Intronically encoded siRNAs improve dynamic range of mammalian gene regulation systems and toggle switch

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ABSTRACT

Applications of conditional gene expression, whether for therapeutic or basic research purposes, are increasingly requiring mammalian gene control systems that exhibit far tighter control properties. While numerous approaches have been used to improve the widely used Tet-regulatory system, many applications, particularly with respect to the engineering of synthetic gene networks, will require a broader range of tightly performing gene control systems. Here, a generically applicable approach is described that utilizes intronically encoded siRNA on the relevant transregulator construct, and siRNA sequence-specific tags on the reporter construct, to minimize basal gene activity in the off-state of a range of common gene control systems. To demonstrate tight control of residual expression the approach was successfully used to conditionally express the toxic proteins RipDD and Linamarase. The intronic siRNA concept was also extended to create a new generation of compact, single-vector, autoinducible siRNA vectors. Finally, using improved regulation systems a mammalian epigenetic toggle switch was engineered that exhibited superior *in vitro* and *in vivo* induction characteristics in mice compared to the equivalent non-intronic system.

INTRODUCTION

An increasing number of therapeutic applications, as well as basic research pursuits, require the highly controlled expression of heterologous genes in a mammalian biological setting (1). Nowhere is this more evident, than in the emerging field of synthetic biology where the modular linking of gene control components has already enabled the creation of many sophisticated functional devices such as an epigenetic toggle switch (2), hysteretic switch (3), logic gates (4,5) and time–delay circuits (6,7) amongst others. However, the creation of further devices, or at least ones with more stringent properties, is still largely dependent upon the underlying gene control systems that are employed. For this reason, the search for new forms of mammalian gene control, and the step-wise improvement of existing systems, remains a valuable pursuit for the synthetic biology community.

Typical mammalian heterologous transcription control systems consist of a DNA-binding protein (usually a bacterial response regulator) fused to an eukaryotic transcriptional regulator such as the *Herpes simplex* virus VP16 transactivation domain, or the KRAB (Kruppel-associated box protein) transsilencing domain (8–10). Binding of the DNA-binding protein to its cognate responsive promoter, which is engineered by adjoining the regulator's DNA-binding site to an eukaryotic promoter, is dependent upon the presence or absence of an appropriate effecter molecule (9,11,12). Depending upon the system and genetic architecture employed, the addition of an effecter either turns gene expression on or off (13). The past decade has seen the continuous emergence of such gene regulation systems that are responsive to an ever-increasing range of effecters (14). The earliest systems were based upon bacterial antibiotic response regulators inducible by common antibiotics such as tetracycline (9), streptogramins (11), macrolides (12) and coumermycin/novobiocin (15). Later systems have been based on response regulators sensitive to other signaling molecules, metabolic compounds and drugs. Non-exhaustive examples include systems sensitive to the immunosuppressive drug rapamycin (16), the hormone estrogen (17), quorum-sensing butyrolactones (18), hypoxia (19,20), the metabolite L-arginine (21), 6-hydroxy-nicotine (22), gaseous acetaldehyde (23) and biotin (vitamin H) (7,24) amongst others.

Despite the development of many systems, and their increasing adoption in gene-function studies, an inherent

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problem in most, if not all, mammalian systems is leaky or residual expression when the systems are in their OFF configuration. This is due to a combination of the promoter's basal activity and, or, the random interaction of binding-incompetent transactivators with their cognate operators. Compared to equivalent prokaryotic gene control systems, this leakiness is responsible for the relatively poor induction factor (ratio of maximal to minimal gene expression) exhibited by mammalian systems. Apart from providing a challenge for simple on versus off gene control in gene function studies or conditional therapeutic scenarios, this also impacts the functionality of synthetic gene networks that are based upon these modalities. Given the primary importance of a strong induction factor for many network applications, there is consequently a strong need to develop more tightly regulable gene control systems. Described here is a new approach that is generically applicable to a wide variety of existing gene control systems. It can be used to not only improve the performance characteristics of transcriptional control systems but also of synthetic gene networks that are based upon these systems. Based upon a differential silencing effect that is dependent upon the ratio of siRNA to target we have been able to generically improve the induction profile of many common gene control systems. These systems were subsequently used to successfully conditionally express highly toxic proteins such as the highly efficient apoptosis inducing death domain of the RIP protein (RipDD) (25,26), and Cassava (*M. esculenta*) derived linamarase which hydrolyses the otherwise innocuous cyanogenic glucoside substrate linamarin into glucose, acetone and gaseous cyanide thereby mediating efficient cell killing (27,28). In addition, the same approach of intronically encoding an siRNA between a heterologous transactivator, when placed downstream of the transactivator's cognate promoter, can also be used to create simple, single-construct, autoregulated siRNA vectors. Finally, it was shown that improved gene control systems could be used to engineer a much improved epigenetic toggle that exhibited superior induction characteristics *in vitro*, and *in vivo* within mice that had been implanted with encapsulated stable toggle transfected cells.

METHODS

Vector design and construction

All plasmids used in this study as well as their cloning strategies are listed in Table 1.

Cell culture, transfection and construction of stable cell lines

Wild-type Chinese hamster ovary cells (CHO-K1, ATCC CCL 61), together with stable cell line derivatives, were cultivated and transiently transfected using an optimized calcium–phosphate-based method as previously described (29). Human embryonic kidney cells transgenic for the simian virus 40 large T antigen [HEK293-T (30)], African green monkey kidney cells (Cos-7, ATCC CRL-1651), human cervical carcinoma cells (HeLa, ATCC CCL-2) and mouse fibroblast cells (NIH/3T3, ATCC CRL-1658) were all cultivated in Dulbecco's modified Eagle's medium

(DMEM; Invitrogen, Basel Switzerland, Cat. No. 52100-39) supplemented with 10% (v/v) fetal calf serum [Pan Biotech GmbH, Aidenbach Germany, Cat. No. 3302 Lot No. P251110] and 1% (v/v) penicillin/streptomycin solution (Sigma, St Louis USA, Cat. No. P4458). Transient transfection of HEK293-T, Cos-7 and HeLa cultures was performed using a standard calcium–phosphate-based method previously described (31). Transient transfection of NIH/3T3 was performed using FuGENE6 transfection reagent (Roche, Mannheim Germany, Cat. No. 11814443001) according to the manufacturer's instructions. All cells were grown in a humidified 5% CO_2, 37°C incubator. Unless otherwise indicated, all co-transfections were performed in equimolar ratio with reporter gene activity assayed 48 h after transfection. Experiments utilizing the linamarase–linamarin prodrug system were conducted as previously described using T25 flasks (TPP, Trasadingen Switzerland) to prevent evaporation of HCN (27). Linamarin (2-OH-isobutyronitrile-β-D-gluco-pyranoside) was used at final concentration of 750 μg/ml.

The monoclonal CHO-K1-derived stable cell line containing pDG168 and pDG161 was created in a two-step sequential process. First, CHO_{DG168} was created by co-transfecting pDG168 (P_{ETR}ON-TAG$_{Luc}$-Pip-siRNA$_{GFP}$-KRAB-IRES-SEAP-pA) and pSV2*neo* in a 10 : 1 ratio into CHO-K1 followed by a 2-week cultivation in G418-containing media (Calbiochem, La Jolla USA, Cat. No. 345810; final concentration 400 μg/ml). Integration of the pDG168 expression cassette and suitability of erythromycin induction kinetics, in the mixed stable population, was tested by transient co-transfection with pWW43 (P_{SV40}-E-KRAB-pA) followed by erythromycin (EM) dose profiling of SEAP reporter gene expression. The mixed stable cell line CHO_{DG168} was subsequently co-transfected with pDG161 (P_{PIR}ON-TAG$_{GFP}$-E-siRNA$_{Luc}$-KRAB-IRES-pA) and pPUR in a 10 : 1 ratio followed by a 2-week cultivation in G418 and puromycin-containing media (Calbiochem, Israel, Cat. No. 540411; final concentration 6 μg/ml) to yield the mixed double stable cell line $CHO_{DG168/DG161}$. One hundred and sixty single cell clones were subsequently isolated, cultivated and screened for pristinamycin (PI)-repressible and EM-inducible SEAP expression. Of these, a subset of six clones were further profiled for their ability to maintain differential expression upon antibiotic removal. Clone 58 was selected for all further work and designated as $CHO_{TOGGLE2}$.

Regulating antibiotics

Pristinamycin (Sanofi-Aventis Inc., Pyostacin®, Zurich Switzerland), erythromycin (Fluka, Buchs Switzerland) and tetracycline and doxycycline (Sigma Chemicals, St Louis USA, Cat. Nos. T3383 & D9891) were prepared as previously described stock solutions (29).

Quantification of reporter gene expression, cell viability and apoptosis

Production of human placental-secreted alkaline phosphatase (SEAP) was quantified using a *p*-nitrophenylphosphate-based light absorbance kinetic assay as previously described (32,33) with results expressed in units per liter

Table 1. Plasmid constructs used and designed in this study

Plasmid	Genotype and/or cloning strategy	Reference or source
pBP62	P_{PIR}ON-E-KRAB-IRES-pA	(2)
pBP139	P_{ETR}ON1-Pip-KRAB-IRES-SEAP-pA (P_{ETR}ON1, P_{hCMV}-ETR)	(2)
pDG1	P_{SV40}-E-siRNA$_{GFP}$-VP16-pA	(29)
pDG54	P_{hEF1a}-TAG$_{Luc}$-SEAP-pA. TAG$_{Luc}$-SEAP created by PCR amplification from pSEAP2-Control using oligos ODG022 (CTTCGACTGAA<u>CTAGT</u>GCGGCCGC*CCGCTGGAGAGCAACTGCA*gaattcac-caccatgctgc; *Spe*I site underlined, annealing sequence in lower case, TAG$_{Luc}$ italicised) and OLM073 (GTACGGA<u>atc</u>ttatcatgtctgctcgaagcgg; *Eco*RV site underlined, annealing sequence in lower case), digested with *Spe*I/*Eco*RV, and cloned into similarly digested pWW276	This work
pDG97	P_{SV40}-E-siRNA$_{GFP}$-KRAB-pA	(29)
pDG98	P_{SV40}-E-siRNA$_{Luc}$-KRAB-pA. E-siRNA$_{Luc}$ fusion with associated splice recognition sequences PCR amplified from pWW35 using oligos OWW18 (TA<u>cgaattc</u>caccatgccccgccccaagctcaa; *Eco*RI site underlined, annealing sequence in lower case) and ODG45 (CTG<u>GCGCGC</u>GGGCACCTGTGG AGAGAAAGGCAAAGTGGATGTCAGTA*CCGCTGGAGAGCAACTGCA*ATCGAT*TGCAGTTG CTCTCCAGCGG*ACTTACCTGggctgtacgcggacgc; *Bss*HII site underlined, annealing sequence in lower case, siRNA$_{Luc}$ italicised), digested with *Eco*RI/*Bss*HII and cloned into similarly digested pWW43	This work
pDG104	P_{ETR}ON1-TAG$_{Luc}$-SEAP-pA. TAG$_{Luc}$-SEAP excised from pDG54 by *Spe*I/EcoRV digest and cloned into *Spe*I/*Sma*I digested pWW72 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG131	P_{PIR}ON-E-siRNA$_{Luc}$-KRAB-IRES-pA. E-siRNA$_{Luc}$-KRAB excised from pDG98 by *Eco*RI/*Hpa*I digestion and cloned into *Eco*RI/*Nae*I digested pTRIDENT11	This work
pDG143	P_{SV40}-Pip-siRNA$_{GFP}$-VP16-pA	(29)
pDG153	P_{SV40}-TetR-siRNA$_{GFP}$-VP16-pA	(29)
pDG156	P_{hCMV^*-1}-E-VP16-pA. P_{hCMV^*-1} excised from pMF111 by *Ssp*I/*Eco*RI digestion and cloned into similarly digested pWW35	This work
pDG159	P_{hCMV^*-1}-E-siRNA$_{GFP}$-VP16-pA. P_{hCMV^*-1} excised from pMF111 by *Ssp*I/*Eco*RI digestion and cloned into similarly digested pDG1	This work
pDG160	P_{ETR}ON1-TAG$_{Luc}$-Pip-pA. P_{ETR}ON1-TAG$_{Luc}$ created by PCR-amplification from pBP139 using oligos ODG053 (cgacacggaaatgttg, entire sequence annealing) and ODG55 (TCCG<u>GAATTC</u>*TGCAGTTGCTCTCCAGCGG*AGAtctgcaggatatccctaaatg; *Eco*RI site underlined, annealing sequence in lower case, TAG$_{Luc}$ italicised), digested with *Ssp*I/*Eco*RI, and cloned into similarly digested pWW87 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG161	P_{PIR}ON-TAG$_{GFP}$-E-siRNA$_{Luc}$-KRAB-IRES-pA. P_{PIR}ON-TAG$_{GFP}$ created by PCR-amplification from pTRIDENT11 using oligos ODG53 (cgacacggaaatgttg, entire sequence annealing) and ODG54 (TCCG<u>GAATTC</u>*GTAGTTGTACTCCAGCTTGTG*AGATCtcgaaatagcgctgtacag; *Eco*RI site under-lined, annealing sequence in lower case, TAG$_{GFP}$ italicised), digested with *Ssp*I/*Eco*RI and cloned into similarly digested pDG131	This work
pDG163	P_{SV40}-Pip-siRNA$_{GFP}$-KRAB-IRES-pA. siRNA$_{GFP}$-KRAB fusion with associated splice recognition sequences PCR-amplified from pWW43 using oligos ODG56 (CTGG<u>GCGCGC</u>CAGGTAAGT*CA CAAGCTGGAGTACAACTAC*ATCGAT*GTAGTTGTACTCCAGCTTGTG*TACTGACATCCACTT-TGCCTTTCTCTCCACAGGTGcgccagatccaaaaaag; *Bss*HII site underlined, annealing sequence in lower case, siRNA$_{GFP}$ italicised) and ODG57 (atc<u>aagctt</u>ggatccttac; *Hind*III site underlined, entire sequence annealing), digested with *Bss*HII/*Hind*III and cloned into similarly digested pMF167	This work
pDG164	P_{ETR}1-E-VP16-pA. P_{ETR}1 excised from pWW37 by *Ssp*I/*Eco*RI digestion and cloned into similarly digested pWW35 (P_{ETR}1, ETR-$P_{hCMVmin}$)	This work
pDG165	P_{ETR}1-E-siRNA$_{GFP}$-VP16-pA. P_{ETR}1 excised from pWW37 by *Ssp*I/*Eco*RI digestion and cloned into similarly digested pDG1 (P_{ETR}1, ETR-$P_{hCMVmin}$)	This work
pDG167	P_{ETR}ON1-TAG$_{Luc}$-Pip-siRNA$_{GFP}$-KRAB-IRES-pA. Pip-siRNA$_{GFP}$-KRAB-IRES excised from pDG163 by *Eco*RI/*Kpn*I digestion and cloned into similarly digested pDG160 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG168	P_{ETR}ON1-TAG$_{Luc}$-Pip-siRNA$_{GFP}$-KRAB-IRES-SEAP-pA. SEAP excised from pLM177 by *Spe*I/*Mlu*I digestion and cloned into similarly digested pDG167 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG178	P_{hCMV^*-1}-TetR-VP16-pA. TetR excised from pSAM200 by *Eco*RI/*Bss*HII digestion and cloned into similarly digested pDG156	This work
pDG179	P_{hCMV^*-1}-TetR-siRNA$_{GFP}$-VP16-pA. TetR excised from pSAM200 by *Eco*RI/*Bss*HII digestion and cloned into similarly digested pDG159	This work
pDG181	P_{ETR}ON1-GFP-pA. GFP was PCR-amplified from pLEGFP-N1 using oligos ODG62 (GATC<u>GC TAGC</u>TTAAGCTTGGTACCGGATCCgccaccatggtgagc; *Nhe*I site underlined, annealing sequence in lower case) and ODG63 (CTCGA<u>GCGGCCGC</u>CACTGTGCTGGATATCgctttacttgtacagctcgtcc; *Not*I site underlined, annealing sequence in lower case), digested with *Nhe*I/*Not*I and cloned into *Spe*I/*Not*I digested pWW72 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG182	P_{ETR}1-GFP-pA. GFP excised from pDG181 by *Eco*RI/*Not*I digestion and cloned into similarly digested pDG183 (P_{ETR}1, ETR-$P_{hCMVmin}$)	This work
pDG183	P_{ETR}1-TAG$_{GFP}$-SEAP-pA. TAG$_{GFP}$-SEAP excised from pLM65 by *Spe*I/*Not*I digest and cloned into similarly digested pWW125 (P_{ETR}1, ETR-$P_{hCMVmin}$)	This work
pDG191	P_{ETR}ON1-TAG$_{GFP}$-SEAP-pA. TAG$_{GFP}$-SEAP excised from pLM65 by *Spe*I/*Not*I digest and cloned into similarly digested pWW72 (P_{ETR}ON1, P_{hCMV}-ETR)	This work
pDG210	P_{SV40}-rTetR-siRNA$_{GFP}$-VP16-pA. rTetR excised from pTET-ON by *Eco*RI/*Bss*HII digestion and cloned into similarly digested pDG1	This work

(continued)

Table 1. Continued

Plasmid	Genotype and/or cloning strategy	Reference or source
pDG211	P_{hCMV^*-1}-TAG$_{GFP}$-SEAP-pA. P_{hCMV^*-1} excised from pMF111 by *Ssp*I/*Xba*I digestion and cloned into *Ssp*I/*Spe*I digested pDG183	This work
pDG213	P_{PIR}-TAG$_{GFP}$-SEAP-pA. P_{PIR} excised from pMF189 by *Ssp*I/*Spe*I digestion and cloned into similarly prepared pDG183	This work
pDG276	$P_{ETR}2$-TAG$_{GFP}$-SEAP-pA. $P_{hsp70min}$ and TAG$_{GFP}$ excised from pDG213 by *Sbf*I/*BssH*II digestion and cloned into similarly prepared pDG183 ($P_{ETR}2$, ETR-$P_{hsp70min}$)	This work
pDG280	$P_{ETR}ON2$-TAG$_{GFP}$-SEAP-pA. ETR operator module and TAG$_{GFP}$ excised from pDG191 by Hind*III*/Nde*I* digestion and cloned into similarly prepared pLM189 ($P_{ETR}ON2$, P_{PGK}-ETR)	This work
pDG284	$P_{ETR}3$-TAG$_{GFP}$-SEAP-pA. $P_{SV40min}$ PCR amplified from pSEAP2-Control using primers ODG092 (ACCGTACAAGGAGCCTGCAGgcgatctgcatctcaattagtc; *Sbf*I site underlined, annealing sequence in lower case) and ODG093 (ggaggcctaggctttttgcaaaCTAGTCACATGCTGG; *Spe*I site underlined, annealing sequence in lower case), digested with *Sbf*I/*Spe*I and cloned into similarly prepared pDG183 ($P_{ETR}3$, ETR-$P_{SV40min}$)	This work
pDG286	$P_{ETR}1$-TAG$_{GFP}$-Lis-pA. $P_{ETR}1$ and TAG$_{GFP}$ PCR amplified from pDG183 using primers ODG085 (ggaaatgttgaatactcatactcttcc, entire sequence annealing) and ODG094 (cacaagctggagtacaactac GCGCGCCCACCA, *BssH*II site underlined, annealing sequence in lower case), digested with *Aat*II/*BssH*II and cloned into similarly prepared pWW315 ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	This work
pDG287	$P_{ETR}ON1$-TAG$_{GFP}$-Lis-pA. $P_{ETR}ON1$ and TAG$_{GFP}$ excised from pDG290 by *Aat*II/*BssH*II digestion and cloned into similarly prepared pWW315 ($P_{ETR}ON1$, P_{hCMV}-ETR)	This work
pDG289	$P_{ETR}1$-TAG$_{GFP}$-RipDD-pA. $P_{ETR}1$ and TAG$_{GFP}$ PCR amplified from pDG183 using primers ODG085 and ODG094 (see above), digested with *Aat*II/*BssH*II and cloned into similarly prepared pWW326 ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	This work
pDG290	$P_{ETR}ON1$-TAG$_{GFP}$-RipDD-pA. $P_{ETR}ON1$ and TAG$_{GFP}$ PCR amplified from pDG191 using primers ODG085 and ODG094 (see above), digested with *Aat*II/*BssH*II and cloned into similarly prepared pWW326 ($P_{ETR}ON1$, P_{hCMV}-ETR)	This work
pLEGFP-N1	P_{hCMV}-GFP-pA	Clontech
pLM65	$P_{hEF1\alpha}$-TAG$_{GFP}$-SEAP-pA	(35)
pLM177	$P_{hEF1\alpha}$-SEAP-pA	(35)
pLM189	$P_{NIC}ON4$-SEAP-pA ($P_{NIC}ON3$, P_{PGK}-O_{NIC})	(49)
pmCMVmpA	mP_{hCMV}-mpA	(34)
pmCMVsiGFPmpA	mP_{hCMV}-siRNA$_{GFP}$-mpA	(34)
pMF111	P_{hCMV^*-1}-SEAP-pA	(61)
pMF167	P_{SV40}-Pip-VP16-pA	(62)
pMF189	P_{PIR}-MCS-pA	(36)
pPUR	P_{SV40}-Puro^r-pA	Clontech
pSAM200	P_{SV40}-TetR-VP16-pA	(60)
pSEAP2-Control	$P_{SV40min}$-SEAP-pA-E_{SV40}	Clontech
pSV2*neo*	P_{SV40}-Neo^r-pA	Clontech
pTet-ON	P_{hCMV}-rTetR-VP16-pA	Clontech
pTRIDENT11	$P_{PIR}ON$-MCS-IRES-MCS-IRES-MCS-pA	(63)
pWW35	P_{SV40}-E-VP16-pA	(12)
pWW37	P_{ETR}-SEAP-pA ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	(12)
pWW43	P_{SV40}-E-KRAB-pA	(12)
pWW56	$P_{ETR}ON1$-SEAP-pA ($P_{ETR}ON1$, P_{hCMV}-ETR)	(12)
pWW72	$P_{ETR}ON$-MCS-pA ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	(36)
pWW87	$P_{ETR}ON$-Pip-pA ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	(2)
pWW125	P_{ETR}-MCS-pA ($P_{ETR}1$, ETR-$P_{hCMVmin}$)	(36)
pWW276	P_{hEF1a}-VEGF-pA	(64)
pWW315	$P_{hEF1\alpha}$-Lis-pA	(27)
pWW326	$P_{hEF1\alpha}$-RipDD-pA	(27)

E, *E. coli*-derived repressor of the macrolide resistance gene *mphA*; E-KRAB, macrolide-dependent transsilencer; E_{SV40}, SV40 enhancer; ETR, operator sequence specific for E binding; E-VP16, macrolide-dependent transactivator; GFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; KRAB, human kox-1 gene transcriptional silencer; Lis, Cassava (*M. esculenta*) linamarase; Luc, firefly luciferase; MCS, multiple cloning site; mP_{hCMV}, modified P_{hCMV} promoter; mpA, synthetic minimal pA; Neo^r, neomycin (G418) resistance conferring gene; pA, virus-derived polyadenylation site; $P_{ETR}1$-3, macrolide-responsive OFF-type promoters containing a single ETR module upstream of either $P_{hCMVmin}$, $P_{hsp70min}$ or $P_{SV40min}$ respectively; $P_{ETR}ON1$-2, macrolide-responsive ON-type promoters containing eight ETR modules downstream of either P_{SV40} or P_{PGK}, respectively; P_{PGK}, constitutive murine phosphoglycerate kinase promoter; P_{hCMV}, constitutive human cytomegalovirus immediate early promoter; P_{hCMV^*-1}, tetracycline responsive promoter containing seven TetO modules upstream of $P_{hCMVmin}$; $P_{hCMVmin}$, minimal P_{hCMV}; $P_{hEF1\alpha}$, constitutive human elongation factor 1α promoter; P_{hsp70}, constitutive *Drosophila* heat-shock gene *hsp*70 promoter; $P_{hsp70min}$, minimal P_{hsp70}; Pip, *S. coelicolor*-derived repressor of the streptogramin resistance operon; PIR, operator sequence specific for Pip binding; P_{PIR}, streptogramin-responsive OFF-type promoter containing a single PIR module upstream of $P_{hsp70min}$; $P_{PIR}ON$, streptrogramin-responsive ON-type promoter containing three PIR modules downstream of P_{SV40}; P_{SV40}, constitutive simian-40 virus derived promoter with E_{SV40}; $P_{SV40min}$, minimal P_{SV40} promoter without E_{SV40}; Puro^r, puromycin resistance conferring gene; RipDD, human RIP death domain; rTetR, mutated TetR exhibiting reverse binding characteristics to tetracycline; SEAP, human placental secreted alkaline phosphatase; siRNA, short interfering RNA (subscript denotes specificity); TAG, siRNA specific target sequence (subscript denotes specificity); TetO, operator sequence specific for TetR binding; TetR, *E. coli*-derived repressor of the TN*10* tetracycline resistance operon; VP16, *Herpes simplex* virus-derived transcriptional activator.

(U/l) or, where relevant, relative to isogenic control vectors (rU/l). Expression profiling of GFP was performed by FACS analysis of harvested cells using a Cytomics FC500 flow cytometer with Beckman CFP analysis software (Beckman Coulter, CA, USA) set for 488 nm excitation and recording at 525 nm, with results expressed in either fluorescence units (FU) or relative to isogenic control vectors (%).

The total cell number and viability of harvested cells, expressed as a percentage of living cells (%), was determined using a Casy1® cell counter (Schaerfe System, Reutlingen, Germany). Apoptosis profiles were determined after staining of harvested cells using an annexin V-FITC/7-AAD apoptosis assay kit (Beckman Coulter, Marseille France, Cat. No. PN IM3614) used according to the manufacturer's protocol. Dye incorporation and quantification of apoptotic cells, expressed as the percentage of cells positive for either or both of annexin V-FITC or 7-AAD, was also performed using the above flow cytometer and analytical software which was set for 488 nm excitation and recording at 525 nm (FITC) and 655 nm (7-AAD).

Encapsulation and *in vivo* methods

$CHO_{TOGGLE2}$ cells were encapsulated in 400 µm alginate-poly-(L-lysine)-alginate beads (alginate-PLL-alginate; 200 cells per capsule) using an Inotech Encapsulator Research IER-20 (Inotech Encapsulation AG, Dottikon, Switzerland) according to the manufacturer's instructions and the following specific parameters: 0.2 mm nozzle, 405 unit flow rate using 20 ml syringe, 1088 Hz nozzle vibration frequency and 900 V for bead dispersion. Seven hundred microliters MOPS-buffered physiological salt solution (Inotech Encapsulation AG, Dottikon, Switzerland) containing 2×10^6 encapsulated cells were injected intraperitoneally into female OF1 mice (oncins france souche 1; Iffa-Credo, Lyon France). Control mice were injected with encapsulated wild-type CHO-K1 cells. Starting 1 h after injection and continuing daily where required, mice were administered either pristinamycin or erythromycin at final doses of 50 mg/kg. Both pristinamycin and erythromycin were formulated for *in vivo* administration by dilution of stock solutions to appropriate concentrations using a 0.9% (w/v) NaCl solution containing 5% (v/v) ethanol. At required time points, blood was collected retro-orbitally and serum obtained using micro-tainer SST tubes (Becton Dickinson, Plymouth, UK). All experiments involving mice were approved by the French Ministry of Agriculture and Fishery (Paris France) and performed by M.D-E at the Institute Universitaire de Technologie (IUTA), F-69200 Villeurbanne Cedex, France.

RESULTS

Differential siRNA silencing improves dynamic range of transgene control systems

The extent of siRNA-mediated gene-silencing during transient transfection is influenced, amongst other factors, by the relative concentration of siRNA to target. This was demonstrated by testing the silencing efficiency of a
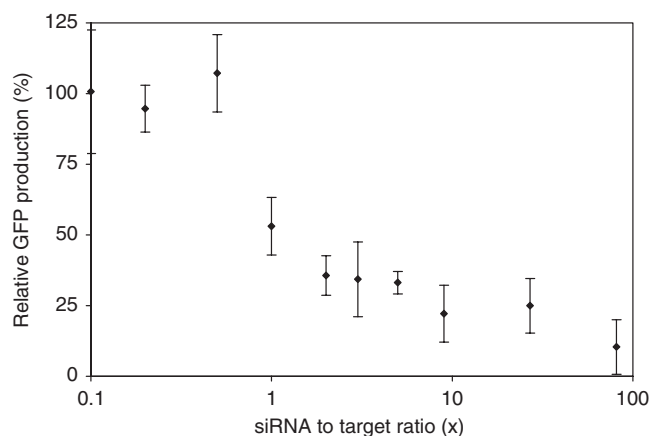
Figure 1. Impact of siRNA to gene target ratio on RNAi-mediated gene silencing. GFP expression of CHO-K1 cells after co-transfection with varying ratios of target GFP (pLEGFP-N1; P_{hCMV}-GFP-pA) and a siRNA directed against GFP (pmCMVsiGFP-mpA; mP_{hCMV}-$siRNA_{GFP}$-mpA). For each ratio, GFP production was assessed relative to equivalent co-transfections of pLEGFP-N1 and an isogenic (non-siRNA containing) control (pmCMVmpA; mP_{hCMV}-mpA).

common GFP-specific siRNA (34), constitutively expressed from its own dedicated promoter (pmCMVsiGFPmpA; mP_{hCMV}-$siRNA_{GFP}$-mpA), at varying siRNA to target (pLEGFP-N1, P_{hCMV}-GFP-pA) ratios in CHO cells (Figure 1). At siRNA to target ratios lower than 1, silencing of GFP was undetectable relative to isogenic control transfections (pmCMVmpA; mP_{hCMV}-mpA). At a 1 to 1 ratio, silencing of ~50% was observed. Increasing ratios thereafter resulted in higher silencing efficiencies with maximal silencing in the range of 80–90% occurring only at ratios greater than 10.

By targeting an siRNA against a reporter gene, itself placed under expression control of a transcription control system, it was speculated that the observed differential silencing efficiency of siRNA could be used to improve the induction ratio of a transcription control system. Assuming siRNA is constitutively produced at a constant level, the relative level of siRNA to reporter mRNA should be much higher in the OFF configuration than in the ON configuration; thus leading to disproportionately higher silencing of residual OFF expression than maximal ON expression. By expressing a functional siRNA as a synthetic intron between the erythromycin (EM)-inducible DNA-binding protein and the relevant transcriptional regulator we sought to test the hypothesis that differential siRNA silencing could be used to improve the induction characteristics of the EM-responsive (E.REX) E_{OFF} and E_{ON} systems (12,29) (Figure 2). Intronically encoded GFP-specific siRNA ($siRNA_{GFP}$), placed within the ET1 (pDG1; P_{SV40}-E-$siRNA_{GFP}$-VP16-pA) and ET4 (pDG97; P_{SV40}-E-$siRNA_{GFP}$-KRAB-pA) transregulators were used to directly target GFP which itself was placed under expression control of either ET1-(pDG182; P_{ETR}1-GFP-pA) or ET4-(pDG181; P_{ETR}ON1-GFP-pA) responsive promoters, respectively. Relative to the native transregulators ET1 (pWW35; P_{SV40}-E-VP16-pA) and ET4 (pWW43; P_{SV40}-E-KRAB-pA), differential siRNA-mediated silencing between the ON and OFF
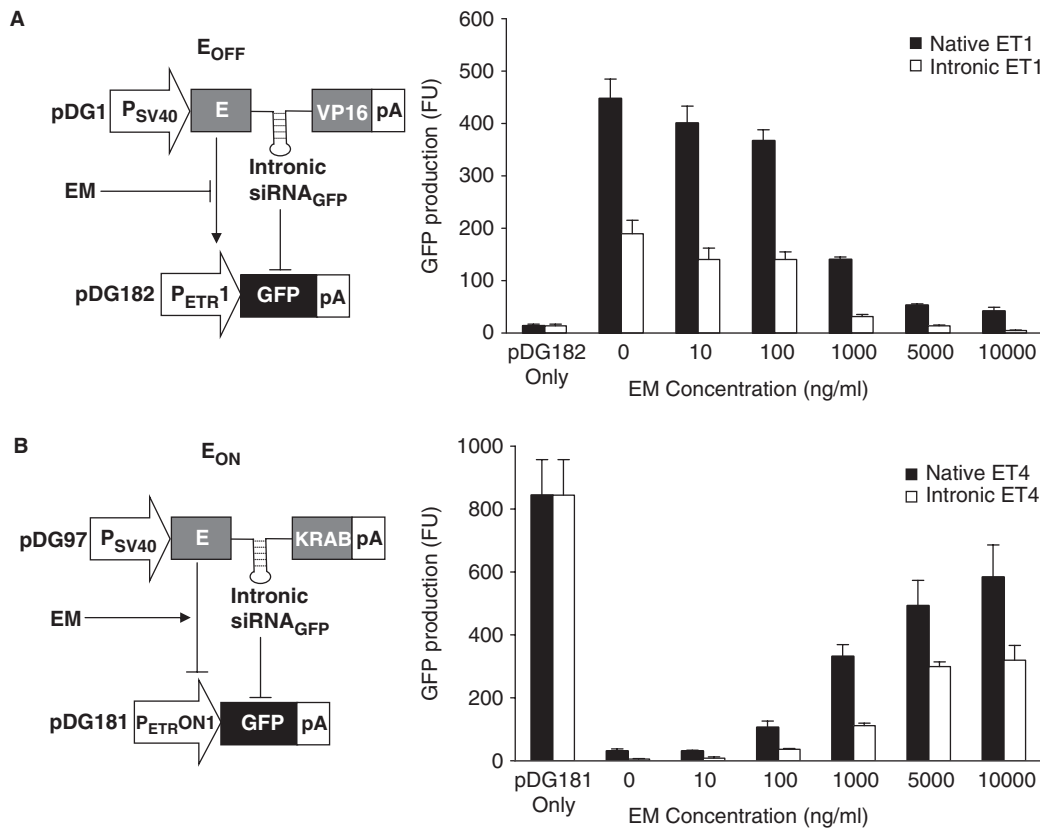
Figure 2. Schematic representation and comparison of E.REX (E_{OFF} and E_{ON}) induction characteristics following inclusion within ET1 (pWW35; P_{SV40}-E-VP16-pA) and ET4 (pWW43; P_{SV40}-E-KRAB-pA) of an intronically encoded siRNA (pDG1; P_{SV40}-E-siRNA$_{GFP}$-VP16-pA and pDG97; P_{SV40}-E-siRNA$_{GFP}$-KRAB-pA, respectively) directed against a GFP reporter gene whose expression was placed under control of the relevant ET1 or ET4 responsive promoter (pDG182; P_{ETR}1-GFP-pA and pDG181; P_{ETR}ON1-GFP-pA, respectively). In each case erythromycin (EM) dose profiles for either the native or intronic siRNA-containing transcriptional regulator ET1 (**A**) or ET4 (**B**) were determined in CHO-KI following co-transfection with the relevant responsive GFP reporter construct. Also shown are expression levels resulting from transfection with just the relevant reporter construct.

configurations improved the induction characteristics of the E_{OFF} and E_{ON} systems from 10- to 39-fold, and 19- to 60-fold, which represents a 4- and 3-fold incremental improvement, respectively.

The system thus described relies upon a specific siRNA targeting a desired reporter gene (in this case GFP). Further application to other reporter genes would require *de novo* design of a reporter gene specific siRNA in each case. To circumvent this issue, and make the system generically applicable to any reporter gene, the siRNA$_{GFP}$ target sequence (TAG) was incorporated into the 5′ untranslated region of another reporter gene, namely SEAP, thereby rendering it capable of siRNA$_{GFP}$ mediated silencing (35) (Figure 3). The performance of the resulting system (For E_{OFF} we used pDG183; P_{ETR}1-TAG$_{GFP}$-SEAP-pA, and for E_{ON} pDG191; P_{ETR}ON1-TAG$_{GFP}$-SEAP-pA) with respect to differential silencing, strong reduction of basal expression, and improvement in induction characteristics (4-fold for ET1 and 3-fold for ET4) was consistent with the improvement observed in the directly targeted reporter system.

To ascertain whether a different intronic siRNA/TAG combination would have the same effect, an E_{ON} system utilizing an intronic siRNA against luciferase (pDG98; P_{SV40}-E-siRNA$_{Luc}$-KRAB-pA) with a corresponding luciferase TAG on the reporter construct was also tested (pDG104; P_{ETR}ON1-TAG$_{Luc}$-SEAP-pA). In direct comparisons of knockdown efficiency, siRNA$_{Luc}$ has been more efficient than siRNA$_{GFP}$ (93 versus 80%) (29). When siRNA$_{Luc}$ was used within the E_{ON} system in transient co-transfections of CHO-K1, maximal and minimal SEAP reporter expression in the presence and absence, respectively, of erythromycin were 6.4 U/l ($\pm$0.4) and 0.06 U/l ($\pm$0.02). In comparison to the native E_{ON} system this corresponded to silencing ratios of 68 and 93%, respectively, and an overall induction ratio of 99-fold which was approximately 5-fold higher than the native E_{ON} system. Despite significantly reducing maximal expression (i.e. 68%), this showed that the use of a more efficient siRNA could also be used to improve induction characteristics because of the higher reduction observed in minimal expression (i.e. 93%). This suggests that differential siRNA-mediated silencing is not dependent upon a particular siRNA *per se*, but rather the ratio of siRNA to target.

To further investigate the impact of different siRNAs upon system characteristics, erythromycin dose response profiles for the native, and intronic GFP- and intronic Luc-containing siRNA E_{ON} systems were compared (Figure 4). Apart from the change in induction characteristics described earlier, a tightening of the inducer
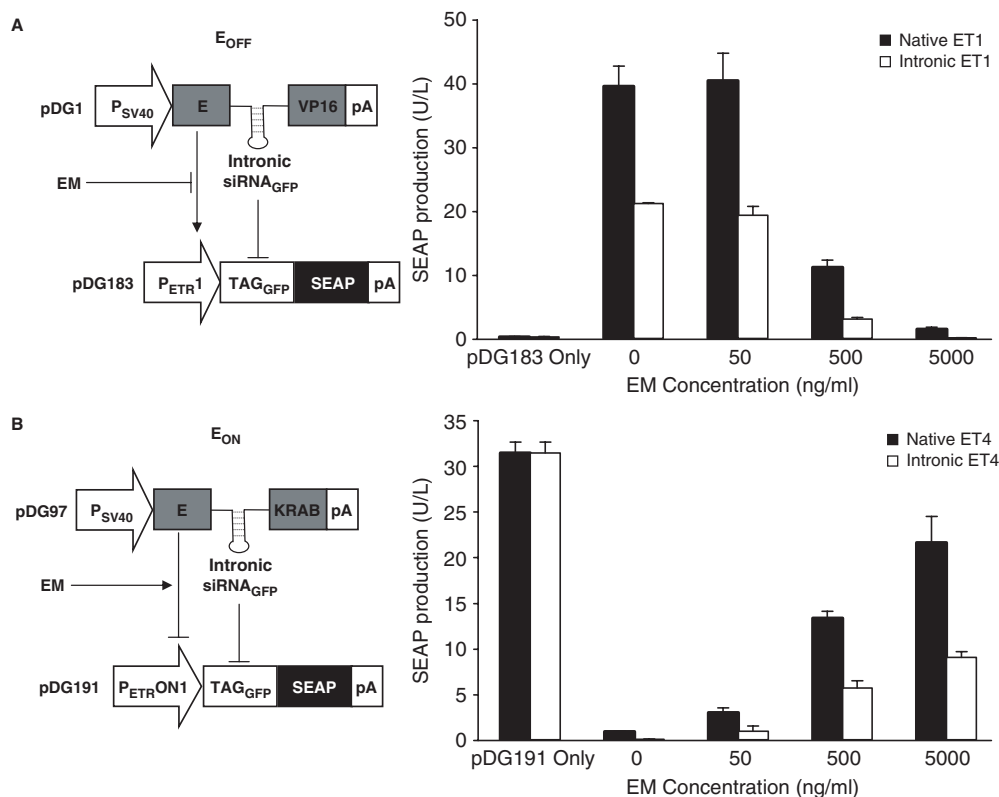
Figure 3. Schematic illustration and comparison of induction performance of E_{OFF} and E_{ON} systems containing intronic siRNA against a target that was placed immediately upstream of a generic reporter gene. Native and intronic siRNA-containing erythromycin (EM) dose profiles for (**A**) E_{OFF} and (**B**) E_{ON} systems were determined after co-transfection of CHO-K1 with the relevant transcriptional regulator (pWW35—native ET1; P_{SV40}-E-VP16-pA, pDG1—ET1 containing intronically encoded siRNA against GFP; P_{SV40}-E-siRNA$_{GFP}$-VP16-pA, pWW43—native ET4; P_{SV40}-E-KRAB-pA or pDG97—ET4 containing intronically encoded siRNA against GFP, P_{SV40}-E-siRNA$_{GFP}$-KRAB-pA) and the corresponding ET1/ET4 responsive SEAP reporter construct containing the siRNA target sequence (TAG$_{GFP}$) immediately upstream of the SEAP reporter gene (E_{OFF} pDG183; P_{ETR}1-TAG$_{GFP}$-SEAP-pA and E_{ON} pDG191; P_{ETR}ON1-TAG$_{GFP}$-SEAP-pA). Also shown are expression levels resulting from transfection with just the relevant reporter construct.
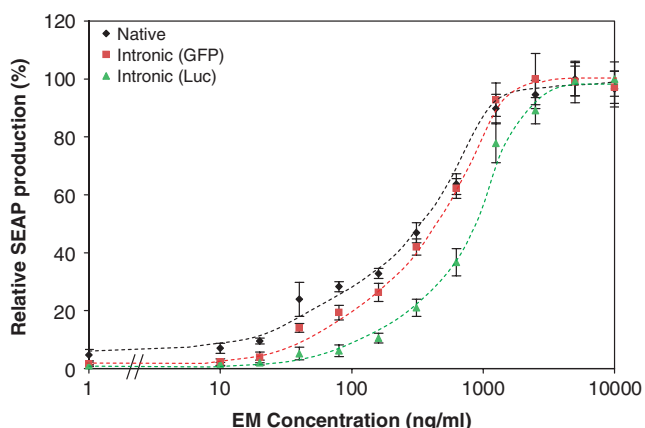
Figure 4. Comparison of erythromycin (EM) dose response profiles for native and two different intronic siRNA-containing E_{ON} systems. Reporter gene expression levels were determined after co-transfection of CHO-K1 with either the native system (pWW43; P_{SV40}-E-KRAB-pA, and pWW56; P_{ETR}ON1-SEAP-pA), an intronic siRNA system utilizing intronic siRNA$_{GFP}$ and TAG$_{GFP}$ upstream of the reporter (Intronic GFP: pDG97; P_{SV40}-E-siRNA$_{GFP}$-KRAB-pA, and pDG191: P_{ETR}ON1-TAG$_{GFP}$-SEAP-pA), or an intronic siRNA system utilizing intronic siRNA$_{Luc}$ (luciferase) and TAG$_{Luc}$ upstream of the reporter (Intronic Luc: pDG98; P_{SV40}-E-siRNA$_{Luc}$-KRAB-pA, and pDG104: P_{ETR}ON1-TAG$_{Luc}$-SEAP-pA). For each respective system, SEAP reporter expression levels are stated relative (%) to the maximal gene expression level observed for that system.

concentration range ('inducer window') was observed for both intronic siRNA containing systems. This was greatest for the intronic siRNA$_{Luc}$ system where the erythromycin concentration at which the system began to switch 'ON' increased from 10 to ~100 ng/ml. For both systems there were minimal changes to the concentration at which the system was fully induced (~1000 ng/ml). Referring back to Figure 1 these results are consistent with the impact of the siRNA to target ratio upon gene silencing. For a relatively efficient siRNA such as siRNA$_{Luc}$, maximum silencing occurs at a relatively lower siRNA to target ratio. Hence, maximal silencing does not require ET4 to be entirely shut-off which accordingly shifts the beginning of the inducer window to the right. However, with respect to the end of the end of the induction window, there is no equivalent shift as once ET4 is completely active there is no further change in siRNA-mediated silencing levels. Taken together this results in an overall compression of the inducer window.

To test the applicability of the approach to other mammalian systems, the performance of the native and intronic siRNA containing versions of the E_{OFF} and E_{ON} control systems were also determined in several other mammalian cell types; in particular the human cell lines HEK293-T and HeLa, the monkey cell line Cos-7, and the mouse cell line NIH/3T3 (Table 2). In each respective case, the

Table 2. Comparison of induction performance of native versus intronic siRNA containing E_{OFF} and E_{ON} systems in different mammalian cell types

	Cell type			
	HEK293-T	Cos-7	HeLa	NIH/3T3
E_{OFF} system				
Reporter alone	28.2 ± 0.4	3.7 ± 0.4	0.3 ± 0.0	0.04 ± 0.01
Native ET1 ($-$EM)	860.5 ± 33.4	653.4 ± 48.8	225.3 ± 10.8	20.66 ± 2.34
Native ET1 ($+$EM)	29.2 ± 1.9	7.6 ± 0.8	0.9 ± 0.1	0.10 ± 0.04
Intronic ET1 ($-$EM)	720.6 ± 20.6	633.9 ± 64.2	109.5 ± 6.4	11.59 ± 2.20
Intronic ET1 ($+$EM)	5.1 ± 0.6	1.9 ± 0.1	0.1 ± 0.1	0.01 ± 0.01
Induction factor ($\times$)				
Native ET1	29.5	86.4	240.2	208.9
Intronic ET1	140.5	333.8	1963.0	2074.5
Change	4.8	3.9	8.2	9.9
E_{ON} system				
Reporter alone	5.28 ± 0.91	14.68 ± 0.64	2.07 ± 0.52	3.29 ± 0.06
Native ET4 ($-$EM)	0.92 ± 0.08	1.56 ± 0.11	0.16 ± 0.00	0.23 ± 0.17
Native ET4 ($+$EM)	5.32 ± 0.77	13.81 ± 1.64	2.26 ± 0.33	1.59 ± 0.42
Intronic ET4 ($-$EM)	0.17 ± 0.27	0.40 ± 0.05	0.02 ± 0.14	0.03 ± 0.02
Intronic ET4 ($+$EM)	2.44 ± 0.32	12.00 ± 1.18	0.86 ± 0.13	0.94 ± 0.17
Induction factor (x)				
Native ET4	5.8	8.9	14.3	7.0
Intronic ET4	14.5	30.2	39.9	29.9
Change	2.5	3.4	2.8	4.3

ET1 or ET4 responsive SEAP reporter constructs containing the siRNA target sequence (TAG_{GFP}) immediately upstream of the SEAP reporter gene (E_{OFF} pDG183; $P_{ETR}1$-TAG_{GFP}-SEAP-pA and E_{ON} pDG191; $P_{ETR}ON1$-TAG_{GFP}-SEAP-pA) were transfected alone or together with either native (ET1, pWW35; P_{SV40}-E-VP16-pA and ET4, pWW43; P_{SV40}-E-KRAB-pA) or intronic $siRNA_{GFP}$ containing (ET1, pDG1; P_{SV40}-E-$siRNA_{GFP}$-VP16-pA and ET4, pDG97; P_{SV40}-E-$siRNA_{GFP}$-KRAB-pA) transregulators into different cell types, and scored for SEAP production (U/l) in the presence or absence of 5 µg/ml erythromycin (EM). The resulting induction factor between OFF and ON expression levels is shown in each case.

intronic siRNA containing versions yielded superior induction performance with the extent of residual expression in the OFF configuration generally approaching the detection limit of the reporter gene assay. This confirmed that differential silencing between ON and OFF configurations occurred irrespective of whether the system was placed in a highly expressing cell line (e.g. HEK293-T or Cos-7) or a much lower expressing and tightly regulating cell line such as NIH/3T3. Indeed for the latter, the reduction in residual expression in the OFF configuration to the detection limit of the reporter assay (6 mU/l) for SEAP (36) resulted in an almost infinite induction ratio (i.e. >2000-fold). Significantly, for the E_{OFF} system the strong siRNA-mediated silencing of leaky expression in the OFF configuration reduced expression levels to well below that of equivalent transfections with just the reporter gene alone. This was never achieved in the OFF configuration with the native E_{OFF} system.

Given the influence of different underlying promoters on gene control system characteristics, the minimal $P_{hCMVmin}$ and constitutive P_{SV40} promoters of the standard E_{OFF} (pDG183) and E_{ON} (pDG191) -TAG_{GFP}-SEAP-pA reporter constructs, were substituted with *Drosophila* heat shock *hsp*70 (pDG276; $P_{ETR}2$-, ETR-$P_{hsp70min}$−) and simian-40 virus derived (pDG284; $P_{ETR}3$, ETR-$P_{SV40min}$−) minimal promoters, and the murine phosphoglycerate kinase (pDG280; $P_{ETR}ON2$-, P_{PGK}ETR-) constitutive promoter, respectively. A comparison of the performance of these systems using both native and intronic siRNA containing transregulators demonstrated that differential silencing between ON and OFF configurations, and resulting improved induction performance, existed for the intronic siRNA systems irrespective of the underlying promoter (Table 3). This occurred when the underlying promoter was weaker (e.g. $P_{hsp70min}$ for E_{OFF}) or stronger (e.g. $P_{SV40min}$ for E_{OFF} or P_{PGK} for E_{ON}) with the overall improvement ranging from 3- to 11-fold.

Exploiting the standard architecture and modular nature of many gene control systems, the generic intronic siRNA concept was extended to improve the induction characteristics of several other common transcription control systems. Therefore, intronically encoded $siRNA_{GFP}$ was placed within the tetracycline (Tet)-dependent transactivator tTA (pDG153; P_{SV40}-TetR-$siRNA_{GFP}$-VP16-pA), the reverse doxycycline (Dox)-dependent transactivator rtTA (pDG210; P_{SV40}-rTetR-$siRNA_{GFP}$-VP16-pA), and the pristinamycin (PI)-dependent transactivator PIT (pDG143; P_{SV40}-Pip-$siRNA_{GFP}$-VP16-pA). Using appropriate reporter constructs containing a TAG_{GFP} (pDG211; P_{hCMV^*-1}-TAG_{GFP}-SEAP-pA, pDG213; and P_{PIR}-TAG_{GFP}-SEAP-pA, respectively) and relative to native transactivators (pSAM200; P_{SV40}-TetR-VP16-pA, pTET-ON; P_{SV40}-rTetR-VP16-pA, pMF167 and P_{SV40}-Pip-VP16-pA, respectively), all systems exhibited between a 3- and 5-fold improvement in regulation performance (Figure 5).

Improved transgene expression allows tight conditional expression of toxic gene products

Irrespective of the cell type, underlying promoter or transcription control system used, the generally observed 3- to
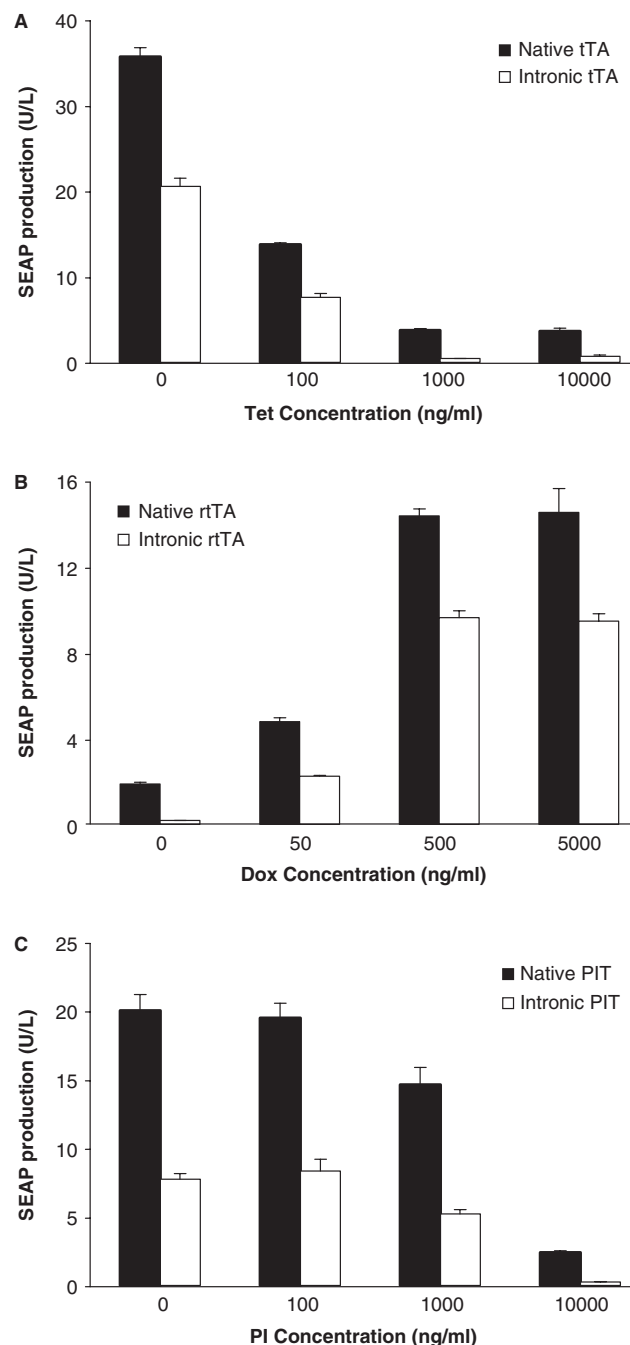
Table 3. Comparison of induction performance within CHO-K1 of native versus intronic siRNA containing E_{OFF} and E_{ON} systems utilizing different minimal and constitutive promoters adjacent to the relevant ETR operator module(s)

	Minimal promoter following single ETR operator		
	$P_{hCMVmin}$	$P_{hsp70min}$	$P_{SV40min}$
E_{OFF} system			
Reporter alone	0.38 ± 0.05	0.12 ± 0.01	1.98 ± 0.10
Native ET1 (−EM)	39.63 ± 3.16	6.59 ± 3.25	80.48 ± 7.97
Native ET1 (+EM)	1.61 ± 0.29	0.78 ± 0.16	6.36 ± 0.47
Intronic ET1 (−EM)	21.24 ± 0.14	2.53 ± 0.07	66.05 ± 0.09
Intronic ET1 (+EM)	0.19 ± 0.04	0.03 ± 0.00	0.84 ± 0.04
Induction factor (×)			
Native ET1	24.7	8.4	12.6
Intronic ET1	109.8	93.8	78.2
Change	4.4	11.2	6.2

	Constitutive promoter preceding octet ETR operator	
	P_{SV40}	P_{PGK}
E_{ON} System		
Reporter alone	31.48 ± 1.16	87.48 ± 15.68
Native ET4 (−EM)	0.98 ± 0.10	3.16 ± 0.66
Native ET4 (+EM)	21.64 ± 2.90	51.91 ± 21.37
Intronic ET4 (−EM)	0.13 ± 0.04	0.15 ± 0.03
Intronic ET4 (+EM)	9.09 ± 0.63	23.06 ± 0.66
Induction factor (×)		
Native ET4	22.0	16.4
Intronic ET4	71.0	158.9
Change	3.2	9.7

E_{OFF} reporter constructs containing either the $P_{hCMVmin}$ (pDG183; $P_{ETR}1$-, ETR-$P_{hCMVmin}$-), $P_{hsp70min}$ (pDG276; $P_{ETR}2$-, ETR-$P_{hsp70min}$−), or $P_{SV40min}$ (pDG284; $P_{ETR}3$-, ETR-$P_{SV40min}$-) minimal promoters, and E_{ON} reporter constructs containing either the P_{SV40} (pDG191; $P_{ETR}ON1$-, P_{SV40}-ETR-) or P_{PGK} (pDG280; $P_{ETR}ON2$-, P_{PGK}-ETR-) constitutive promoters, which drive transcription of a siRNA target sequence (TAG_{GFP}) immediately upstream of a SEAP reporter gene, were transfected alone or together with native (ET1, pWW35; P_{SV40}-E-VP16-pA and ET4, pWW43; P_{SV40}-E-KRAB-pA) or intronic $siRNA_{GFP}$ containing (ET1, pDG1; P_{SV40}-E-$siRNA_{GFP}$-VP16-pA and ET4, pDG97; P_{SV40}-E-$siRNA_{GFP}$-KRAB-pA) transregulators, and scored for SEAP production (U/l) in the presence or absence of 5 μg/ml erythromycin (EM). The resulting induction factor between OFF and ON expression levels for each system is shown in each case.

10-fold improvement in induction characteristics of the intronic siRNA approach was sufficient to improve residual leakiness to less than, or close to, 1% of maximal gene expression. To determine whether this improvement in reducing basal leakiness was of significance we sought to compare intronic siRNA mediated versus native transcription control of two toxic gene products. In the first, the highly toxic RIP death domain (RipDD) (25,26) was cloned immediately downstream of TAG_{GFP} containing E_{OFF} and E_{ON} responsive promoters (for E_{OFF} we used pDG289; $P_{ETR}1$-TAG_{GFP}-RipDD-pA and for E_{ON} pDG290; $P_{ETR}ON1$-TAG_{GFP}-RipDD-pA), and expression of RipDD in HeLa controlled with either native or intronic siRNA containing ET1 (pWW35 or pDG1) or

Figure 5. Extension of intronic siRNA/TAG concept to other gene regulation systems in CHO-K1. For each system the induction performance of the native transactivator was compared against the same transactivator containing an intronically encoded $siRNA_{GFP}$, the target (TAG_{GFP}) of which was incorporated immediately upstream of a SEAP reporter gene that was placed under control of the requisite transactivator responsive promoter. (**A**) Tet_{OFF} tetracycline (Tet) dose response in which either native tTA (pSAM200; P_{SV40}-TetR-VP16-pA) or intronic $siRNA_{GFP}$ containing tTA (pDG153; P_{SV40}-TetR-$siRNA_{GFP}$-VP16-pA) was co-transfected with pDG211 ($P_{hCMV*−1}$-TAG_{GFP}-SEAP-pA). (**B**) Tet_{ON} doxycycline (Dox) dose response in which either native rtTA (pTET-ON; P_{SV40}-rTetR-VP16-pA) or intronic $siRNA_{GFP}$ containing rtTA (pDG210; P_{SV40}-rTetR-$siRNA_{GFP}$-VP16-pA) was co-transfected with pDG211. (**C**) PI_{OFF} pristinamycin (PI) dose response in which either native PIT (pMF167; P_{SV40}-Pip-VP16-pA) or intronic $siRNA_{GFP}$ containing PIT (pDG143; P_{SV40}-Pip-$siRNA_{GFP}$-VP16-pA) was co-transfected with pDG213 (P_{PIR}-TAG_{GFP}-SEAP-pA).
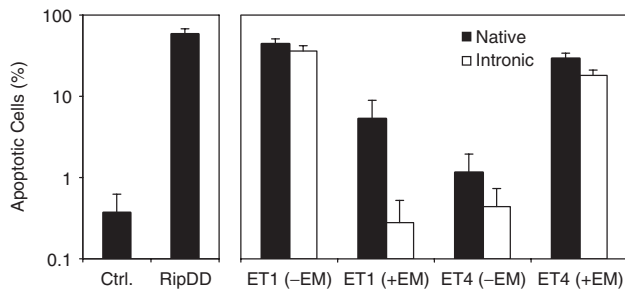
Figure 6. Comparison of constitutive, and conditional native and intronic siRNA containing E_{OFF} and E_{ON}, RIP death domain (RipDD) expression in HeLa cells. The proportion of apoptotic cells (cells positively stained for either or both of annexin V or 7-AAD) was determined after transient co-transfection of ET1/ET4 responsive RipDD expression constructs, containing an siRNA target sequence (TAG_{GFP}) immediately upstream of the RipDD gene (E_{OFF} pDG289; $P_{ETR}1$-TAG_{GFP}-RipDD-pA and E_{ON} pDG290; $P_{ETR}ON1$-TAG_{GFP}-RipDD-pA), and the relevant native (Nat. ET1 pWW35; P_{SV40}-E-VP16-pA and Nat. ET4 pWW43; P_{SV40}-E-KRAB-pA) or intronic $siRNA_{GFP}$ containing (Int. ET1 pDG1; P_{SV40}-E-$siRNA_{GFP}$-VP16-pA and Int. ET4 pDG97; P_{SV40}-E-$siRNA_{GFP}$-KRAB-pA) transregulator, and grown for 72 h in the presence or absence of 5 µg/ml erythromycin (EM). Mock-transfected cells (Ctrl.) and cells transfected with constitutively expressed RipDD (pWW326; $P_{hEF1\alpha}$-RipDD-pA) were used as respective controls.

ET4 (pWW43 or pDG97). Apoptosis profiling demonstrated that only the intronic siRNA containing transregulators enabled sufficiently tight OFF expression in which apoptosis was at the same level as that observed in negative control transfections (Figure 6). Importantly, maximal expression of the intronic siRNA systems was not overly compromised as evidenced by the roughly equivalent levels of apoptosis in native versus intronic siRNA systems in their respective ON configurations.

The utility of the intronic siRNA containing systems was further tested in CHO-K1 using the linamarase–linamarin prodrug system (27). In this system linamarase expression enables conversion of the otherwise harmless prodrug linamarin into highly toxic and readily diffusible gaseous cyanide. While linamarase is only expressed intracellularly, any produced gaseous cyanide can readily diffuse to neighboring cells thereby resulting in highly efficient cell killing through a 'bystander effect (27)'. Similarly to RipDD, Linamarase (Lis) was expressed downstream of the requisite TAG_{GFP} containing E_{OFF}/E_{ON} responsive promoters (E_{OFF} pDG286; $P_{ETR}1$-TAG_{GFP}-Lis-pA and E_{ON} pDG289; $P_{ETR}ON1$-TAG_{GFP}-Lis-pA) and assessed for native and intronic siRNA containing ET1 and ET4 performance (Figure 7). As evidenced both by microscopic imagery (Figure 7A) and cell viability assessment (Figure 7B), the difference between native and intronic siRNA containing transregulation in either OFF configuration (+ EM for E_{OFF} and –EM for E_{ON}) was highly evident. It was only by using intronic siRNA-mediated regulation that basal expression of linamarase was sufficiently abrogated to prevent high cell death. Indeed for the E_{OFF} system, and consistent with results described earlier using the quantitative SEAP reporter gene, the intronic siRNA containing system in its OFF configuration resulted in lower cell death than equivalent experiments with the linamarase expressing

plasmid alone (Rpt. Only, Figure 7). This succinctly demonstrated the ability of intronic siRNA to reduce basal expression levels below that inherent to the regulable promoter, and most critically, how this relatively small effect can equate to a major difference at a biological process level.

Simple, one-step autoinducible siRNA vectors

To fully explore the concept of intronically encoded siRNA within a transregulatory control system the constitutive P_{SV40} promoter of pDG1 and pDG153 (intronic $siRNA_{GFP}$ containing ET1 and tTA, respectively) were switched for the respective cognate promoters for each transactivator thereby generating EM- (pDG165; $P_{ETR}1$-E-$siRNA_{GFP}$-VP16-pA) and Tet- (pDG179; $P_{hCMV}^*{}_{-1}$-TetR-$siRNA_{GFP}$-VP16-pA) responsive autoregulatory vectors (Figure 8). In each case the respective transregulator not only positively drives its own expression, but also its intronically encoded siRNA. Addition of the respective inducer shuts off both transactivator and siRNA expression. Significantly, each vector contains all the necessary elements to enable controllable siRNA expression within a single compact format. The ET1-based vector is encoded within 3.5 kb; the tTA-based vector within 3.9 kb. In co-transfections with a GFP target (pLEGFP-N1), and relevant to the appropriate isogenic controls (pDG164; $P_{ETR}1$-E-VP16-pA, and pWW35), the autoregulated EM-responsive system was capable of mediating GFP silencing to almost the same degree as constitutively expressed intronic siRNA (pDG1). Importantly, the addition of EM was sufficient to shut-off ET1 expression with a consequent decrease in siRNA expression and GFP silencing. Similarly, the Tet-responsive system was capable of mediating strong silencing of a co-transfected GFP TAGed reporter construct (pLM65; $P_{hEF1\alpha}$-TAG_{GFP}-SEAP-pA), relative to isogenic controls (pDG178; $P_{hCMV}^*{}_{-1}$-VP16-pA), which could be alleviated across a relatively broad Tet concentration range.

A higher-fold *in vitro* and *in vivo* epigenetic toggle switch using intronically encoded siRNA

The utility of tighter gene control systems was demonstrated by applying the earlier concepts to improve the performance of a synthetic gene network; in particular, the mammalian epigenetic toggle switch (2). In its native configuration this network consists of the PIP_{ON} and E_{ON} antibiotic-inducible transrepressor control systems arranged such that they repress each other's expression (Figure 9). The system is capable of two stable expression states depending upon which transrepressor is actively repressing the other. Critically, and to distinguish it from a typical on versus off gene control system, the switch from one state to the other only requires the transient administration of an appropriate inducer. Co-transfection of native toggle components pBP62 ($P_{PIR}ON$-E-KRAB-IRES-pA) and pBP139 ($P_{ETR}ON1$-Pip-KRAB-IRES-SEAP-pA) into CHO cells, followed by incubation with either EM or PI for 24 h, results in two distinct expression levels which are maintained for the subsequent 24 h after substitution with inducer-free
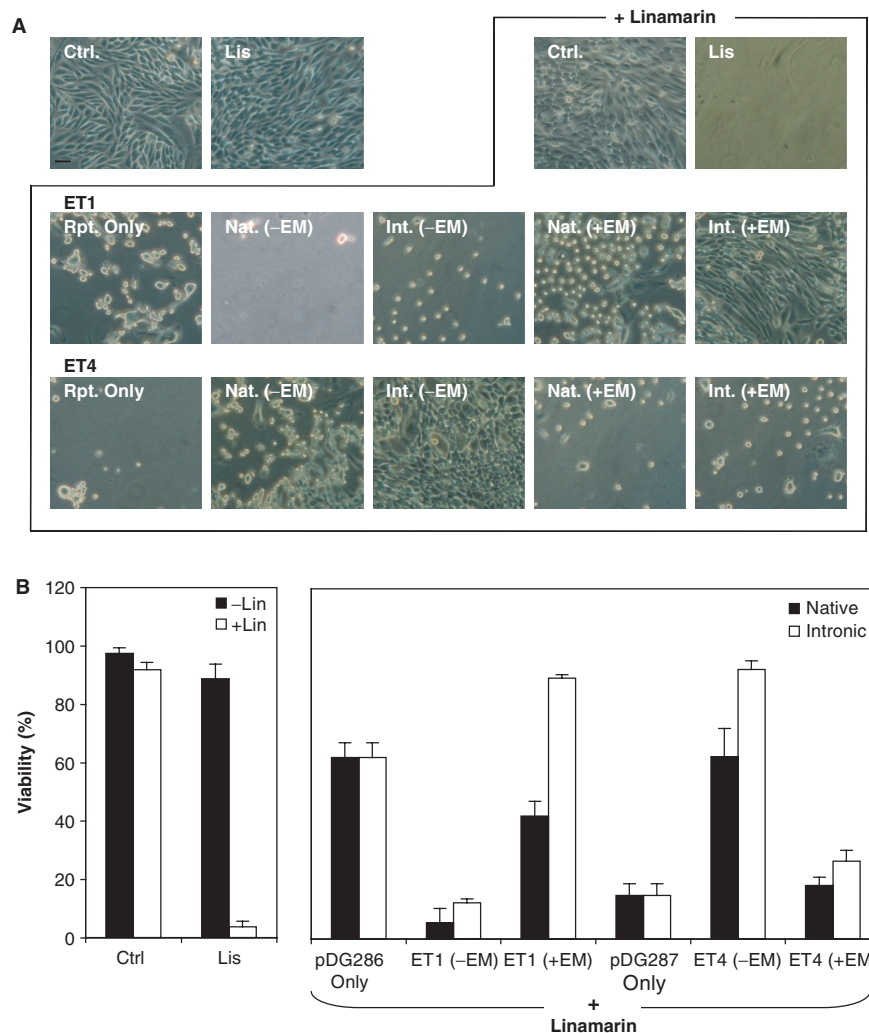
Figure 7. Impact of constitutive and E_{OFF}/E_{ON} regulated linamarase (Lis) expression, with linamarin (Lin) application, on CHO-K1 cell viability. (**A**) Light microscopy (scale bar 50 μM) and (**B**) cell viability of harvested cells after ET1/ET4 responsive Lis expression constructs containing an siRNA target sequence (TAG_{GFP}) immediately upstream of the Lis gene (E_{OFF} pDG286; $P_{ETR}1$-TAG_{GFP}-Lis-pA and E_{ON} pDG287; $P_{ETR}ON1$-TAG_{GFP}-Lis-pA) were transfected alone (Rpt. Only) or together with the relevant native (Nat. ET1 pWW35; P_{SV40}-E-VP16-pA and Nat. ET4 pWW43; P_{SV40}-E-KRAB-pA) or intronic $siRNA_{GFP}$ containing (Int. ET1 pDG1; P_{SV40}-E-$siRNA_{GFP}$-VP16-pA and Int. ET4 pDG97; P_{SV40}-E-$siRNA_{GFP}$-KRAB-pA) transregulator and grown for 96 h in the presence of Lin, and the presence or absence of 5 μg/ml erythromycin (EM). Mock-transfected cells (Ctrl.) and cells transfected with constitutively expressed Lis (pWW315; $P_{hEF1\alpha}$-Lis-pA), grown in either the presence or absence of Lin were used as respective controls.

media (Figure 9B). Using intronically encoded $siRNA_{GFP}$ and $siRNA_{Luc}$ [luciferase-specific siRNA (29)] placed between each transrepressor, and targeting a siRNA-specific TAG placed immediately upstream of each opposing transrepressor, a variant of the native toggle was engineered which used both transrepression as well as gene silencing to control expression of the opposing construct (Figure 9A). Upon co-transfection of this network, encoded on pDG161 ($P_{PIR}ON$-TAG_{GFP}-E-$siRNA_{Luc}$-KRAB-IRES-pA) and pDG168 ($P_{ETR}ON1$-TAG_{Luc}-Pip-$siRNA_{GFP}$-KRAB-IRES-SEAP-pA), into CHO a bistable expression pattern also resulted which was maintained following the withdrawal of relevant inducers (Figure 9B). Significantly, the use of gene-silencing through intronically encoded siRNA increased the dynamic range of the two stable states. The initial presence of PI (for the first 24 h only), which induces the lower

expression state, resulted in SEAP expression levels of 0.25 and 0.47 U/l after 24 and 48 h, respectively, whereas the native toggle resulted in 0.55 and 0.76 U/l, respectively. This demonstrated that intronically encoded $siRNA_{Luc}$ reduced Pip-KRAB and SEAP expression further than E-KRAB-mediated repression could alone, thus effectively lowering the leakiness of the system. Conversely, in the initial presence of EM (for the first 24 h only), the intronic toggle yielded SEAP levels of 1.83 and 3.03 U/l, and the native toggle 1.71 and 1.51 U/l, again at 24 and 48 h. In this instance, intronic $siRNA_{GFP}$ minimized leaky E-KRAB expression, thus enabling higher Pip-KRAB and SEAP expression compared to the native configuration. In relation to the dynamic range, the intronic toggle exhibited an induction factor of 7- and 6-fold, compared to 3- and 2-fold for the native toggle at 24 and 48 h, respectively.
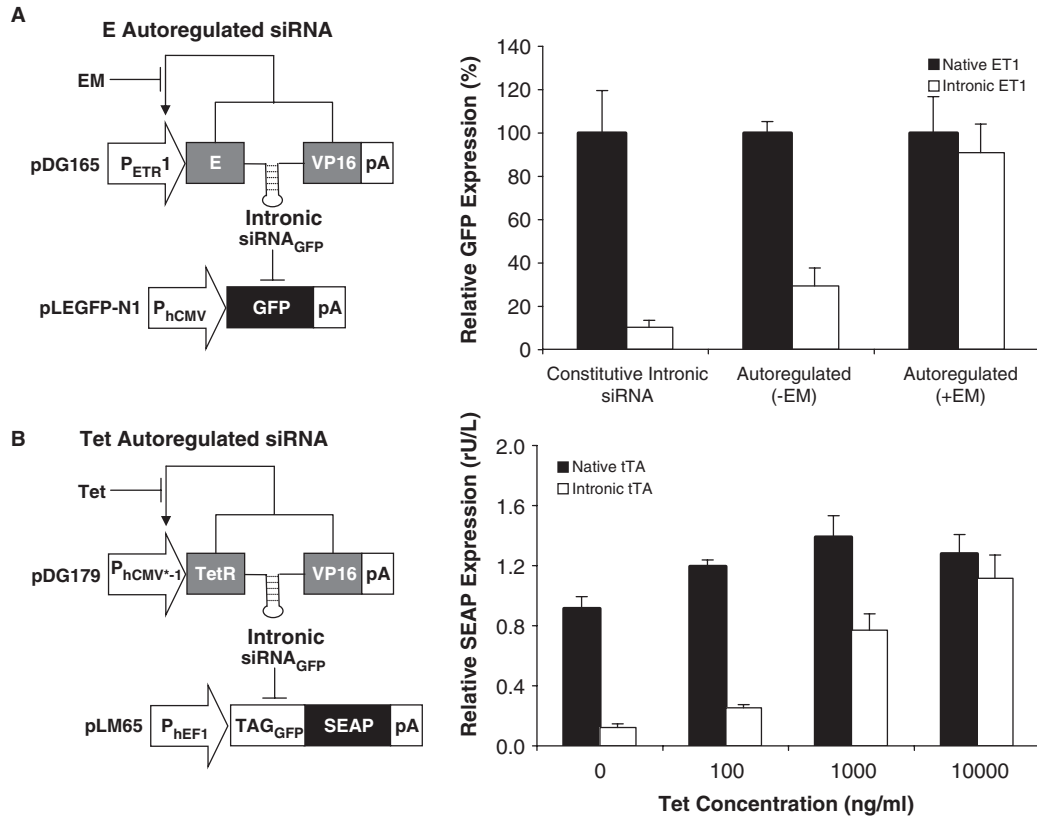
Figure 8. Schematic illustration and regulation performance within CHO-K1 of autoregulated intronic siRNA systems responsive to either erythromycin or tetracycline. In either system, each transactivator is under the transcriptional control of its own cognate promoter thereby providing positive autofeedback control for not only the transactivator itself, but also the intronically encoded siRNA contained within the respective transactivator. (**A**) Erythromycin responsive autoregulated siRNA in which GFP silencing was assessed after co-transfection of pLEGFP-N1 (P_{hCMV}-GFP-pA) with either constitutively produced intronic siRNA (pDG1; P_{SV40}-E-siRNA$_{GFP}$-VP16-pA) or autoregulated intronic siRNA (pDG165; P_{ETR}1-E-siRNA$_{GFP}$-VP16-pA) in the presence or absence of 5 µg/ml erythromycin (EM). In each case gene expression was assessed relative to equivalent transfections with isogenic (Native) non-siRNA containing controls (pWW35; P_{SV40}-E-VP16-pA and pDG164; P_{ETR}1-E-VP16-pA). (**B**) Tetracycline dose response in which silencing of a GFP tagged SEAP reporter gene (pLM65; $P_{hEF1\alpha}$-TAG$_{GFP}$-SEAP-pA) was determined after co-transfection with autoregulated tTA containing an intronic siRNA against GFP (pDG179; P_{hCMV^*-1}-TetR-siRNA$_{GFP}$-VP16-pA). Again, gene activity was assessed relative to pLM65 co-transfection with an isogenic (Native) non-siRNA control (pDG178; P_{hCMV^*-1}-TetR-VP16-pA).

To enable longer-term validation, reversibility and *in vivo* studies we constructed a double transgenic cell line, CHO$_{TOGGLE2}$, stable for pDG161 and pDG168 (Figure 10). After an initial 3-day period to set populations to either a high or low SEAP expression state (using EM or PI), each population was subjected to a further 9-day incubation phase in inducer-free media (Figure 10A). Analysis of expression levels every 3 days showed that the differential expression state of each population was maintained over this period. In a similar experimental setup, cell populations were initially exposed to one inducer to set expression patterns before multiple switching of inducer regimes (Figure 10B). In each case the cell populations exhibited full reversibility in the presence of a different inducer thus indicating that the network retained its superior bistable characteristics even after repeated switching between states. Finally, to demonstrate that the intronic toggle was also capable of improved *in vivo* performance CHO$_{TOGGLE2}$ cells were microencapsulated and intraperitoneally implanted into mice. All mice were initially dosed with either PI and EM, following which only a subset were exposed to ongoing inducer dosing (Figure 10C). For either PI or EM dosed mice, serum SEAP expression levels were similar regardless of whether dosing was continuous or only transient. Secondly, the induction ratio between transient EM versus PI dosed mice of 7- (3d), 9- (6d) and 7-fold (9d) remained higher at all times in the intronic siRNA network compared to the previously published native toggle network (2).

DISCUSSION

Typical mammalian heterologous gene control systems consist of a regulable promoter controlled by a constitutively expressed transregulator (13). In this work an intronically encoded siRNA was simultaneously co-expressed with a functional transregulator that controlled expression of a reporter gene that was itself targeted by the siRNA. Significantly, in the ON configuration the ratio of siRNA to target mRNA was lower than where the same promoter is in the OFF configuration. Computational models, referenced to the molecular and biochemical parameters of RISC-based siRNA interference processes, and experimentally validated using synthetic transcription-
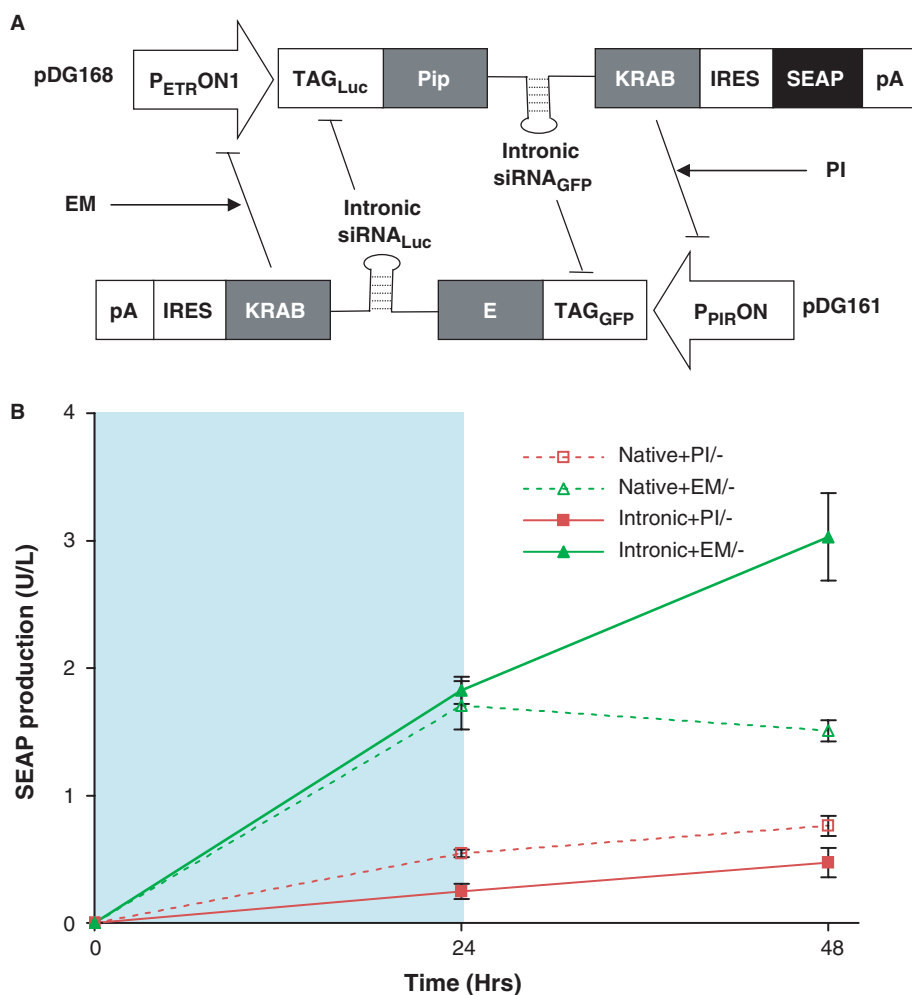
Figure 9. (A) Schematic depiction of an intronic siRNA-containing genetic toggle network consisting of two transpressors [pristinamcyin (PI)-inducible Pip-KRAB and erythromycin (EM)-inducible E-KRAB] configured in a mutually oppressing manner whereby each repressor is under the transcriptional control of the opposing repressors cognate promoter (P_{ETR}ON1 and P_{PIR}ON, respectively). Each transrepressor contains an intronically encoded siRNA [either GFP- ($siRNA_{GFP}$) or Luc- ($siRNA_{LUC}$) specific] directed against a TAG placed immediately upstream of the opposing transrepressor. SEAP, encoded downstream of Pip-KRAB via an internal ribosome entry site (IRES), provides a read-out of the network's expression status. (B) Regulation performance of the native (non-siRNA intron nor TAG containing) toggle switch (pBP62; P_{PIR}ON-E-KRAB-IRES-pA, and pBP139; P_{ETR}ON1-Pip-KRAB-IRES-SEAP-pA) versus the intronic siRNA toggle switch (pDG161 and pDG168) following transient co-transfection in CHO-K1. In each case only a transient pulse of effecter (presence indicated by shading) is required to enable the opposing repressor to be maximally transcribed until, in a self-perpetuating manner, it stably represses the originally active promoter. Either of the two steady states are maintained in a following removal of relevant effecter molecules (non-shaded region) by substitution with fresh media.

translation networks, have previously shown that both the rate of mRNA disappearance and eventual mRNA steady state level are directly influenced by the relative concentrations of siRNA to target mRNA (35,37). This feature was exploited in the above configuration with the resulting differences in gene silencing used to increase the induction window of several common gene control systems. Significantly, this differential silencing effect was observed across many tested mammalian cell types, and occurred irrespective of the underlying regulable promoter thus demonstrating the broad applicability of the approach.

Since the development of mammalian gene control systems, many strategies have focused upon improving induction characteristics by eliminating residual expression. Given its early development and subsequent widespread adoption much of this work has been specifically aimed at the Tet-responsive system. Thus incremental improvements to the original Tet configuration have been achieved by mutational enhancement of the Tet DNA-binding domain to improve its binding and sensitivity (38–40), by modifying the Tet-cognate promoter (41,42), and finally by combining non-heterodimerizing reverse Tet-transactivators with antagonizing Tet-repressors (43–47). While successful on their own or in combination, each of these enhancements is specific to the Tet-system and not easily reproducible for other gene control systems. At a more general level it is possible to alter the properties of any gene control system through multimerization and alternate spacing of operator sites, choice of underlying promoter, as well as selection of transregulatory protein (41,48–51). However, the systems described to date have generally explored these avenues and are already optimized. An alternate, but time-consuming and poorly scalable, avenue is to stably transfect a gene
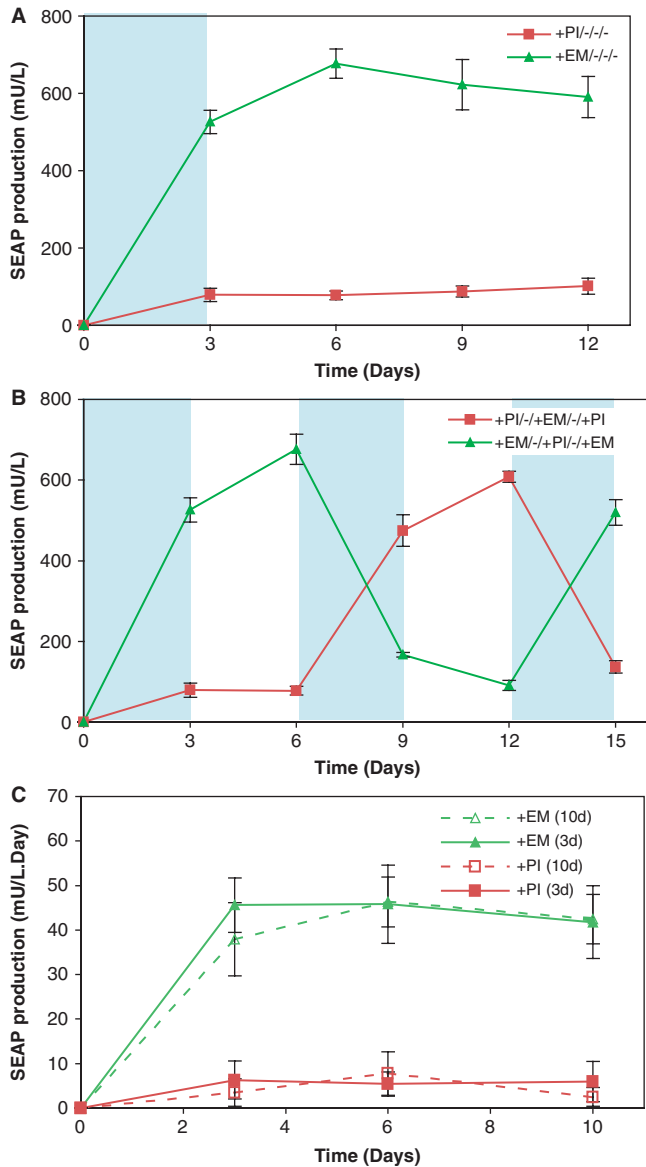
Figure 10. (**A**) Long-term bistability of transgenic CHO-K1 cells (CHO$_{TOGGLE2}$) engineered for an intronic siRNA containing genetic toggle network by stable transfection of pDG161 (P$_{PIR}$ON-TAG$_{GFP}$-E-siRNA$_{Luc}$-KRAB-IRES-pA) and pDG168 (P$_{ETR}$ON1-TAG$_{Luc}$-Pip-siRNA$_{GFP}$-KRAB-IRES-SEAP-pA). Two cell populations were grown for 3 days in the presence of either pristinamycin (PI) or erythromycin (EM) to set initial SEAP expression levels (presence of effecters indicated by shading). Cells were subsequently harvested, reseeded in effecter-free media, and assayed for SEAP activity every 3 days over a total period of 9 days. (**B**) Expression reversibility in CHO$_{TOGGLE2}$. Again, two cell populations were initially grown for 3 days in the presence of either PI or EM to set expression levels, followed by a further 3-day cultivation in effecter-free media. At days 6 and 12, effecter dosing was switched. SEAP was scored immediately prior to each media change and at day 15. (**C**) *In vivo* expression control of microencapsulated CHO$_{TOGGLE2}$ cells implanted intraperitoneally into mice. Serum SEAP levels were quantified at days 3, 6 and 10 for four groups of mice which were initially exposed to 3 days of either daily PI or EM effecter injections. Effecter injections were subsequently halted for two groups of mice (3d) whereas the remaining two groups served as controls in which respective effecter administration was maintained daily over the following 7 days (10d). Shown are mean values including SD of at least six mice per timepoint.

control system and select cells with high regulation performance by single cell cloning (11,12). Finally, several attempts have also been made to explicitly reduce leakiness through the engineering of synthetic gene networks. Early networks were based on transcriptional cascades of several independently operating gene control systems (52,53). Later attempts have built upon these networks and sought to utilize conditional RNA interference to actively eliminate basal reporter expression (35,54). In some cases, however, the required number of interacting elements and associated complexity has rendered each system relatively user unfriendly and not readily adaptable to new systems. In contrast a far simpler generic solution is presented here that does not entail significant added complexity and which can be applied to most gene control systems that share the same essential architecture. This was illustrated by improving the performance characteristics of three common antibiotic responsive control systems for which the only manipulation required was the insertion of an intronically encoded siRNA on the transregulator and placement of a siRNA target immediately upstream of the desired reporter gene.

By placing a transactivator containing an intronically encoded siRNA under transcriptional control of the transactivator's cognate promoter it was also possible to engineer a highly effective autoregulable silencing vector. This tool enables one-step genetic engineering of eukaryotic cells for adjustable expression of a siRNA. Significantly, these vectors contain all the necessary elements for self-regulation within a single cistron on a single plasmid that is less than 4 kb in size. Over the past 5 years mammalian gene regulation technology has been progressively applied to siRNA expression (55–59) and indeed autoregulated siRNA expression vectors have also been developed (58). However, such vectors typically encode the regulatory component (e.g. tTA) and the siRNA responsive component as two independent cistrons, with associated promoters and mRNA processing sequences, on the one plasmid. Here, issues associated with cross-promoter competition or interaction are circumvented, and the number of introduced genetic elements minimized, by simultaneously expressing the siRNA and regulatory component from the same cistron. Importantly, by applying the design using two different systems (i.e. E$_{OFF}$ and Tet$_{OFF}$) it was demonstrated that the architecture is also generically applicable. Thus, it could used to create autoinducible vectors responsive to other effective molecules by simply switching both the DNA-binding protein and cognate promoter for one specific to the desired gene control system. This technology could also easily be combined with multicistronic expression technology to enable autoregulated expression control of several siRNAs and transgenes all off a single vector (29,60). This could be a useful tool for the study or engineering of combinatorial effects of different genes in mammalian cells.

While the generic strategy described earlier was used to improve the induction window of several gene control systems it did nonetheless also result in a reduction in maximum expression levels. Arguably the most common problem with existing mammalian gene control systems is not with maximum expression levels, but with leaky

residual expression. To that end, foregoing some maximal expression for tighter residual expression may be an acceptable compromise that will be of significant benefit to many applications. We have demonstrated this by using intronically encoded siRNA-based regulation to conditionally express highly biologically toxic genes such as the RipDD and linamarase–linamarine prodrug system. Critically in both cases, siRNA silencing of residual expression enabled tight suppression in OFF configurations which produced outcomes equivalent to control experiments. While siRNA silencing also occurred during the respective ON configurations it was not sufficient to unduly compromise the affect of highly potent gene products such as RipDD and linamarase. To further illustrate the utility of differential silencing we have also utilized intronic siRNA technology to engineer an improved epigenetic toggle that exhibits a far greater induction window than the equivalent native (non-intronic) version (2). This was demonstrated in not only transiently transfected mammalian cells, but also encapsulated stably transfected cells that were implanted into mice. Significantly, we believe that the *in vivo* demonstration of the superior mammalian toggle characteristics substantiates its future utility for therapeutic and other applied usage.

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