, CCAAT/enhancer-binding protein alpha; cPPT, central polypurine tract; ECMV, enhancer derived from P$_{hCMV}$; EIAV, equine infectious anemia virus; EYFP, enhanced yellow fluorescent protein; HIV-1, human immunodeficiency virus type 1; gag, lentiviral core protein; IRES, internal ribosome entry site of polioviral origin; MCS, multiple cloning site; ori$_{SV40}$, simian virus 40-derived origin of replication; pA, SV40-derived polyadenylation site; pA$_I$, pA$_{II}$, minimal synthetic polyadenylation sites; P$_{hCMV}$, cytomegalovirus immediate early promoter; P$_{hEFIα}$, promoter of the human elongation factor 1α; P$_{hCMVmin}$ minimal version of P$_{hCMV}$; P$_{hsp70min}$ minimal version of the *Drosophila* heat-shock protein 70 promoter; PIT, streptogramin-dependent transactivator; pol, virion-associated polymerase; P$_{PIR8}$, streptogramin-responsive promoter; PTR, PIT-specific operator module; R-U5, elements of 5′-/3′-LTRs; Rev, specific for nuclear export of viral RNA; RRE, Rev response element; S2, EIAV-specific determinant critical for viral replication and pathogenic properties *in vivo*; SAMY, *B. stearothermophilus-derived* secreted α-amylase; SEAP, human secreted alkaline phosphatase; tat, LTR-specific transactivator; VEGF$_{121}$, human vascular endothelial growth factor 121; VSV-G, vesicular stomatitis virus protein G; Δ Env, truncated EIAV envelope-encoding gene; and ψ⁺, extended lentiviral packaging signal.

Table 2. Oligonucleotides designed for this study

Primer	Sequence	Restriction sites (5′→3′)
OBM27	CGGTTTAAACgacgtcggagaaatagcg	PmeI
OBM28	CGTGATCAGTTTAAACAgctcgaattccgcgga	BelI, PmeI
OBM37	CGGGCGCGCCtaagcttggtaccgagct	AscI
OBM38	CGACGCGTATTTAAATaagcttcaagcataatct	MluI, SwaI
OBM39	CGGGGCGCGCCgacgtcggagaaatagcg	AscI
OBM40	CGTGATCAACGCGTagctcgaattccgcgga	BelI, MluI

Annealing sequence in lower case; sites for indicated restriction endonucleases are underlined.

three times with ddH$_2$O for 30 s and the nuclei stained for 60 s with Mayer's hematoxylin (Sigma Chemicals). Finally, cells were rinsed in ddH$_2$O for 10 min and covered with cover slips using 10% glycerol in PBS (35). Alkaline phosphatase-specific staining of osteogenic cell phenotypes was visualized following a five-day cultivation using the Sigma Kit 85 according to the manufacturer's instructions (Sigma Chemicals).

Transduction of chicken embryos

Experiments involving chicken embryos were conducted following the shell-free cultivation protocols by Djonov *et al.* (36). After three days of incubation at 37°C, Brown Leghorn eggs were opened and their contents were carefully poured into 80 mm plastic Petri dishes. The chicken embryos were incubated at 37°C in a humidified atmosphere. Recombinant lentiviral particles (2 µl of pBM104 (8.7 × 10⁶ cfu)- and 1 µl of pMF392 (1.5 × 10⁵ cfu)-derived) were co-applied locally on top of the growing chorioallantoic membrane (CAM) at embryonic day 9, together with 0.5 µl CellTracker Orange CMTMR (Molecular Probes Inc., Eugene, OR;

Cat.No. C-2927) in 100 µl DMEM (final concentration 0.5 nM). To modulate heterologous VEGF$_{121}$ expression, different amounts (0, 6, 30, 60 µg) of pristinamycin were administered for 1 h post-transduction. On embryonic day 12, the CAMs were examined by *in vivo* fluorescence microscopy following intravenous injection of 100 µl of 2.5% fluorescein isothiocyanate dextran (FITC, 2 000 000; Sigma Chemicals) (37).

Fluorescence microscopy

EYFP expression in cell culture as well as FITC-stained CAM blood vessels were visualized at 50× and 100× magnifications using a Leica DM-RB fluorescence microscope equipped with a Leica digital fluorescence camera DC300 FX (Leica Microsystems AG, Heerbrugg, Switzerland) and a XF114 filter (Omega Optical Inc., Brattleboro, VT). EYFP-mediated fluorescence was quantified using a GeniusPro (Tecan Group Ltd, Maennedorf, Switzerland) and/or the ImageJ software (Wayne Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij/).

Regulating antibiotics

The pristinamycin antibiotic Pyostacin® (PI, Aventis Inc., Paris, France: lot no. 27404) was prepared as stock solution of 500 µg/ml in dimethyl sulfoxide and used at a final concentration of 2 µg/ml unless indicated otherwise.

RESULTS

HIV- and EIAV-derived lentivector configurations for streptogramin-responsive EYFP expression

Based on the recently developed HIV-1-derived (modular) SIN lentivector design (29), we have constructed pBM57
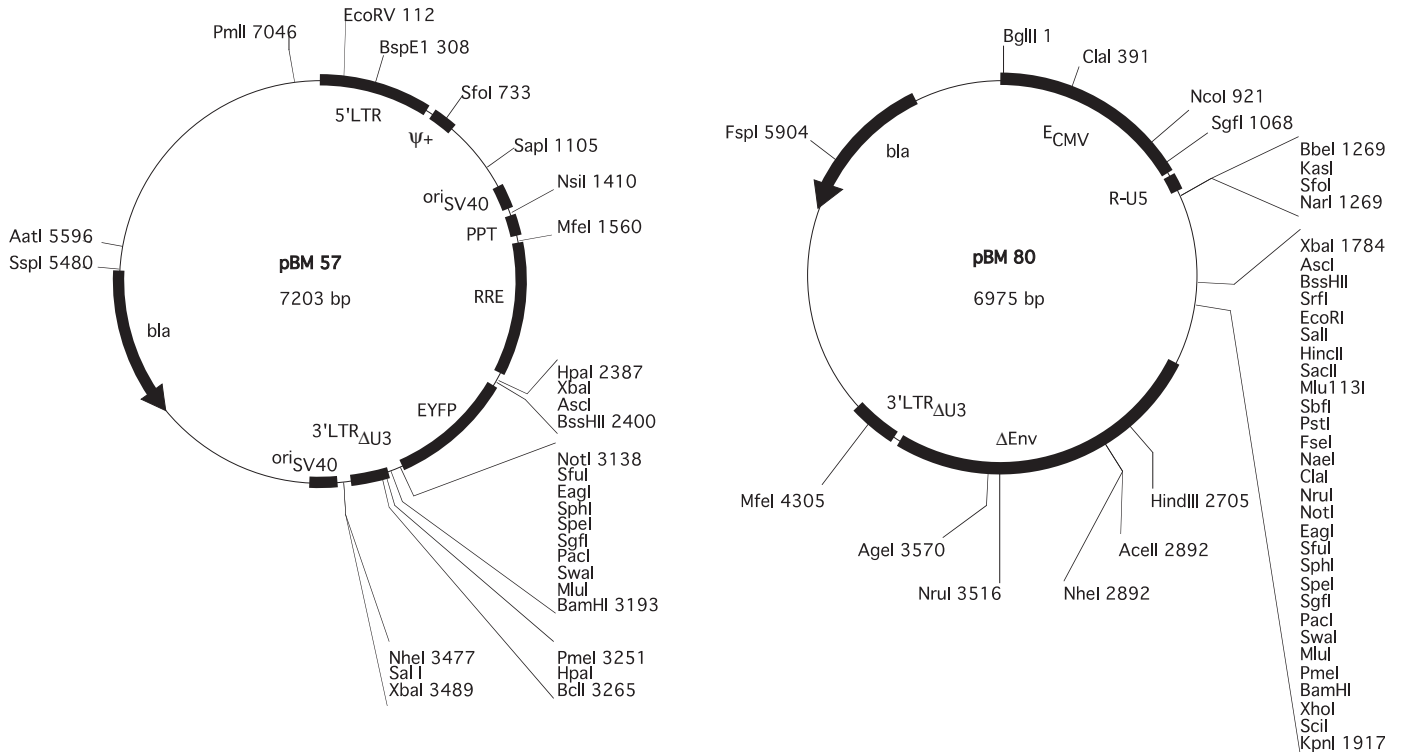
Figure 1. Plasmid maps of basic SIN lentiviral expression vectors derived from HIV (pBM57) and EIAV (pBM80). pBM57 contains a 5′-LTR, an extended packaging signal (ψ^+), SV40 origins of replication (ori_{SV40}), a central polypurine tract (cPPT), the RRE, anEYFP and an enhancer-free 3′LTR$_{\Delta U3}$). pBM80 harbors a modified 5′-LTR consisting of a CMV-derived enhancer element (E_{CMV}) cloned adjacent to the R-U5 module, an extensive polylinker, a truncated envelope-encoding gene (ΔEnv) and an enhancer-free 3′-LTR (3′LTR$_{\Delta U3}$). pBM57 is fully compatible with previously reported lentiviral expression vectors and enable straightforward module swapping (29).

(5′LTR-ψ^+-ori$_{SV40}$-PPT-RRE-MCSI-EYFP-MCSII-3′LTR$_{\Delta U3I}$-MCSIII-3′LTR$_{\Delta U3II}$), which provides convenient multiple cloning sites (MCSI and MCSIII) for integration of promoter elements driving an EYFP transgene (Figure 1). The optimized streptogramin-responsive promoter [P$_{PIR8}$; (28)] was either placed within the ΔU3 region to support directional copy into the 5′-LTR following lentivector reverse transcription and integration (pBM60; 5′LTR-ψ^+-ori$_{SV40}$-PPT-RRE-MCSI-EYFP-MCSII-3′LTR$_{\Delta U3I}$-P$_{PIR8}$-3′LTR$_{\Delta U3II}$) or cloned immediately 5′ of EYFP (pBM94; 5′LTR-ψ^+-ori$_{SV40}$-PPT-R R E - P$_{PIR8}$-EYFP-MCSII-3′LTR$_{\Delta U3I}$-MCSIII-3′LTR$_{\Delta U3II}$) (Figure 2A). pBM60- and pBM94-derived lentiviral particles were transduced into CHO-TWIN1$_{108}$ constitutively expressing the streptogramin-dependent transactivator [PIT; (31)], and cultivated in the presence or absence of the regulating streptogramin antibiotic pristinamycin I (PI). Both lentivectors provided high-level EYFP expression in the absence of PI whereas transgene production was completely repressed following addition of the regulating antibiotic (Figure 2B). Although both promoter-transgene configurations enabled tight regulation, qualitative assessment of EYFP expression profiles revealed higher maximum expression levels from P$_{PIR8}$ placed 5′ of the desired transgene rather than in the ΔU3 region (Figure 2B). Following the direct P$_{PIR8}$-EYFP design concept, a non-primate EIAV-based pONY8.0G-derived lentivector (19) (pBM80; E$_{CMV}$-R-U5-MCS-ΔEnv-3′LTR$_{\Delta U3}$; Figure 1) was engineered for streptogramin-responsive EYFP expression (pBM99; E$_{CMV}$-R-U5-P$_{PIR8}$-EYFP-ΔEnv-3′LTR$_{\Delta U3}$)

and transduced into CHO-TWIN1$_{108}$. Fluorescence micrograph-based analysis suggested EIAV-mediated EYFP expression to be significantly lower compared to levels resulting from HIV-based transduction (Figure 2B), which likely confirms reports on decreased transduction efficiency of this non-human lentivector type in CHO cells (18). Nevertheless, this EIAV-based lentivector design pioneers transcription modulation in a non-human lentivirus configuration and combines tight regulation with maximum expression levels acceptable for most applications.

Comparative transgene expression profiling of different promoter positions

To quantify P$_{PIR8}$-driven transgene regulation profiles associated with promoter positions immediately 5′ of the transgene or within the 3′LTR$_{\Delta U3}$, we replaced EYFP of pBM60 and pBM94 by the intracellular *Bacillus stearothermophilus*-derived heat-stabile α-amylase [amyS$_{\Delta S}$; (33)]. pBM84 (5′LTR-ψ^+-ori$_{SV40}$-PPT-RRE-MCSI-amyS$_{\Delta S}$-MCSII-3′LTR$_{\Delta U3I}$-P$_{PIR8}$-3′LTR$_{\Delta U3II}$)- and pBM97 (5′LTR-ψ^+-ori$_{SV40}$-PPT-RRE-P$_{PIR8}$-amyS$_{\Delta S}$-MCSI-3′LTR$_{\Delta U3I}$-MCSIII-3′LTR$_{\Delta U3II}$)-derived lentiviral particles were transduced into CHO-TWIN1$_{108}$ and intracellular α-amylase levels quantified 72 h post-transduction (Figure 3A). Both lentivector configurations enabled tight α-amylase control and showed similar induction factors [pBM84, (32); pBM97, (35)]. Yet, the P$_{PIR8}$-amyS$_{\Delta S}$ configuration outperformed maximum expression
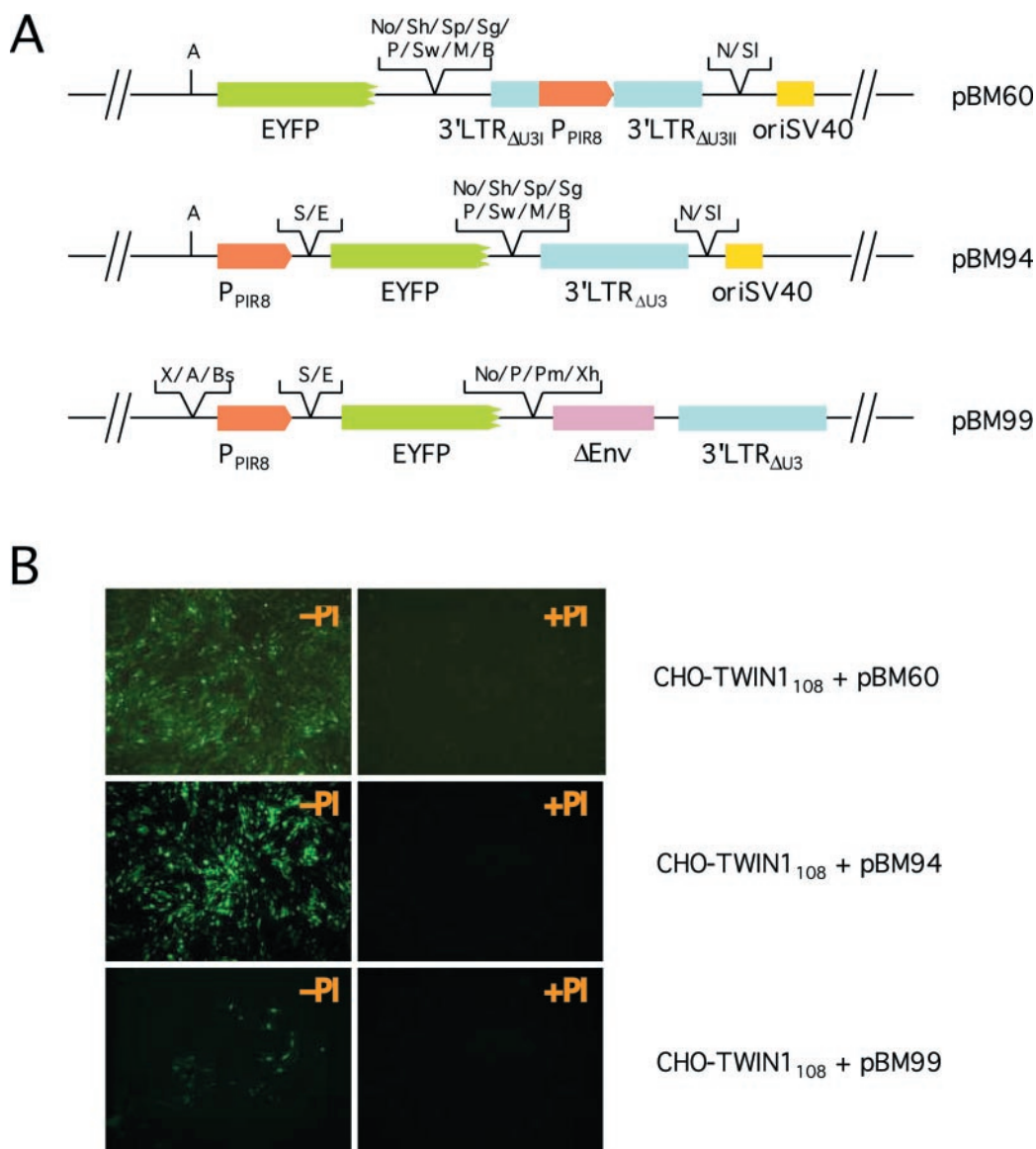
Figure 2. Lentivector-based regulated expression of fluorescent proteins. (**A**) Detail representation of streptogramin-responsive transgene modules of HIV- (pBM60; pBM94) and EIAV- (pBM99) derived lentivectors. $3'LTR_{\Delta U3}$; enhancer-less $3'$-LTR; $3'LTR_{\Delta U3I}$/$3'LTR_{\Delta U3II}$, modified $3'LTR_{\Delta U3}$; P_{PIR8}, streptogramin-responsive promoter; ΔEnv, truncated EIAV envelope-encoding gene. Abbreviation of restriction endonucleases: A, AscI; B, BamHI; Bs, BssHII; C, ClaI; E, EcoRI; F, FseI; H, HpaI; K, KpnI; M, MluI; N, NheI; No, NotI; Nr, NruI; P, PacI; Pm, PmeI; S, SacII; Sb, SbfI; Sl, SalI; Sg, SgfI; Sh, SphI; Sp, SpeI; Sr, SrfI; Sw, SwaI; X, XbaI; and Xh, XhoI. (**B**) EYFP-specific fluorescence micrographs of PIT (pristinamycin-dependent transactivator)-expressing CHO-TWIN1_{108} transduced with pBM60, pBM94 and pBM99 cultivated for 48 h in the presence (+PI) and absence (−PI) of the streptogramin antibiotic PI.

levels of $3'LTR_{\Delta U3}$-located P_{PIR8} 80-fold (Figure 3B). Therefore, we chose to use direct promoter-transgene configurations for further analysis.

Lentivectors engineered for streptogramin-responsive control of secreted protein production

Since tight control of secreted protein therapeutics is a standard challenge in current gene therapy scenarios, we have evaluated conditional expression performance of lentivectors engineered for streptogramin-responsive expression of the *B. stearothermophilus*-derived secreted α-amylase [SAMY; (33)], human VEGF_{121} (29) and the human model glycoprotein

SEAP (38). pBM96 ($5'LTR$-ψ^{+}-ori_{SV40}-cPPT-RRE-P_{PIR8}-SAMY-$3'LTR_{\Delta U3}$)-, pBM104 ($5'LTR$-ψ^{+}-ori_{SV40}-cPPT-RRE-P_{PIR8}-VEGF_{121}-$3'LTR_{\Delta U3}$)- and pBM105 ($5'LTR$-ψ^{+}-ori_{SV40}-cPPT-RRE-P_{PIR8}-SEAP-$3'LTR_{\Delta U3}$)-derived lentiviral particles were transduced into CHO-TWIN1_{108} and respective reporter protein secretion quantified 72 h post-transduction cultivation in the presence and absence of regulating streptogramin antibiotics. All reporter proteins showed equivalent tight pristinamycin-responsive secretion substantiating the fact that this lentivector design enables robust expression control [pBM96 (induction factor 36), −PI: 4031 ± 480 U/l, +PI: 113 ± 14 U/l; pBM104 (induction factor 28), −PI: 43 ± 3.4 ng/ml, +PI: 1.5 ± 0.3 ng/ml; pBM105 (induction factor 30), −PI: 2758 ± 325 ng/ml, +PI: 91 ± 18.64 ng/ml]. Furthermore,
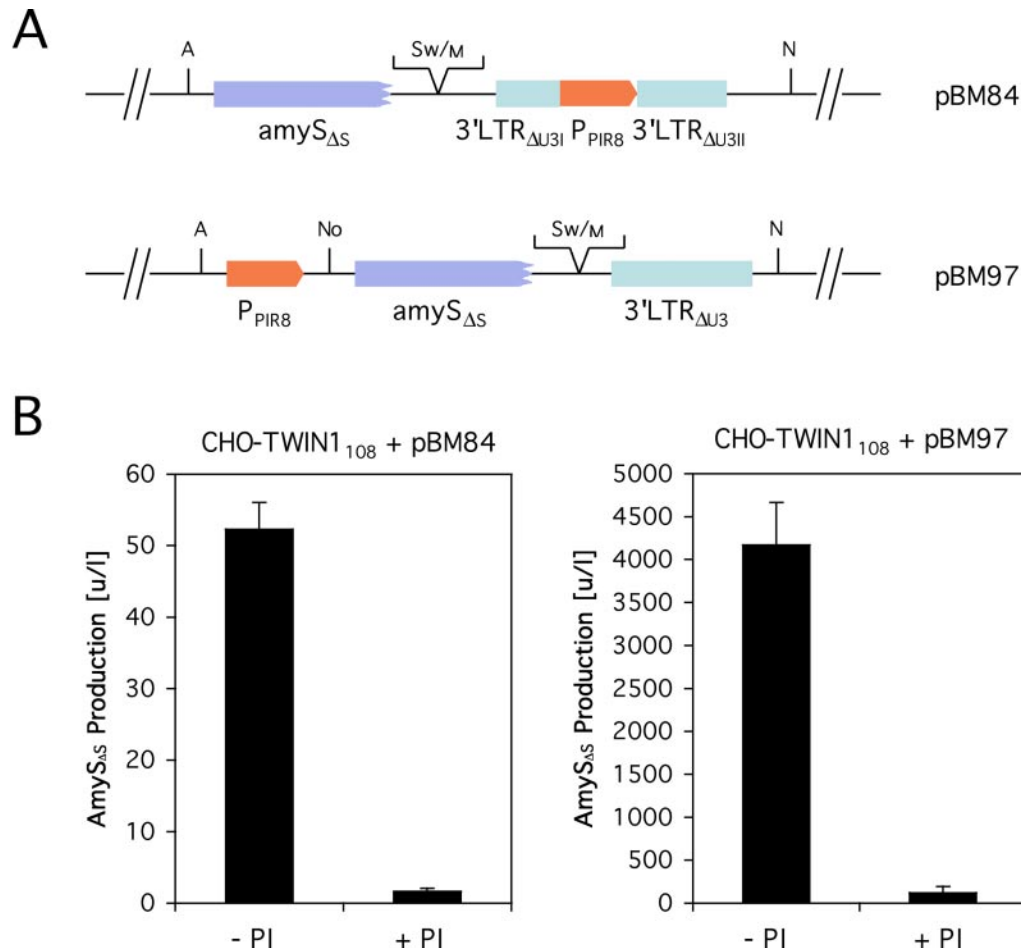
Figure 3. Comparative streptogramin-responsive expression profiling of lentivectors with different promoter-transgene configurations. (**A**) Detail projection of isogenic lentivectors pBM84 and pBM97. Although pBM84 contains the streptogramin-responsive promoter (P_{PIR8}) within a modified 3′-LTR (3′LTR$_{\Delta U3}$; 3′LTR$_{\Delta U3I}$; 3′LTR$_{\Delta U3II}$) and requires reverse transcription and chromosomal integration for P_{PIR}-driven expression of the intracellular reporter gene amyS$_{\Delta S}$ (signal peptide-free *B.stearothermophilus*-derived α-amylase), pBM97 contains a central P_{PIR8}- amyS$_{\Delta S}$ expression configuration. (see caption of Figure 2 for abbreviation of restriction endonucleases). (**B**) Quantitative α-amylase profiling of PIT-producing CHO-TWIN1$_{108}$ transduced with pMF84-/pMF97-derived lentiviral particles and cultivated for 48 h in the presence (+) and absence (−) of PI. pBM84-derived lentiviral particles reached 32-fold induction whereas cells transduced with pBM97 lentivectors showed an induction factor of 35.

pBM392/pBM104-derived lentiviral particles enabled transduction and VEGF$_{121}$ regulation in human PBMC (induction factor 11, −PI: 1.2 ± 0.1 ng/ml, +PI: 0.1 ± 0.0 ng/ml) and mouse CD4$^+$ cells (induction factor 13, −PI 0.8 ± 0.1 ng/ml, +PI: 0.1 ± 0.0 ng/ml).

Transgene expression switching and adjustability of streptogramin-responsive SAMY-encoding lentivectors

Switch kinetics, reversibility of expression status and adjustability of transgene expression are key characteristics of any heterologous transcription control configuration. We have therefore subjected CHO-TWIN1$_{108}$ cultures transduced with pBM96 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{PIR8}$-SAMY-3′LTR$_{\Delta U3}$)-derived lentiviral particles to changing streptogramin dosing regimes (+PI/+PI/+PI; +PI/+PI/−PI; −PI/−PI/−PI; −PI/−PI/+PI; +PI/−PI/−PI; +PI/−PI/+PI; −PI/+PI/+PI; −PI/+PI/−PI) and SAMY expression readouts were taken at corresponding days 4/7/10. pMF364-derived lentiviral particles (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-P$_{hEF1\alpha}$-SAMY-3′LTR$_{\Delta U3}$) were used as constitutive control. SAMY

expression was characterized by tight expression switches as well as complete reversibility of transgene control (Figure 4A). Also, streptogramin-responsive SEAP expression encoded on pBM105-derived lentiviral particles can be precisely titrated to desired levels by defined PI dosing (Figure 4B).

Streptogramin-controlled adipocyte versus osteoblast differentiation following lentivector-based transduction of muscle precursor cells

Current gene therapy and tissue engineering initiatives evaluate the potential of heterologous gene control systems for rational reprogramming of desired cell phenotypes following conditional expression of differentiation-modulating transgenes. Transdifferentiation studies require particularly tight control and set a high standard for artificial transcription control modalities. Following a multi-step design strategy, we have configured pBM57 for streptogramin-responsive expression of the human CCAAT/enhancer-binding protein alpha (C/EBP-α) (pBM103; 5′LTR-ψ$^+$-ori$_{SV40}$-PPT-RRE-P$_{PIR8}$-c/ebp-α-MCSII-3′LTR$_{\Delta U3I}$-MCSIII-3′LTR$_{\Delta U3II}$) (Figure 5A).
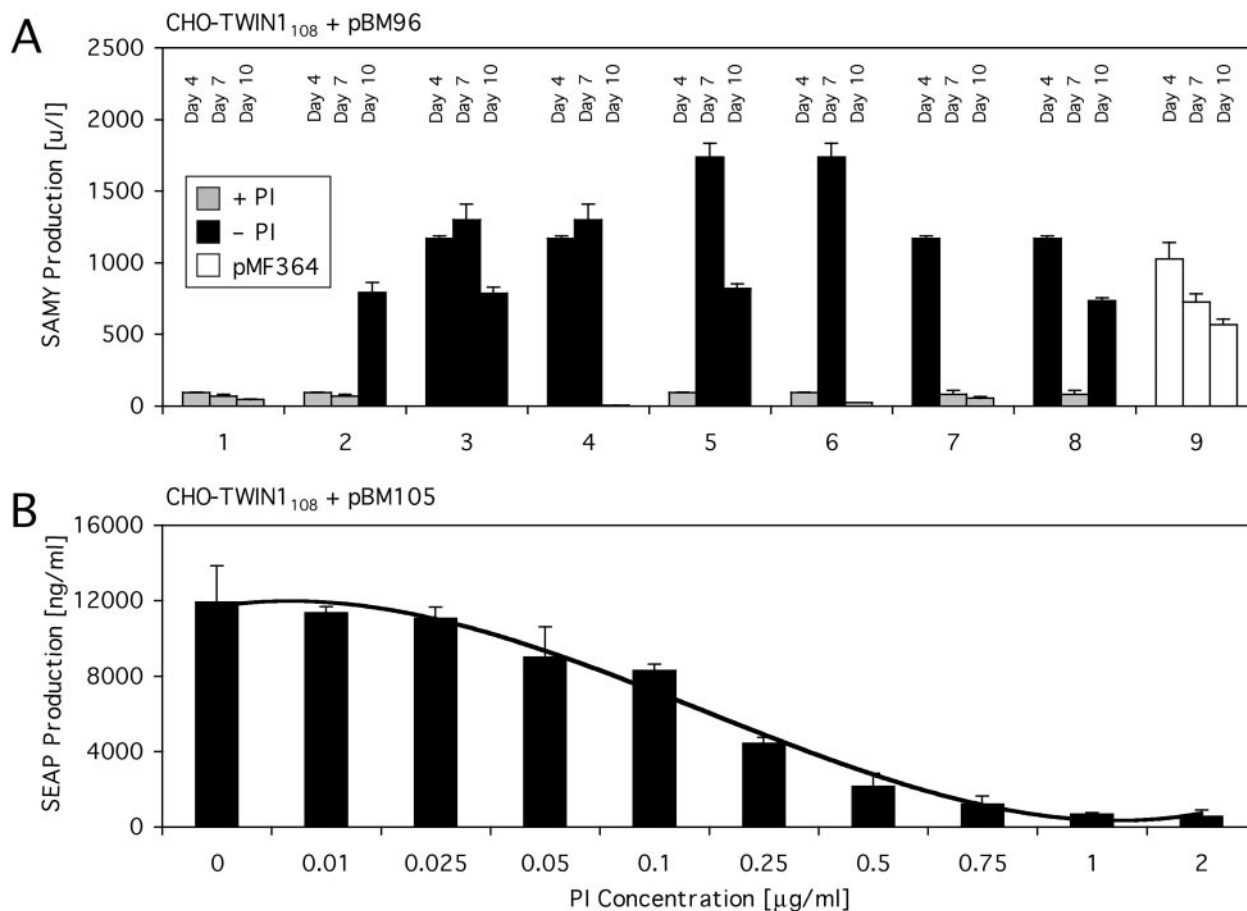
Figure 4. Lentivector-based streptogramin-responsive transgene expression switching kinetics and adjustability in CHO-K1 cell derivatives. (**A**) SAMY expression of different cultures of CHO-TWIN1$_{108}$ transduced with pBM96-derived lentiviral particles (1–8) was switched at days 4, 7 and 10 post-transduction (maximum induction factor 209). CHO-TWIN1$_{108}$ transduced with pMF364-derived lentiviral particles was used as constitutive control. (**B**) Adjustable SEAP expression of CHO-TWIN1$_{108}$ transduced with pBM105-derived lentiviral particles and cultivated for 48 h in the medium supplemented with indicated PI concentrations (maximum induction factor 20).

To induce differential differentiation pBM103-transduced C2C12$_{PIT/tTA}$' myoblasts were cultivated in the presence (repression of *c/ebp*-α) and absence (induction of *c/ebp*-α) of PI. Indeed, following repression of *c/ebp*-α, C2C12$_{PIT/tTA}$ differentiated into adipocytes following 14 days cultivation period in DEI-containing medium [Figure 5B; (30)]. In contrast, maintenance in streptogramin-free medium resulted in ongoing C/EBP-α expression, which differentiated engineered C2C12$_{PIT/tTA}$ cell populations into osteoblasts (Figure 5C).

Transduction of chicken embryos using lentiviral particles transgenic for streptogramin-adjustable expression of human VEGF

To validate lentivector-based streptrogramin-responsive transgene expression *in vivo*, we co-transduced chicken CAM by distinct lentiviral particles encoding (i) constitutive PIT (pMF392) and (ii) PIT-responsive P$_{PIR8}$-driven VEGF$_{121}$ (pBM104) expression, and scored neovascularization as well as vessel morphology after administration of different amounts of pristinamycin. CAMs cultivated under PI-free conditions exhibited VEGF-mediated induction of new blood vessels

exemplified by increased formation of numerous arterioles and venules (Figure 6A and B). Also, the hierarchical, tree-like structure of the supplying vessels was disturbed and the multitude of arterioles and venules adopted an irregular tortuous shape associated with atypical delta- or brush-like vessel end points. The VEGF-induced effect was confined to a 4 mm radius around the lentiviral particle application sites and could not be observed on the same CAM beyond this perimeter (Figure 6C). Following administration of increasing amounts of PI (6, 30, 60 μg), VEGF$_{121}$ expression was gradually decreased to full repression that resulted in native vascularization profiles and vessel morphologies at the application site (Figure 6D–F).

Autoregulated streptogramin-responsive lentivectors

Owing to the binary nature of the streptogramin-responsive transcription control modality lentivector-based engineering of desired target cells requires co-transduction of lentiviral particles transgenic for constitutive PIT (pMF392) and P$_{PIR8}$-driven EYFP expression (pBM60, pBM94). However, as exemplified by infection of primary NHDF and CHO-K1 cells co-transduction provided excellent regulation profiles but at lower efficiencies
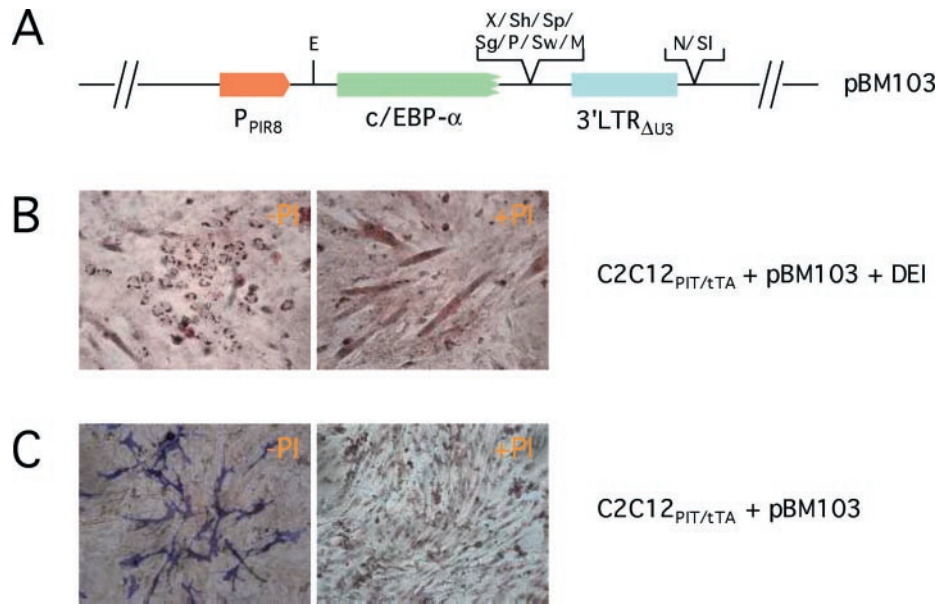
Figure 5. Streptogramin-responsive differential adipocyte versus myoblast versus osteoblast differentiation of C2C12-derived cell lines transduced with pBM103-derived lentiviral particles engineered for streptogramin-responsive expression of the CCAAT/enhancer-binding protein alpha (C/EBP-α). (**A**) Schematic detail representation of pBM103 harboring a *c/ebp-α* cassette driven by the streptogramin-responsive promoter (P_{PIR8}) and terminated by an enhancer-free 3′-LTR (3′LTR$_{\Delta U3}$) (see caption of Figure 2 for abbreviation of restriction endonucleases). (**B**) Light micrographs of C2C12$_{PIT/tTA}$ transduced with pBM103-derived lentiviral particles and cultivated for 14 days in DEI-supplemented PI-containing (+PI) or PI-free medium. Cultures were stained with Oil Red O to visualize triglyceride droplets exclusively produced by adipocytes ($-$PI, induction of *c/ebp-α*). (**C**) Microscopic analysis of C2C12$_{PIT/tTA}$ transduced with pBM103-derived lentiviral particles and cultivated for 5 days in the presence (+) and absence ($-$) of PI prior to specific staining of osteoblasts (blue; $-$PI, induction of *c/ebp-α*). C/EBP-α has recently been shown to induce osteogenesis in C2C12-derived myoblast cultures (30).
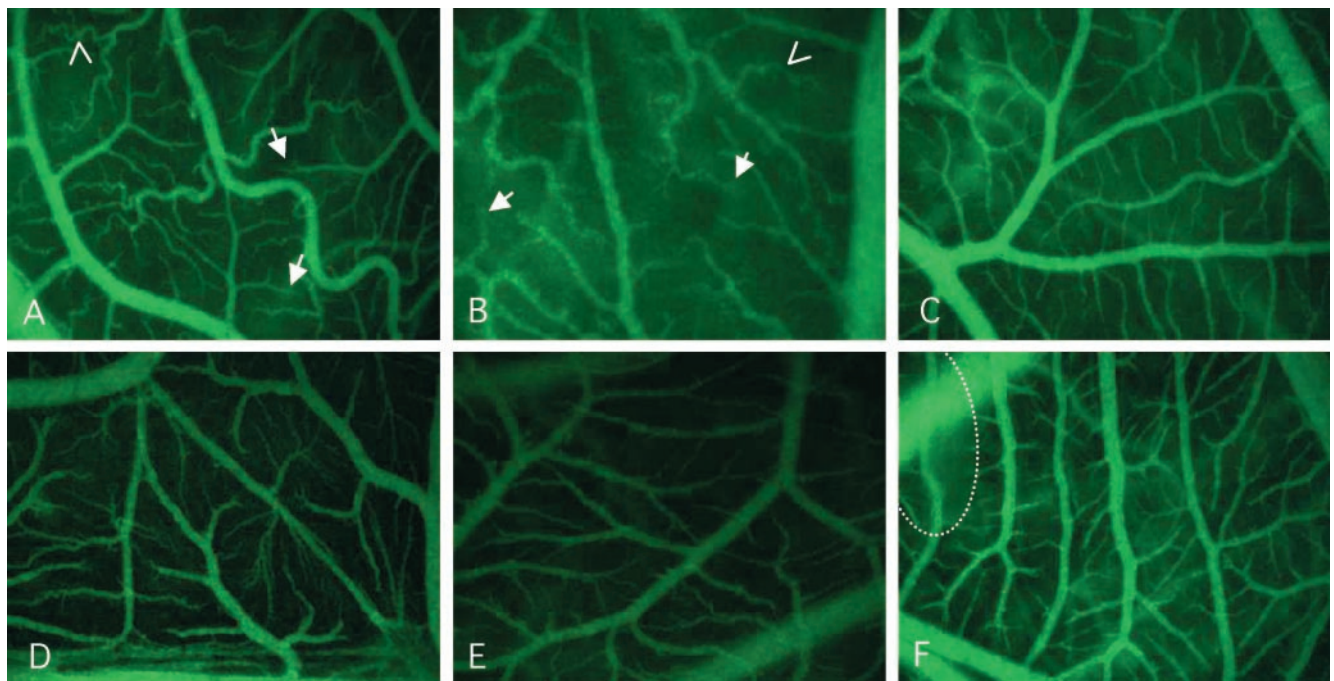

Figure 6. *In vivo* examination of microvascular growth in the CAM of 12-day-old chicken embryos 72 h post co-transduction with pBM104- [2 μl (8.7×10^6 cfu) producing 106.66 ± 7.84 ng/ml VEGF$_{121}$ in CHO-K1-derived cells in 48 h] and pMF392- (1 μl; 1.5×10^5 cfu) derived lentiviral particles encoding the human VEGF$_{121}$. Lentivirus application induced an angiogenic response with atypical (brush- and delta-like) end point patterns (arrows) and irregular tortuous vessel shape (arrowhead) within a perimeter of 4 mm when no antibiotic was present [(**A**) 50× magnification; (**B**) 100× magnification]. Beyond this boundary, the VEGF$_{121}$-based effects fade in a distance-dependent manner and are undetectable at 15 mm (**C**). Following administration of increasing amounts of VEGF$_{121}$-repressing PI concentrations post-transduction VEGF$_{121}$-induced angiogenesis gradually decreased [(**D**) 6 μg (50× magnification); (**E**) 30 μg (50× magnification)] to full repression which resulted in native vascularization profiles and vessel morphologies at the application site [(**F**) 60 μg (50× magnification); dashed line].
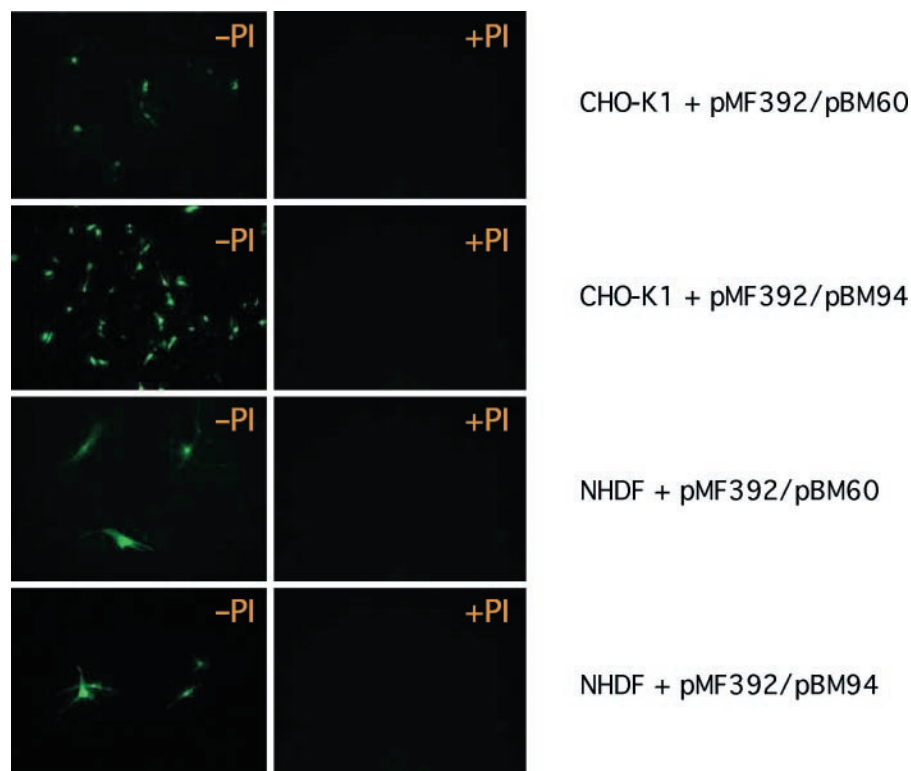
Figure 7. Fluorescence micrographs of streptogramin-responsive EYFP expression in CHO-K1 and primary NHDF following co-transduction of pMF392-[engineered for constitutive expression of the pristinamycin-dependent transactivator (PIT)] and pBM60- [encoding the streptogramin-responsive promoter (P_{PIR8}) within a modified 3′-LTR (3′$LTR_{\Delta U3}$)] or pBM94- (harboring a direct P_{PIR8}-EYFP expression unit) derived lentiviral particles and cultivation in the presence (+) and absence (−) of PI.

(Figure 7) compared to mono-transductions (Figure 2B) (24). In addition to compromising transduction efficiencies, co-integration of transactivator- and transgene-encoding proviruses may result in deregulated transgene expression owing to interference between the operator contained in the transactivator-driving promoter and the antibiotic-responsive promoter. To provide interference-free streptogramin-responsive transgene expression following transduction of a single lentiviral particle type, we designed the following classes of bidirectional autoregulated lentiviral expression vectors: (i) pBM93 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-EYFP←$P_{hCMVmin}$-PTR-$P_{hsp70min}$→PIT-pA$_{II}$-3′$LTR_{\Delta U3}$) and pBM126 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-SEAP←$P_{hCMVmin}$-PTR-$P_{hsp70min}$→PIT-pA$_{II}$-3′$LTR_{\Delta U3}$), (ii) pBM98 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-EYFP←$P_{hCMV-hCMVmin}$-PTR-$P_{hsp70min}$→PIT-3′$LTR_{\Delta U3}$) and pBM127 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-pA$_I$-SEAP←$P_{hCMVmin}$-PTR-$P_{hsp70min}$→PIT-3′$LTR_{\Delta U3}$) and (iii) pBM106 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-EYFP←$P_{hCMVmin}$-PTR-$P_{hsp70min}$→PIT-3′$LTR_{\Delta U3}$) and pBM128 (5′LTR-ψ$^+$-ori$_{SV40}$-cPPT-RRE-SEAP←$P_{hCMVmin}$-PTR-$P_{hsp70min}$→PIT-3′$LTR_{\Delta U3}$) (Figure 8A). All autoregulated lentiviral expression vectors contain a central asymmetric bidirectional streptogramin-responsive promoter consisting of a central PIT-specific PTR operator site flanked by minimal versions of the human cytomegalovirus immediate early ($P_{hCMVmin}$) and the *Drosophila* heat-shock protein 70 ($P_{hsp70min}$) promoters. The stronger $P_{hCMVmin}$ was arranged to drive EYFP or SEAP expression whereas $P_{hsp70min}$ was set to maintain the autoregulatory circuit via PIT expression. pBM93/pBM126, pBM98/pBM127 and pMF106/pBM128

differ in their configuration of synthetic minimal polyadenylation sites. Although the divergent expression units of pBM93 and pBM126 are terminated by synthetic size-optimized pA sites (pA$_I$, pA$_{II}$), only the EYFP/SEAP expression unit of pBM98 and pBM127 harbor a pA (pA$_I$) site whereas pBM106 and pBM128 are devoid of any pA. Autoregulated expression is kicked off by leaky expression of the bidirectional promoter which produces PIT at regulation-effective concentrations which is either inactivated in the presence of PI or may bind and activate the bidirectional promoter in the absence of regulating streptogramins thereby initiating a positive feed-forward circuit expressing PIT as well as divergently encoded EYFP/SEAP. Following transduction of human HT-1080 and CHO-K1 with pBM93-, pBM98- and pBM106-derived lentiviral particles, streptogramin-responsive EYFP expression could be observed for all lentivectors (Figure 8B and C; Table IV). Furthermore, streptogramin-regulatable SEAP expression was quantified following transduction of pBM126-, pBM127- and pBM128- derived lentiviral particles into K-562 [pBM126, −PI: 345.5 ± 18.5 ng/ml, +PI: 35.1 ± 0.5 ng/ml (induction factor 10); pBM127, −PI: 219.7 ± 9.5 ng/ml, +PI: 27.4 ± 2.1 ng/ml (induction factor 8); pBM128, −PI: 122.3 ± 6.1 ng/ml, +PI, 15.0 ± 0.4 ng/ml (induction factor 8)].

DISCUSSION

In the past decade, viral transduction systems have witnessed dramatic advances including implementation of heterologous
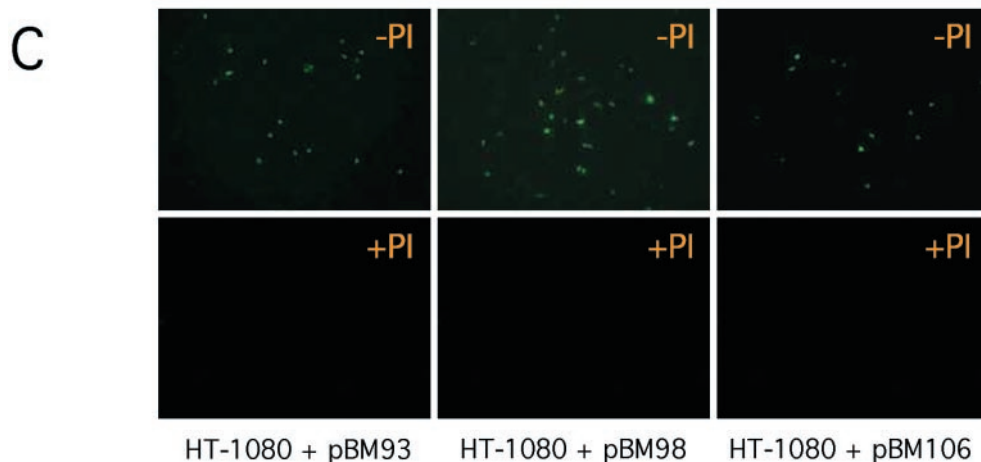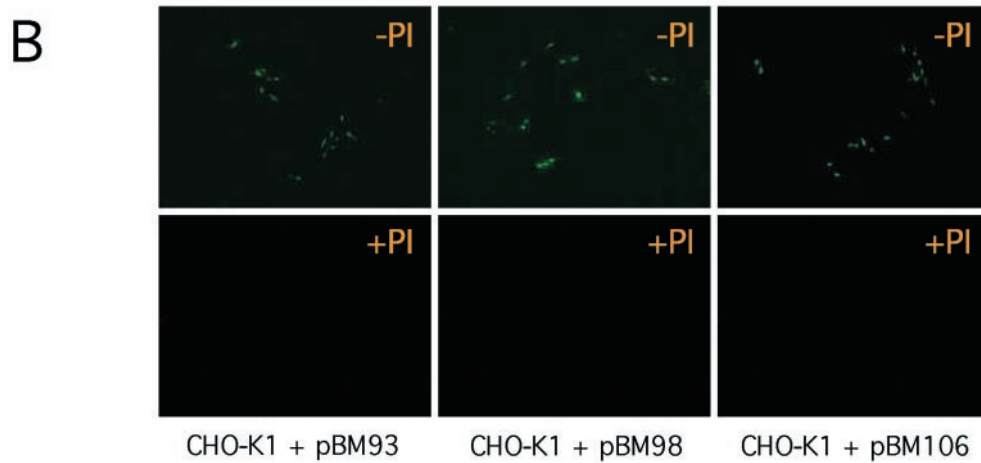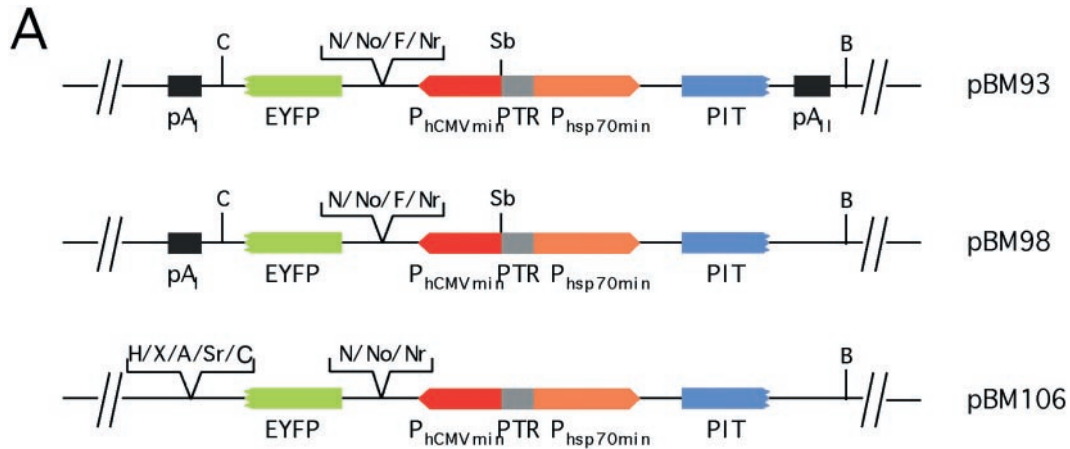
Figure 8. Autoregulated streptogramin-responsive transgene expression following transduction of a single lentiviral particle type into CHO-K1 and human HT-1080 cells. (**A**) Schematic representation of the bidirectional streptogramin-responsive autoregulated EYFP expression units of lentivectors pBM93, pBM98 and pBM106. At the center of these lentivectors resides an asymmetric bidirectional promoter containing a PIT (pristinamycin-dependent transactivator)-specific operator module (PTR) flanked by minimal versions of the human CMV immediate early ($P_{hCMVmin}$) and the *Drosophila* heat-shock protein 70 ($P_{hsp70min}$) promoters. Although PTR-$P_{hsp70min}$ initiates and maintains the autoregulated feedback circuit by driving PIT expression, PTR-$P_{hCMVmin}$ modulates EYFP transcription. pBM93, pBM98 and pBM106 differ in the configuration of their synthetic size-optimized polyadenylation sites (pA_I, pA_{II}). Whereas pBM93 contains two pAs flanking the bidirectional expression unit, only pA_I terminates EYFP transcripts in pBM98 and pBM106 remains pA-free (see caption of Figure 2 for abbreviation of restriction endonucleases). pBM93-, pBM98- and pBM106-derived lentiviral particles were transduced into CHO-K1 (**B**) and HT-1080 cells (**C**) and grown for 48 h in the absence (−) or presence (+) of the regulating streptogramin antibiotic PI prior to microscopical and quantitative analyses of EYFP expression profiles: CHO-K1 (pBM93, −PI: 12.3 ± 1.0, +PI: 0.9 ± 0.3 RLU; pBM98, −PI: 15.6 ± 1.7, +PI: 0.9 ± 0.2 RLU; pBM106, −PI: 9.2 ± 0.2, +PI: 1.0 ± 0.2 RLU), HT-1080 (pBM93, −PI: 11.8 ± 0.8, +PI: 1.3 ± 0.3 RLU; pBM98, −PI: 21.1 ± 1.8, +PI: 1.9 ± 0.1 RLU; pBM106, −PI: 17.4 ± 0.5, +PI: 2.1 ± 0.5 RLU).

transgene modulation (25,39–48). For lentivirus-based transduction arrangements, systems responsive to tetracycline antibiotics (the TET systems) have been most popular and were used in the following basic/optimized configurations: (i) classic TET_{OFF} system (24,49–54), (ii) reverse tetracycline-dependent TET_{ON} system (55–59) and (iii) the TetR-based TET_{ON} technology (14,60,61). Lentiviral transduction systems have been engineered for tetracycline-responsive gene expression to (i) enable construction of stable helper cell lines producing pseudotyping VSV-G in a timely manner and/or at subtoxic doses (15,52,62), (ii) design chimeric adeno-lentiviral vectors which produce lentiviral particles from transgenic lentiviruses in a tetracycline-responsive manner (57), (iii) generate conditionally replicating HIV-based vectors for vaccination (56,59), (iv) mediate tetracycline-inducible/repressible transgene expression (24,50,51,53,54,58,60,63) and (v) modulate siRNA-based knockdown of endogenous genes (14,63).

Pioneering tetracycline-responsive expression configurations were based on two separate vectors one of which included a constitutive tetracycline-dependent transactivator and the other contained a tetracycline-responsive promoter-driven transgene expression unit engineered into a non-SIN lentiviral backbone. Owing to their non-SIN structure first-generation transgene-controlled lentivectors exhibited increased leakiness resulting from LTR-associated enhancer interference with tetracycline-responsive promoters (50–52). SIN-based tetracycline-responsive lentivectors containing an enhancer deletion in the 3′ $LTR_{\Delta U3}$ provided improved induction factors based on interference-free repression.

Following the proven design concept of the tetracycline-responsive expression technology, we have recently developed a human-compatible mammalian transcription control system responsive to clinically licensed streptogramin antibiotics (26). This so-called PIP system included several inducible/repressible configurations all of which showed excellent regulation performance in a variety of different cell lines and human primary cells as well as in mice (26–28,31, 42,64). Also, PIP systems were fully compatible with TET and other antibiotic-adjustable transcription control systems and could be used for differential fine-tuning of different transgene activities (28,31,42). Following configuration into a lentiviral expression context, the PIP system showed excellent streptogramin-responsive transgene regulation performance, including tight repression, high-level expression, reversibility and adjustability in a variety of mammalian/human cell lines and primary cells. Efficiency of the streptogramin-responsive lentivector design was exemplified by differential differentiation reprogramming of muscle cells into fat or bone phenotypes as well as conditional vascularization of chicken embryos. Comparative regulation profiling of isogenic lentivectors either encoding a direct P_{PIR8}-transgene expression unit or harboring P_{PIR8} within a SIN-modified 3′$LTR_{\Delta U3}$ revealed the former configuration to enable almost two orders of magnitude higher transgene expression levels at comparable induction factors. Direct comparison of isogenic HIV- and EIAV-derived lentiviral vectors engineered for direct P_{PIR8}-EYFP expression suggested lower EIAV-mediated transduction rates although both systems provided excellent regulation performance in the CHO-K1-derived cell lines. Although outperformed by HIV-derived lentivectors at first sight, this pioneering example of regulated transgene expression in non-primate lentiviruses may hold great promise for clinical application because of minimal risk associated with EIAV-based therapeutic interventions.

Owing to the binary nature of currently available transcription control systems, transduction of regulated transgene expression is typically realized by simultaneous co-infection of two separate lentiviral particles, one encoding the antibiotic-responsive transactivator and the second one harboring the transactivator-responsive transgene expression unit. Since co-transduction rates are typically lower and require a higher multiplicity of infection compared to transduction using a single lentiviral particle type design of single lentivector-based strategies for adjustable therapeutic interventions is the current clinical priority. First-generation one-vector-based regulated lentiviral transgene configurations included placement of independent transactivator- and transgene-encoding expression units on the same lentivector (51). However, this configuration mediated significant leaky expression due to combinatorial interference of the enhancers contained in the transactivator-driving promoter and the 3′-LTR. Auto-regulated expression configurations in which transactivator and transgene are co-expressed in a coordinated manner represent the only a priori interference-free regulated expression configuration (42,65). Recently, Ogueta *et al.* (60) have engineered an autoregulatory lentiviral one-vector design by cloning the tetracycline repressor (TetR) and the gene of interest into a dicistronic configuration driven by the tetR-repressible promoter (P_{hCMV}-$tetO_2$-EGFP-IRES-TetR; $tetO_2$, dimeric TetR-specific operator; EGFP, enhanced green fluorescent protein; and IRES, internal ribosome entry site). Although this inducible autoregulated lentiviral expression system enabled excellent regulation performance, the restricted tissue tropism of IRES elements as well as the lag phase required to accumulate repression-effective TetR concentrations may limit some applications. The bidirectional autoregulated cassette presented here addresses aforementioned limitations by enabling streptogramin-responsive transgene expression in the most compact and efficient format.

All of the currently available HIV-1-based expression vectors tailored for adjustable transgene expression in mammalian cells exhibit residual transcription activity under repressed conditions (42,65). Although such basal expression remains an inherent characteristic of any heterologous transcription control system (42,65), leaky expression is accentuated by HIV-1's integration preference for transcriptionally active hot spots, which may interfere with desired transgene control to a significant extent (42,65).

Combination of robust transgene control modalities with latest generation lentiviral transduction systems will further advance molecular interventions for gene therapy and tissue engineering.

ACKNOWLEDGEMENTS

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