Quorum-Sensing-Based Toolbox for Regulatable Transgene and siRNA Expression in Mammalian Cells

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Technologies for regulated expression of multiple transgenes in mammalian cells have gathered momentum for bioengineering, gene therapy, drug discovery, and gene-function analyses. Capitalizing on recently developed mammalian transgene modalities (QuoRex) derived from *Streptomyces coelicolor*, we have designed a flexible and highly compatible expression vector set that enables desired transgene/siRNA control in response to the nontoxic butyrolactone SCB1. The construction-kit-like expression portfolio includes (i) multicistronic (pTRIDENT), (ii) autoregulated, (iii) bidirectional (pBiRex), (iv) oncoretro- and lentiviral transduction, and (v) RNA polymerase II-based siRNA transcription-fine-tuning vectors for straightforward implementation of QuoRex-controlled (trans)gene modulation in mammalian cells.

Introduction

Bacteria are particularly efficient and flexible in their molecular adaptation/response to (micro-) environmental changes (*1*, *2*). For rapid molecular coordination of intra- and interpopulation communication, highly sophisticated global regulatory networks known as quorum-sensing systems have evolved to manage control of growth (*3*), secondary metabolite production (*4*), differentiation (*5*), and pathogenesis (*6*, *7*). In many *Streptomyces* spp. (intra-/inter-) population-wide molecular crosstalk is based on small molecules of the butyrolactone class that diffuse within/among populations, bind to specific receptor molecules, and modulate target regulons in a dose-dependent manner (*3*, *4*, *8*, *9*).

Recent advances in mammalian transgene control design have capitalized on prokaryotic quorum-sensing systems by engineering receptors for small-molecule-adjustable transactivation of transgenic mammalian target promoters (*10*−*12*). Quorum-sensing-derived heterologous transgene-modulating arrangements join a growing family of conditional molecular intervention systems in mammalian cells, including those responsive to immunosuppressive drugs (*13*), antibiotics (*14*−*16*), or hormone analogues (*17*, *18*). QuoRex is accessing a vast number of conditional receptor-operator interactions that can be fine-tuned by nonclinical molecules many of which have a long history of man-bacteria coevolution (*19*).

The *Streptomyces coelicolor*-derived QuoRex technology exemplified quorum-sensing-based transgene regulation in a mammalian configuration (*11*, *12*). QuoRex provided robust high-precision transcription fine-tuning in a variety of mammalian cell lines as well as in mice using nontoxic butyrolactone dosing regimes (*11*, *12*). Because the most basic QuoRex configuration lacks flexibility for straightforward implementation of butyrolactone-adjustable transcription control, we have designed a portfolio of versatile mammalian expression vectors for (i) expression of multiple genes in an operon-like configuration based on track-record pTRIDENT design (*20*−*24*), (ii) autoregulated gene expression in a compact one-vector format, (iii) stoichiometric expression of two transgenes from a single compact bidirectional promoter (pBiRex), (iv) oncoretro-/lentiviral installation of QuoRex control, and (v) conditional siRNA transcription for target-specific translation engineering. We are convinced that the QuoRex-controlled mammalian expression vector systems greatly improve therapeutic, gene-function-focused, and metabolic-engineering-centered molecular interventions in mammalian cells.

Material and Methods

Vector Design. All plasmids and oligonucleotides used in this study are listed in Table 1. Detailed information on plasmid construction is also provided.

Cell Culture, Transfection, and Reporter Gene Assays. Chinese hamster ovary cells (CHO-K1, ATTC CCL-61) were cultivated in FMX-8 medium (Cell Culture Technologies, Zurich, Switzerland) supplemented with 10% FCS (PAN Biotech GmbH, Aidenbach, Germany, lot P231902) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO, cat. no. P4458). CHO-K1 cells were transfected using an optimized protocol based on calcium phosphate. In brief, 12 h prior to transfection, 60 000 CHO-K1 cells were seeded per well of a 24-well plate. A total of 1.2 μg of plasmid DNA per well (in case of cotransfection, equal amounts of each plasmid were used) in 0.5 M $CaCl_2$ (final volume 12 μl) was mixed with 12 μL of phosphate solution (50 mM HEPES, pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4), vortexed for 5 s, and incubated for 25 s prior to addition of 0.4 mL FMX-8

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Table 1. Oligonucleotides and Plasmids Used and Designed in This Study[a]

plasmid/oligo	description and cloning strategy	reference or source
OWW22	5′-gctagaattccgcggaggctggatcgg-3′	(*15*)
OWW49	5′-gatcgacgtctaagatacagactgagcggttttttttcctgcaggtcgagctcggtacccgggtc-3′	(*11*)
OWW50	5′-gacgtcttcgaagttaactctagataagatacagactgagcggttttttttgctagcgttaaccctg cagggaattccaccatgggggtgcccgaacgt-3′	this work
OWW51	5′-gcgcgcatcgattcacctgtcccctctcctgcag-3′	this work
OWW505	5′-ggcccaagcttggggccattgacaaaccgaccgtgc-3′	this work
OWW506	5′-ccggaattccatatgggaagcggtccatggc-3′	this work
pcDNA3.1/V5-His TOPO	mammalian expression vector	Invitrogen, Carlsbad, CA
pEF6/V5-His TOPO	mammalian expression vector	Invitrogen, Carlsbad, CA
pLEGFP-N1	EGFP expression vector	Clontech, Palo Alto, CA
pRevTRE	oncoretroviral expression vector	Clontech, Palo Alto, CA
pRevSCA	Oncoretroviral SCA-encoding expression vector (5′LTR-Ψ^+-SCA-P_{PGK}-neo-3′LTR) (pWW158)	(11)
pLM43	Macrolide-responsive siRNA expression vector (mP_{ETR4}-$siRNA_{GFP}$-mpA; mP_{ETR4}, ETR4-$mP_{hCMVmin}$)	(*55*)
pmCMVsiGFPmpA	Vector containing a modified P_{hCMV} promoter (mP_{hCMV}) and a synthetic minimal polyadenylation site (mpA) and a GFP mRNA-specific siRNA ($siRNA_{GFP}$) (mP_{hCMV}-$siRNA_{GFP}$-mpA)	(26)
pMF242	Constitutive EPO expression vector (P_{hCMV}-EPO-pA)	(13)
pMF365	Lentiviral expression vector (5′LTR-Ψ^+-ori_{SV40}-cPPT-RRE-$P_{hEF1\alpha}$-EYFP-3′$LTR_{\Delta U3}$)	(24)
pWW122	Constitutive SCA expression vector (P_{SV40}-SCA-pA)	(11)
pWW124	QuoRex-controlled SEAP expression vector (P_{SPA}-SEAP-pA)	(11)
pWW125	Macrolide-responsive expression vector (P_{ETR3}-MCS-pA)	(23)
pWW135	QuoRex-controlled SEAP expression vector (P_{SCA}-SEAP-pA)	(11)
pWW156	QuoRex-controlled EPO expression vector (P_{SCA}-EPO-pA)	(11)
pUA44	Macrolide-responsive tricistronic expression vector (P_{ETR3}-EPO-IRES-SAMY-CITE-SEAP-pA)	(23)
pWW134	$P_{hCMVmin}$ was amplified from pRevTRE using primers OWW49/OWW22 and ligated in sense orientation into pcDNA3.1/V5-His TOPO	this work
pWW139	EPO was PCR-amplified from pMF242 using primers OWW50/51 and ligated into pEF6/V5-His TOPO (P_{hCMV}-O_{ScbR}-EPO-pA)	this work
pWW159	O_{ScbR}-EPO was excised from pWW139 by *Xba*I/*Cla*I and ligated into compatible sites (*Nhe*I/*Cla*I) of pWW139 (P_{hCMV}-$(O_{ScbR})_2$-EPO-pA)	this work
pWW160	$(O_{ScbR})_2$-EPO was excised from pWW159 by *Xba*I/*Cla*I and ligated into compatible sites (*Nhe*I/*Cla*I) of pWW159 (P_{hCMV}-$(O_{ScbR})_4$-EPO-pA)	this work
pWW161	$(O_{ScbR})_4$-EPO was excised from pWW160 by *Xba*I/*Cla*I and ligated into compatible sites (*Nhe*I/*Cla*I) of pWW160 (P_{hCMV}-$(O_{ScbR})_8$-EPO-pA)	this work
pWW431	$(O_{ScbR})_8$ was excised from pWW161 by *Aat*II/*Sbf*I and ligated into the corresponding sites (*Aat*II/*Sbf*I) of pLM43 (mP_{SCA}-$siRNA_{GFP}$-mpA; mP_{SCA}, $(O_{ScbR})_8$-$mP_{hCMVmin}$)	this work
pTRIDENT20	Macrolide-responsive tricistronic expression vector (P_{ETR3}-MCSI-IRESI-MCSII-IRESII-MCSIII-pA) (pWW73)	(24)
pTRIDENT24	P_{SCA} was excised from pWW135 by *Ssp*I/*Eco*RI and ligated into the corresponding sites (*Ssp*I/*Eco*RI) of pTRIDENT20 (P_{SCA}-MCSI-IRESI-MCSII-IRESII-MCSIII-pA) (pWW516)	this work
pTRIDENT27	P_{SCA} was excised from pWW156 by *Pvu*I/*Nhe*I and ligated into the corresponding sites (*Pvu*I/*Nhe*I) of pUA44 (P_{SCA}-EPO-IRESI-SAMY-IRESII-SEAP-pA) (pWW527)	this work
pTRIDENT-SCA	SCA was excised from pWW122 and ligated (*Eco*RI/*Hin*dIII) into pWW516 (P_{SCA}-SCA-IRESI-MCSI-IRESII-MCSII-pA) (pWW520)	this work
pBiRex8	Bidirectional vector: pA_I-MCSI → $P_{hCMVmin1}$-ETR-$P_{hCMVmin2}$ → MCSII-pA_{II} (pCF85)	(*38*)
pBiRex13	Bidirectional vector: pA_I-SEAP → $P_{hCMVmin1}$-ETR-$P_{hCMVmin2}$ → EPO-pA_{II} (pCF152)	(*38*)
pBiRex15	P_{SCA} was excised from pWW134 by *Hind*III/*Eco*RI and ligated into the corresponding sites (*Hind*III/*Eco*RI) of pBiRex8 (pA_I-MCSI → $P_{hCMVmin1}$-O_{ScbR}-$P_{hCMVmin2}$ → MCSII-pA_{II}) (pWW524)	this work
pBiRex17	P_{SPA} was PCR-amplified from pWW124 using primers OWW505/OWW506, restricted by *Hind*III/*Nde*I and ligated into the corresponding sites (*Hind*III/*Nde*I) of pBiRex13 (pA_I-SEAP → $P_{hCMVmin1}$-O_{SpbR}-$P_{hCMVmin2}$ → EPO-pA_{II}) (pWW528)	this work
pLentiSCA	SCA was excised from pWW122 by *Not*I/*Xba*I and ligated into the corresponding sites (*Not*I/*Xba*I) of pMF365 (5′LTR-Ψ^+-ori_{SV40}-cPPT-RRE-$P_{hEF1\alpha}$-SCA-3′$LTR_{\Delta U3}$) (pWW435)	this work
pQuoRex	P_{SCA} was excised from pWW135 by *Ssp*I/*Eco*RI and ligated into the corresponding sites (*Ssp*I/*Eco*RI) of pWW125 (P_{SCA}-MCS-pA) (pWW515)	this work

[a] Abbreviations: 3′LTR, 3′ long terminal repeat; $3'LTR_{\Delta U3}$, 3′ long terminal repeat devoid of the enhancer; 5′LTR, 5′ long terminal repeat; CITE, cap-independent translation enhancer of the encephalomyocarditis virus; cPPT, central polypurine tract; EPO, erythropoietin; ETR, operator module specific for MphR(A) and macrolide-dependent transactivators; ETR4, quadruplex ETR tandem repeats; EYFP, enhanced yellow fluorescent protein; IRES, internal ribosome entry site of polioviral origin; MCS, multiple cloning site; mP_{ETR4}, modified macrolide-responsive promoter (ETR4-$mP_{hCMVmin}$); mP_{hCMV}, modified P_{hCMV}; $mP_{hCMVmin}$, minimal mP_{hCMV} variant; mP_{SCA}, modified P_{SCA} ($(O_{ScbR})_8$-$mP_{hCMVmin}$); $P_{hCMVmin}$, minimal variant of P_{hCMV}; ori_{SV40}; simian virus 40-derived origin of replication; O_{ScbR}, ScbR-specific operator module; $(O_{ScbR})_n$, tandem O_{ScbR} operator modules; neo, G418 resistance-conferring gene; pA, simian virus 40-derived polyadenylation site; pA_I/pA_{II}, synthetic polyadenylation site; P_{ETR3}, macrolide-responsive promoter (ETR-$P_{hCMVmin}$); P_{hCMV}, human cytomegalovirus immediate early promoter; $P_{hEF1\alpha}$, promoter of the human elongation factor 1 alpha; P_{PGK}, promoter of the phosphoglycerate kinase; P_{SCA}, butyrolactone-responsive SCA-dependent promoter; P_{SPA}, butyrolactone-responsive SPA-dependent promoter; P_{SV40}, simian virus 40-derived promoter; Rev, specific for nuclear export of viral RNA; RRE, Rev response element; SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase; SCA, *Streptomyces coelicolor*-derived ScbR-based transactivator (ScbR-VP16); ScbR, butyrolactone-responsive quorum-sensing receptor of *Streptomyces coelicolor*; SEAP, human secreted alkaline phosphatase; siRNA, short interfering RNA; $siRNA_{GFP}$, GFP mRNA-specific siRNA; SPA, *Streptomyces pristinaespiralis*-derived SpbR-based transactivator (SpbR-VP16); SpbR, butyrolactone-responsive quorum-sensing receptor of *Streptomyces pristinaespiralis*; VP16, *Herpes simplex* virus-derived transactivation domain; Δ^+, extended lentiviral packaging signal

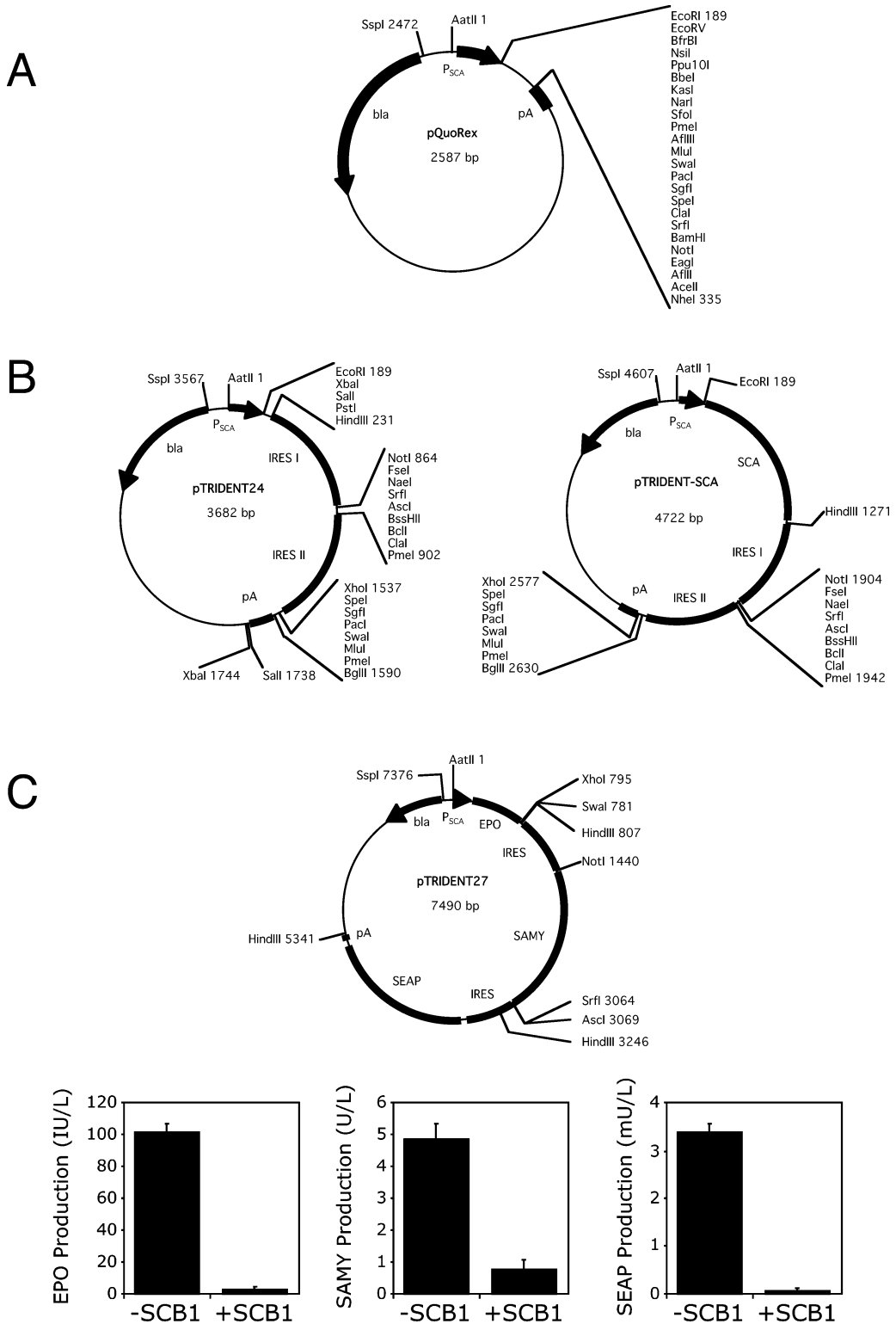

Figure 1. QuoRex-controlled mammalian expression vectors. (A) Basic butyrolactone-responsive multipurpose expression vector pQuoRex containing an extensive polylinker including several sites for rare-cutting 8 bp-recognizing restriction endonucleases for complication-free insertion of desired transgenes. (B) Multicistronic expression vectors pTRIDENT24 and pTRIDENT-SCA enable coordinated/autoregulated expression of several transgenes. pTRIDENT24 mediates cocistronic expression of up to three genes under control of the butyrolactone-responsive P_{SCA} promoter. Whereas the first cistron is translated in a classical cap-dependent manner, translation-initiation of subsequent cistrons requires cap-independent IRES-mediated translation-initiation. pTRIDENT-SCA, a pTRIDENT24 derivative encoding the butyrolactone-dependent transactivator SCA by default provides autoregulated control of up to two transgenes inserted as cistrons two and three. Leaky transcripts resulting in undetectable SCA quantities enable butyrolactone-responsive kick-off of an autoregulated expression circuit resulting in high-level SCA expression and cocistronically encoded transgenes only in the absence of regulating molecules. Expression from the autoregulated unit is gradually reduced to complete repression in the presence of regulation-effective SCB1 concentrations. (C) P_{SCA}-controlled tricistronic EPO, SAMY, and SEAP expression profiling of CHO-K1 cotransfected with pTRIDENT27 and SCA-encoding pWW122 followed by cultivation for 48 h in the presence and absence of SCB1 (±SCB1). Abbreviations: bla, β-lactamase conferring ampicilin resistance in prokaryotes; IU/L: international units per liter; U/L, units per liter. See caption of Table 1 for genetic determinants.

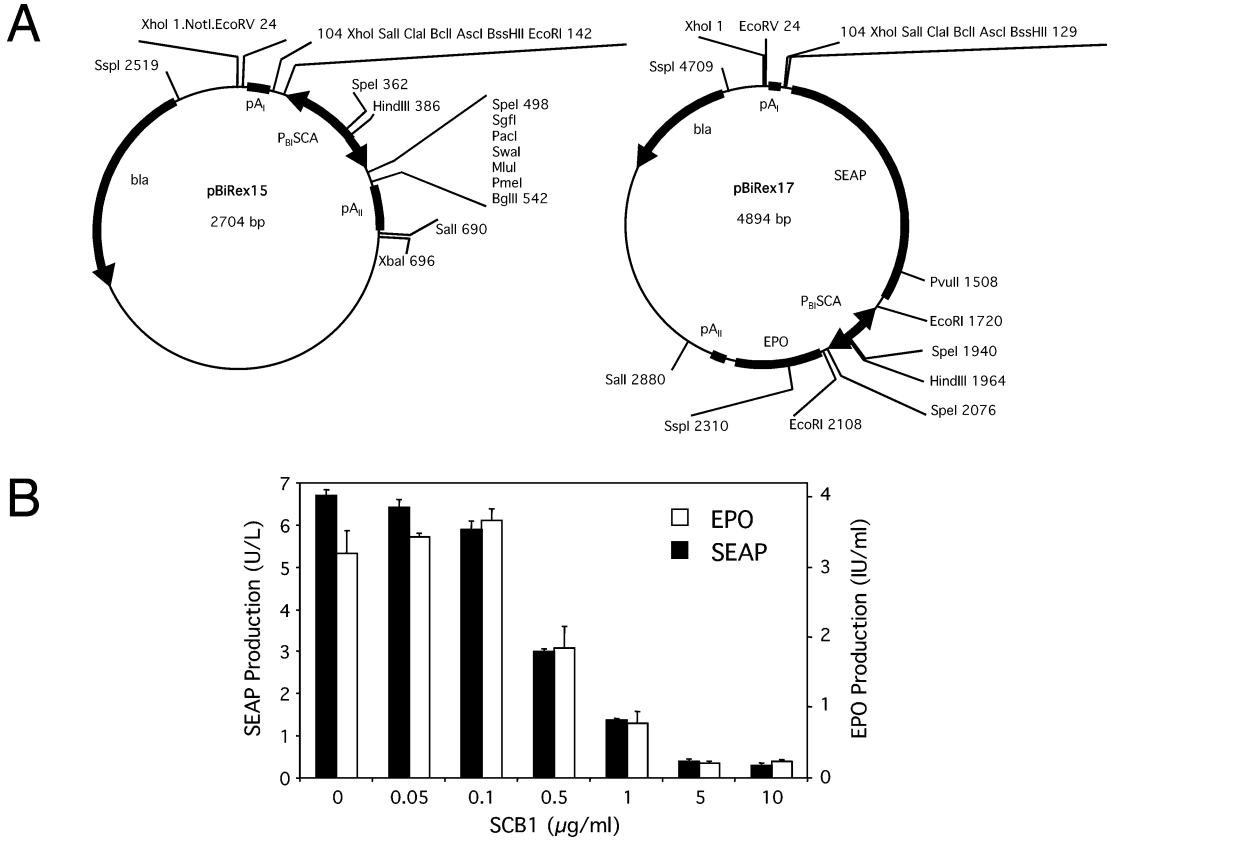

Figure 2. Bidirectional butyrolactone-responsive expression vector for stoichiometric expression of two genes. (A) The bidirectional expression vector pBiRex15 harbors the promoter $P_{BI}SCA$ consisting of a central O_{ScbR} operator site flanked by two divergent minimal human cytomegalovirus-derived promoters ($P_{hCMVmin}$). $P_{BI}SCA$-driven expression units were equipped with two multiple cloning sites as well as two minimal synthetic polyadenylation sites. pBiRex16 encodes bidirectional $P_{BI}SCA$-driven SEAP and EPO expression. (B) SCB-1-adjustable pBiRex16-based bidirectional expression. CHO-K1 cells were cotransfected with pBiRex16 and the SCA expression vector (pWW122), cultivated for 48 h at different SCB1 doses, and profiled for SEAP and EPO production. See captions of Figure 1 and Table 1 for abbreviations.

medium containing 2% (v/v) FCS. The culture medium was replaced by the transfection mixture, and cells were incubated for 5 h prior to performing a 30 s glycerol shock (0.5 mL, 15% (v/v) glycerol in FMX-8 medium containing 2% (v/v) FCS) and one washing step (1 mL FMX-8 + 10% FCS). The cells were cultivated in the presence and absence of the regulating butyrolactone for 48 h prior to quantification of reporter gene expression. Neomycin-resistant CHO-K1 derivatives were selected in the presence of 400 μg/mL G418.

Oncoretro- and lentiviral particles were produced by transfection of GP-293 (*22*) and HEK 293T (*25*) cells with desired expression and appropriate helper plasmids (VSV-G-based pseudotyping) following a previously established protocol (*22*, *25*).

Expression of the human model glycoprotein SEAP was quantified by heat inactivating nonspecific phosphatases in the cell culture supernatant at 65 °C for 30 min prior to scoring of SEAP-catalyzed hydrolysis of *p*-nitrophenyl phosphate at 405 nm and 37 °C in a reaction mix containing 10 mM homoarginine, 0.5 mM $MgCl_2$, 6 mM *p*-nitrophenyl phosphate, and 11.5% diethanolamine/HCl, pH 9.8. SEAP activity was calculated according to the Lambert−Beer law using the specific absorption coefficient for the reaction product *p*-nitrophenolate (18 600 M^{-1} cm^{-1}). The *Bacillus stearothermophilus*-derived secreted α-amylase SAMY was quantified by measuring SAMY-catalyzed starch hydrolyzation using the Phadebas amylase kit (Pharmacia-Upjohn, Uppsala, Sweden, cat. no. 10-5380-32) at 70 °C as detailed by Schlatter and co-workers (*26*). EPO production was quantified using a Quantikine EPO ELISA kit according to the supplier's protocol (R&D Systems, Minneapolis, MN). EGFP production was assessed by FACS-mediated single-cell analysis using a Becton Dickinson FACStar Plus and standardized to pmCMVsiGFPmpA-mediated repression (*27*).

Regulating Butyrolactone. The racemic butyrolactone inducer 2-(6′-methyl-1′-hydroxyheptyl)-3-(hydroxymethyl)-butanolide (IM-2-type SCB1), referred to as SCB1 throughout this article, was synthesized using an optimized multistep reaction protocol (*12*). SCB1 was diluted from a stock solution of 10 mg/mL in DMSO and used at final concentrations of 10 μg/mL unless stated otherwise.

Results and Discussion

Versatile and Multicistronic Butyrolactone-Responsive Expression Vectors. In its most basic configuration the mammalian QuoRex design consists of the butyrolactone-responsive transactivator (SCA; ScbR-VP16) and the cognate promoter (P_{SCA}) assembled by juxtaposition of an SCA-specific operator module O_{ScbR} to the minimal human cytomegalovirus immediate early promoter, $P_{hCMVmin}$. Although this generic design provided excellent regulation performance in a variety of mammalian cell types and in mice, its molecular implementation in a single QuoRex-controlled transgene expression vector set limits the flexibility of QuoRex for other transgene-cell phenotype configurations (*11*, *12*). In order for QuoRex to be operational at the forefront of next-generation functional genomic research, target validation, and tissue engineering and gene therapy initiatives

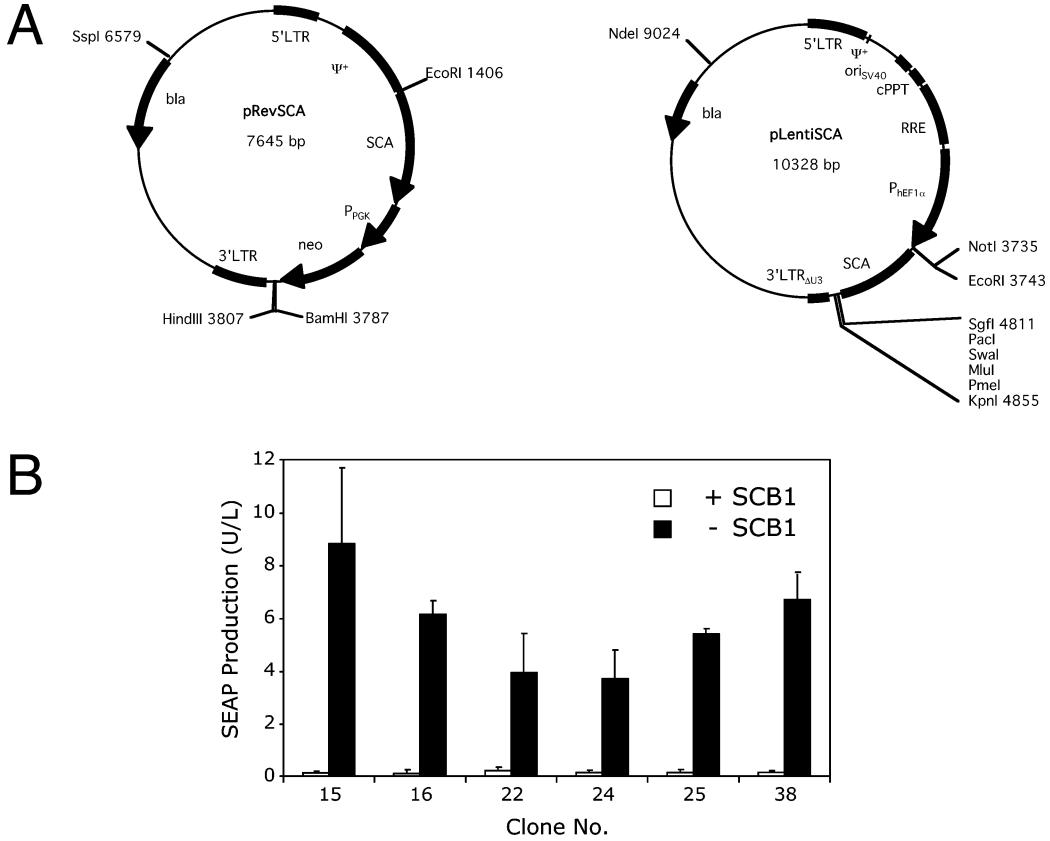

Figure 3. Oncoretro- and lentiviral vectors for transduction of SCA-expression units into mitotically active and proliferation-inert cell phenotypes. (A) The oncoretroviral vector pRevSCA encodes 5′LTR-driven SCA as well as P_{PGK}-controlled G418 resistance-conferring gene. The third-generation self-inactivating lentiviral vector pLentiSCA expresses SCA under $P_{hEF1\alpha}$ control. See caption of Table 1 for abbreviations. (B) Analysis of individual cell clones stably transduced with pRevSCA-derived retroviral particles. Following transduction of CHO-K1 with pRevSCA-derived retroviral particles, selection for G418 resistance and single-cell cloning individual clones were transfected with a P_{SPA}-driven SEAP expression unit and cultivated for 48 h in the presence and absence of SCB1 prior to SEAP quantification.

(*13*, *28*−*32*), we have designed a molecular construction kit of P_{SCA}-containing expression vectors equipped with extensive multiple cloning sites for straightforward assembly of up to tricistronic expression units (Figure 1A).

pQuoRex features a butyrolactone-responsive promoter P_{SCA} followed by 24 unique sites for restriction endonucleases for efficient target gene transfer. Furthermore, QuoRex was engineered into multicistronic pTRIDENT-based (*20*, *23*, *24*) vectors for coordinated expression of several target genes driven by a single P_{SCA} promoter to enable sophisticated biopharmaceutical manufacturing (*33*), multiregulated gene network engineering (*34*), or multigene-based therapeutic interventions (*35*). In multicistronic expression units a single promoter controls transcription of a multicistronic mRNA. Cistrons two and three are preceded by internal ribosome entry sites (IRES), which mediate ribosome assembly and translation initiation in a 5′cap-independent manner (*36*). QuoRex-controlled multicistronic expression units exemplified by pTRIDENT24 are shown in Figure 1B. pTRIDENT's multicistronic expression unit harbors a butyrolactone-responsive promoter (P_{SCA}) and two polioviral IRES elements, each of which is flanked by extensive multiple cloning sites for ease of integration of transgenes one, two, and three (Figure 1B). All multiple cloning sites are compatible with the ever-growing pTRIDENT multicistronic expression vector platform and enable one-step swapping of key modules (*20*−*24*).

Multicistronic expression units can also be engineered for autoregulated expression of several transgenes in a one-vector format (*37*). Autoregulated QuoRex-controlled vectors contain a P_{SCA}-driven expression unit that also encodes the butyrolactone-dependent transactivator as well as desired transgenes (Figure 1B). P_{SCA}-driven leaky expression results in the production of undetectable SCA amounts that can either be inactivated by addition of regulating butyrolactone doses or kick off the autoregulatory expression circuit by binding and activating P_{SCA} under butyrolactone-free conditions. This results in ongoing expression of SCA as well as cocistronically encoded transgenes. pTRIDENT-SCA exemplifies autoregulated multicistronic expression and provides a generic platform for QuoRex-controlled multicistronic transgene expression in a compact one-vector format (Figure 1B). Autoregulated expression of SCA from pTRIDENT-SCA was validated by cotransfection with an expression vector encoding a P_{SPA}-controlled SEAP (human placental secreted alkaline phosphatase) expression unit (pWW124). In the absence of regulating SCB1 high-level SEAP expression (38.3 ± 0.3 U/L) was observed, whereas SCB1 addition resulted in background SEAP levels (1.5 ± 0.1 U/L).

Butyrolactone-responsive tricistronic transgene expression was validated by construction of pTRIDENT27 (Figure 1C), which encodes the glycohormone erythropoietin (EPO), *Bacillus stearothermophilus*-derived α-amylase (SAMY), and SEAP in cistrons one to three, respectively. Transfection of pTRIDENT27 into Chinese hamster ovary cells resulted in high-level expression of all three secreted transgenes in the absence of regulating substances while those genes were coordinatedly repressed

to near detection limits following addition of regulating doses of the butyrolactone SCB1 (Figure 1C).

Bidirectional Butyrolactone-Responsive Expression Units. Despite the success of operon-like multicistronic expression units the pTRIDENT design may (i) become size-restricted as a result of extensive *cis*-acting IRES elements, (ii) suffer from IRES-based transgene interferences (*38*), (iii) be compromised by IRES-cell phenotype incompatibilities (*39*), and/or (iv) limited by position effects that impact on multicistronic transcript stability (*38*).

As an alternative to multicistronic expression, we designed a bidirectional QuoRex-controlled promoter (P_{BI}-SCA), which co-fine-tunes expression of two transgenes. P_{BI}SCA was constructed by flanking a central O_{ScbR} operator module by two divergently oriented minimal versions of the human cytomegalovirus immediate early promoter ($P_{hCMVmin}$) (*39*). Following SCA binding to O_{ScbR}, divergent expression units are cotranscribed in a SCB1-adjustable manner (Figure 2A). The bidirectional expression vector pBiRex15 contains two extensive multiple cloning sites for trouble-free sequential integration of two desired transgenes, e.g., (i) two unlinked genes of interest, (ii) two subunit-encoding determinants, (iii) one transgene and one reporter gene for accurate dosing assessment, or (iv) one transgene and SCA for autoregulated expression control. The bidirectional expression concept was validated by construction of pBiRex17 encoding P_{BI}SCA-driven EPO and SEAP expression units. Cotransfection of pBiRex17 with the constitutive SCA expression vector (pWW122) into CHO-K1 cells resulted in high-level production of both (model) product proteins in butyrolactone-free medium, which could be gradually decreased to near-complete repression following addition of increasing SCB1 doses (Figure 2B).

Oncoretro- and Lentiviral Vectors for Rapid Installation of Butyrolactone-Responsive Gene Expression. Retroviral vectors are valuable tools for efficient transfer of desired transgenes into difficult-to-transfect cell phenotypes including primary cells or tissues (*25*). For straightforward installation of QuoRex-controlled gene expression in those cell types we have designed a murine stem cell virus-derived (*40*) oncoretroviral vector (pRevSCA) engineered for constitutive SCA expression driven by the 5′long terminal repeat (5′LTR) (Figure 3A). pRevSCA-derived oncoretroviral particles are replication-incompetent as a result of deletion of *gag*, *pol*, and *env* genes and thus compatible with highest safety standards. For simplified selection of stable SCA-expressing cell lines, pRevSCA harbors a separate neomycin-resistance-conferring unit driven by the phosphoglycerate kinase promoter. Because oncoretroviral transduction remains limited to mitotically active cells, we also provide a HIV-1-derived third-generation self-inactivating lentivector (pLentiSCA), which encodes SCA under control of the human elongation factor 1 alpha promoter ($P_{hEF1\alpha}$) and could be used to transduce proliferation as well as mitotically inert cell phenotypes (Figure 3A) (*25*). Functionality of the oncoretroviral pRevSCA vector also harboring a neomycin-resistance determinant was validated by transduction of CHO-K1 cells followed by selection of neomycin-resistant cells. The mixed stable population was cloned, and individual clones were assessed for SCA expression by transfection of a P_{SPA}-controlled SEAP expression vector (pWW124) followed by SEAP quantification in cell cultures grown in the presence and absence of SCB1. The expression pattern of six representative clones is shown in Figure 3B,

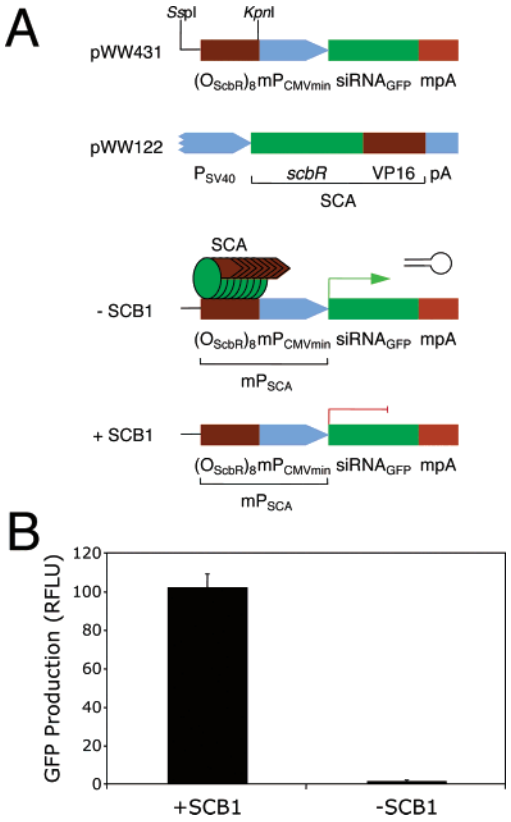

Figure 4. QuoRex-controlled RNA polymerase II-based siRNA transcription. (A) In the absence of SCB1 (−SCB1) SCA binds to the octameric operator module $(O_{ScbR})_8$ and activates siRNA$_{GFP}$ transcription from $P_{mCMVmin}$, which results in specific interference with GFP-encoding transcripts. In the presence of SCB1 (+SCB1), SCA binding and siRNA$_{GFP}$ transcription are prevented and GFP-mediated fluorescence can be observed. (B) SCB1-regulated siRNA expression. CHO-K1 cells were cotransfected with pWW122, pWW431, and the EGFP expression vector pLEGFP-N1, cultivated for 48 h in SCB-1-containing/-free media and assessed for green fluorescence (RFLU, relative fluorescence units) See caption of Table 1 for further abbreviations.

confirming pRevSCA's potential for rapid construction of stable SCA-expressing cell lines.

The lentiviral vector pLentiSCA designed for in vivo applications and therefore devoid of an antibiotic resistance determinant was validated by transduction of CHO-K1 cells with pLentiSCA-based viral particles. SCA expression was assessed by subsequent transfection of pLentiSCA-transgenic cells with the P_{SPA}-controlled SEAP expression vector pWW124 followed by SEAP quantification of cultures grown for 48 h in the presence and absence of SCB1 (−SCB1, 4.6 ± 1.1 U/L; +SCB1, 1.5 ± 0.1 U/L).

Regulatable Expression of Short Interfering RNAs. In recent years, short interfering RNAs (siRNA) have gathered momentum for sequence-specific knockdown of mammalian cell transcripts (*41*). This technology has significantly advanced (i) functional genomics (*8*), (ii) drug target validation (*42*), (iii) therapeutic interventions for treatment of dominantly inherited diseases (*43*), and (iv) rational differentiation reprogramming of embryonic stem cells (*44*).

Today, technology for adjustable transcription of siRNAs is still in its infancy and based on special RNA polymerase III promoters that are incompatible with the standard (tissue-specific) expression vectors/units, most of which rely on RNA type II polymerases for transcription (*45*, *46*). Recent development of RNA polymerase II-

driven siRNA expression fostered the design of QuoRex-controlled transcription of desired siRNAs (*27*). The human cytomegalovirus immediate early promoter modified for constitutive siRNA transcription (mP_{hCMV}) was adapted for QuoRex control by replacing mP_{hCMV}'s enhancer by an octameric SCA-specific operator module ($O_{ScbR})_8$ (mP_{SCA}) (Figure 4A). In the absence of regulating SCB1, SCA binds to ($O_{ScbR})_8$ and transactivates transcription of desired siRNAs, resulting in knockdown of a specific target gene. However, in the presence of SCB1 SCA-($O_{ScbR})_8$ binding and thus siRNA transcription is prevented, which enables high-level transgene expression.

For validation of QuoRex-controlled siRNA expression we designed pWW431 harboring a GFP transcript-specific siRNA ($siRNA_{GFP}$) driven by the modified QuoRex-controlled mP_{SCA} (Figure 4A). Cotransfection of pWW431 and the SCA-encoding pWW122 into EGFP-producing CHO-K1 cells resulted in high-level green fluorescence in the presence of SCB1 ($siRNA_{GFP}$ transcription repressed) and quasi-undetectable EGFP production in butyrolactone-free culture medium ($siRNA_{GFP}$ transcription maximally induced (Figure 4B)).

Conclusion

The QuoRex-controlled construction kit including (i) multicistronic, (ii) autoregulated, (iii) bidirectional, (iv) onco-/lentiviral, and (v) RNA polymerase II-driven siRNA expression vectors enables high-precision fine-tuning of transgene transcription/translation using quorum-sensing butyrolactones. For maximum flexibility and module swapping all QuoRex-controlled vectors are equipped with extensive multiple cloning sites that are mutually compatible and attuned to previously reported expression configurations (*21*−*24*, *39*, *47*−*49*). The QuoRex-controlled expression vector portfolio provides bioengineers with a molecular toolbox for the development of next-generation metabolic engineering (*33*, *50*), drug discovery (*28*), prototype gene therapy and tissue engineering initiatives (*13*, *51*−*53*), sophisticated gene-function analyses (*54*), design of complex human-disease-mimicking animal models (*29*), or therapeutic gene network engineering (*34*, *37*).

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