BioLogic Gates Enable Logical Transcription Control in Mammalian Cells

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Abstract: The architecture of gene regulatory networks is reminiscent of electronic circuits. Modular building blocks that respond in a logical way to one or several inputs are connected to perform a variety of complex tasks. Gene circuit engineers have pioneered the construction of artificial gene regulatory networks with the intention to pave the way for the construction of therapeutic gene circuits for next-generation gene therapy approaches. However, due to the lack of a critical amount of eukaryotic cell-compatible gene regulation systems, the field has so far been limited to prokaryotes. Recent development of several mammalian cell-compatible expression control systems laid the foundations for the assembly of transcription control modules that can respond to several inputs. Herein, three approaches to evoke combinatorial transcription control have been followed: (i) construction of artificial promoters with up to three operator sites for regulatory proteins, and (ii) parallel and (iii) serial linking of two gene regulation systems. We have combined tetracycline-, streptogramin-, macrolide-, and butyrolactone transcription control systems to engineer BioLogic gates of the NOT IF-, AND-, NOT IF IF-, NAND-, OR-, NOR-, and INVERTER-type in mammalian cells, which are able to respond to up to three different small molecule inputs. BioLogic gates enable logical transcriptional control in mammalian cells and, in combination with modern transduction technologies, could serve as versatile tools for regulated gene expression and as building blocks for complex artificial gene regulatory networks for applications in gene therapy, tissue engineering, and biotechnology.

Keywords: gene regulation; transcription control; gene regulatory networks; gene therapy; E.REX; PIP; TET; quorum sensing

INTRODUCTION

Increasing knowledge on how cell phenotypes are shaped by the expression interplay of different sets of genes (Bolouri and Davidson, 2002; Milo et al., 2002; Shen-Orr et al., 2002) reveals complex regulatory transcription networks that show response characteristics reminiscent of logic gate-driven electric circuits. The expression output of mammalian cell-based regulatory networks is often a logic response modulated by one or several input signals (Simpson et al., 2001; Buchler et al., 2003). With a vision to enable sophisticated therapeutic interventions for next-generation gene therapy and tissue engineering, gene circuit engineers focused on the combination of compatible heterologous gene control modules to configure artificial regulatory networks in a rational (Elowitz and Leibler, 2000; Gardner et al., 2000), combinatorial (Guet et al., 2002), or evolutionary (Yokobayashi et al., 2002) manner. Lack of available human cell-compatible heterologous transcription control modalities was one factor limiting the design of artificial regulatory networks to prokaryotic systems (Weiss et al., 2003). Furthermore, transduction of complex multicomponent networks to mammalian cells and establishing stable cell lines expressing all the necessary components remains a major challenge. Improvements in this area will be fundamental for clinical applications.

The transfer of these artificial regulatory networks to mammalian cells requires a variety of heterologous transcription control units that can be arranged to integrate internal and external signals and modulate desired biologic responses (Gossen and Bujard, 1992; Fussenegger et al., 2000; Weber et al., 2002, 2003). These control units capitalize on a binary design concept consisting of chimeric transcription modulators, assembled by fusing procaryotic response regulators to mammalian transactivation (Triezenberg et al., 1988) or transrepression (Moosmann et al., 1997) domains, which bind modulator-specific operator-containing promoters in a small molecule-adjustable manner. In most configurations, the presence of regulating agents abolishes transactivator/transrepressor–promoter interaction and results in repression/derepression of desired transgene expression. Owing to their sigmoid-shaped dose–response curves, gene regulation systems could be considered genetic analog–digital converters. Their output is either ON or OFF for a wide range of input inducer concentrations, except for a concentration window typically between 10 and 1,000 ng/mL in which the systems gradually

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change from the ON to the OFF state. In this study, we used ''digital'' inputs, which resulted in either maximum or minimum output signals. In Boolean terms, pharmacologic control resulting in induction of target gene expression, so-called ON systems, represent IF gates, whereas systems configured for transcription–repression, known as OFF systems, show a NOT-type signal integration.

In this study, we pioneer a variety of different two- and three-input BioLogic gates in mammalian cells by combining several compatible heterologous gene control units responsive to tetracycline, streptogramin, macrolide, and butyrolactone input signals in the following configurations: (i) chimeric promoters containing operators specific for up to three different transactivators/transrepressor enable NOT IF-, NOT IF IF-, and AND-type regulation profiles with three molecular intervention levels; (ii) configurations involving two independent IF or NOT regulation units in parallel create OR and NAND transcription logic; and (iii) small artificial regulatory networks in series which provide NOR- and INVERTER-type signal cascade-like progression of input signals.

METHODS

Promoter and Plasmid Constructions

pBP103 (P_{PIR}ON-SAMY-pA) was constructed by cloning the P_{PIR}ON-promoter of pTRIDENT11 (Moser et al., 2000) *Nhe*I/*Sma*I into pCF87 (C. Fux et al., unpublished) (*Xba*I/*Swa*I). The $tetO_7$-ETR_8-$P_{hCMVmin}$ promoter was assembled by cloning the ETR_8 operator contained on pWW56 (Weber et al., 2002) (*Stu*I/*Eco*RV) into the *Stu*I site of pTRIDENT1 (Fussenegger et al., 1998) to result in pTRIDENT38. Subsequently, SEAP was excised from pCF64 (C. Fux et al., 2003) by *Eco*RI/*Not*I and cloned into pTRIDENT38 (*Eco*RI/*Not*I) resulting in pBP187 ($tetO_7$-ETR_8-$P_{hCMVmin}$-SEAP-pA). The triple-responsive promoter $tetO_7$-PIR_3-ETR_8-$P_{hCMVmin}$ was constructed by cloning the PIR_3 operator encoded on pTRIDENT11 (*Hin*dIII) into the corresponding site of pBP187, thus resulting in $tetO_7$-PIR_3-ETR_8-$P_{hCMVmin}$-SEAP-pA (pBP215). The ETR_8 sequence of pWW56 (Weber et al., 2002) (*Aat*I/*Sse*I) was replaced by the $scbR_8$ operator excised from pWW161 (Weber et al., 2003) by *Aat*I/*Sse*I to result in pTRIDENT39 ($scbR_8$-$P_{hCMVmin}$-IRES-IRES-pA). Construction of pTRIDENT40 ($scbR_8$-PIR_3-$P_{hCMVmin}$-IRES-IRES-pA) involved *Stu*I/*Pvu*I excision of PIR_3 from pTRIDENT11, blunting, and cloning into the *Stu*I site located between $scbR_8$ site and $P_{hCMVmin}$ ofpTRIDENT39. SAMY excised from pDuoRex19 (Fux and Fussenegger, 2003) by *Pme*I/*Xho*I was placed under control of the $scbR_8$-PIR_3-$P_{hCMVmin}$ promoter (pBP211, $scbR_8$-PIR_3-$P_{hCMVmin}$-SAMY-pA). pBP232 (P_{ETR}ON-PIP-KRAB-pA) was constructed by excising P_{ETR}ON (*Ssp*I/*Eco*RI) from pWW56 (Weber et al., 2002) and inserting it into the corresponding sites of pMF207 (Fussenegger et al., 2000).

Cell Culture and Transfection

Chinese hamster ovary cells (CHO-K1, ATCC CCL 61) were grown in FMX-8 medium (Cell Culture Technologies, Zurich, Switzerland), supplemented with 10% calf serum (FCS, PAA Laboratories, Linz, Austria; lot number A01129-242). Transfection was performed using an optimized calcium phosphate transfection protocol (Weber et al., 2003). Reporter gene activities were quantified 48 h post transfection, as previously described (Schlatter et al., 2002).

Chemicals

Erythromycin (Fluka, Buchs, Switzerland) was prepared as a stock solution of 2 mg/mL in ethanol. The streptogramin antibiotic pristinamycin (Pyostacin, Aventis Inc., Paris, France) as well as tetracycline (catalog number T3383, Sigma, St. Louis, MO) were prepared as stock solutions of 500 μg/mL in DMSO. All antibiotics were used at final concentrations of 2 μg/mL, and SCB1 at a concentration of 1 μg/mL.

RESULTS

Recently reported NOT IF gates in *Escherichia coli* provide an IF-type output in the presence of a single input signal but a NOT-like integration when a second, both, or no signals are entered into the system (Guet et al., 2002). We have engineered NOT IF gates in mammalian cells by combining constitutive expression of the butyrolactone (QuoRex [Weber et al., 2003])- and streptogramin (PIP [Fussenegger et al., 2000])-responsive gene regulation systems' transactivator SCA (P_{hCMV}-SCA-pA; pWW122) and transrepressor PIP-KRAB (P_{hCMV}-PIP-KRAB-pA; pMF207) with a chimeric SCA- and PIP-KRAB-specific promoter driving the secreted α-amylase output reporter gene (SAMY [Schlatter et al., 2002]) ($scbR_8$-PIR_3-$P_{hCMVmin}$-SAMY-pA, pBP211) (Fig. 1A). Signal integration profiles resulting from co-transfection of pWW122, pMF207, and pBP211 into CHO-K1 cells and different butyrolactone and streptogramin inputs are shown in Figs. 1B and C. Maximum $scbR_8$-PIR_3-$P_{hCMVmin}$-driven SAMY expression results from exclusive SCA binding. In this configuration the SCA–$scbR_8$ interaction is enabled by the absence of the butyrolactone SCB1 (racemic 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl)butanolide); the presence of the streptogramin pristinamycin (PI) prevents binding of PIP-KRAB to its cognate PIR_3 operator module. Upon challenging this NOT IF biologic gate with SCB1 and PI, transgene expression is shut down since SCA and PIP-KRAB no longer bind their target promoter. Active higher-level SAMY repression is achieved in the +SCB1/−PI configuration when only PIP-KRAB is binding competent and actively represses $scbR_8$-PIR_3-$P_{hCMVmin}$. Co-transfection of pBP211 with pWW122 resulted in a 14-fold regulation in dependence of SCB1, while the

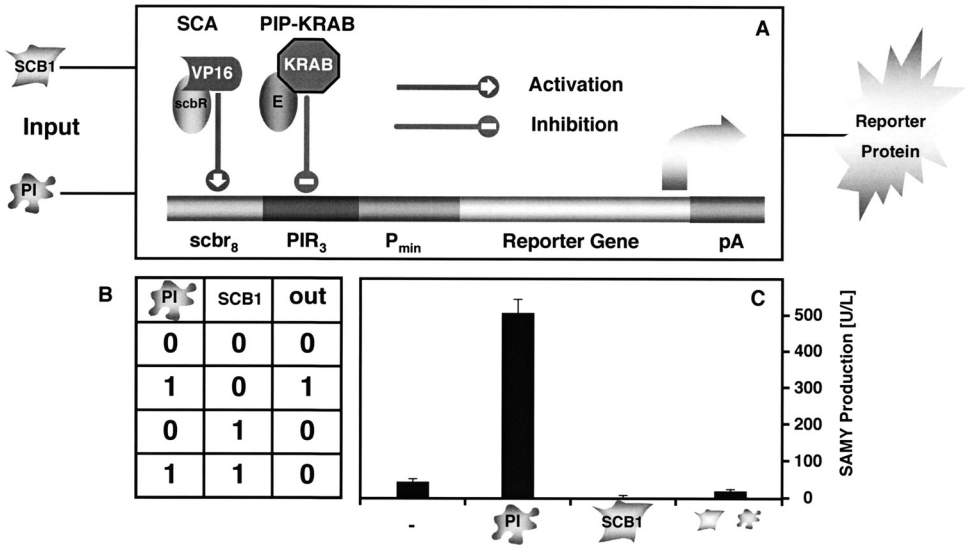

Figure 1. Molecular setup, truth table, and conditional expression levels of a NOT IF gate. (**A**) Butyrolactone-dependent transactivator SCA (scbR-VP16) and the streptogramin-dependent transrepressor PIP-KRAB are constitutively expressed and modulate reporter gene expression driven by a chimeric promoter containing specific operator modules $scbr_8$ (SCA) and PIR_3 (PIP-KRAB) in response to 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide (SCB1) and/or pristinamycin (PI). (**B**) Boolean representation of the NOT IF gate. (**C**) SAMY production profiles CHO-K1 cells containing NOT IF gate plasmids (P_{hCMV}-SCA-pA, P_{hCMV}-PIP-KRAB-pA, and $scbR_8$-PIR_3-$P_{hCMVmin}$-SAMY-pA) cultivated for 48 h in the absence (−) or presence of 2 mg/mL indicated small molecule input signals. Cells transiently transfected with the NOT IF gate plasmids under several input conditions.

presence of PIP-KRAB yielded 350-fold regulation between the input cases +SCB1 and +PI, while maintaining the absolute expression level, as can be seen in Fig. 1C. In the presence of both input signals, neither SCA nor PI interacts with their specific promoter and transgene expression remains silent.

Isogenic NOT IF gates could also be assembled using tetracycline (Gossen and Bujard, 1992)- and macrolide (Weber et al., 2002)-responsive gene regulation systems. Co-transfection of constitutive expression vectors encoding the tetracycline-dependent transactivator (tTA; P_{hCMV}-tTA-pA: pSAM200) and the erythromycin-dependent transrepressor (E-KRAB; P_{hCMV}-E-KRAB-pA; pWW43) with a reporter construct driven by a tTA/E-KRAB-specific promoter ($tetO_7$-ETR_8-$P_{hCMVmin}$-SEAP-pA; pBP187) resulted in similar signal integration performance in CHO-K1 cells compared to the QuoRex/PIP configuration: −tetracycline (TET)/ −erythromycin (EM), 0.2 ± 0.18 U/L; −TET/+EM, 6.25 ± 0.15 U/L; +TET/−EM, 0.001 ± 0.13; and +TET/+EM, 0 ± 0.004. The forementioned NOT IF gate can be converted into an AND-type BioLogic gate by replacing tTA with its reverse tetracycline-dependent homologue, which binds the *$tetO_7$* operator only in the presence of tetracycline. Co-transfection of pTetON (P_{hCMV}-rtTA-pA, Clontech, Palo Alto, CA), pWW43, and pBP187 resulted in typical AND-like signal integration profiles: −TET/−EM, 0 ± 0.04 U/L; −TET/+EM, 0 ± 0.07 U/L; +TET/−EM, 0.08 ± 0.01 U/L; +TET/+EM, 1.4 ± 0.17 U/L.

The generic two-signal input NOT IF gates could be extended to higher-order control networks (NOT IF IF) responsive to three external signals: tetracycline, erythromycin, and pristinamycin. Multi-level control of a chimeric SEAP-driving promoter containing $tetO_7$, PIR_3, and ETR_8 operator sites ($tetO_7$-PIR_3-ETR_8-$P_{hCMVmin}$-SEAP-pA, pBP215) in CHO-K1 cells constitutively expressing tTA (pSAM200), PIP-KRAB (pMF207), and E-KRAB (pWW43) provides high-level SEAP expression when both transrepressors (PIP-KRAB, E-KRAB) are locked by erythromycin and pristinamycin in an operator binding-incompetent allosteric configuration (Fig. 2A). In all other configurations, including tetracycline, input signals that prevent binding of the only transactivator tTA and/or binding of one of the two transrepressors PIP-KRAB or E-KRAB SEAP expression remain silent (Fig. 2B). NOT IF (IF) type of BioLogic gates are widely found in nature. Many key decisions in development depend on the presence of an activator and the absence of one or several repressors (McAdams and Shapiro, 1995; Bolouri and Davidson, 2002).

NAND-like gene regulation profiles following a two-signal input can be achieved by parallel arrangement of two NOT gates consisting of the PIP and the E.REX systems (Fig. 3). Co-transfection of constitutive transactivator-encoding vectors pMF156 (P_{hCMV}-PIT-pA; PIT, streptogramin-dependent transactivator) and pWW35 (P_{hCMV}-ET-pA; ET, macrolide-dependent transactivator) with PIT and ET-responsive SEAP expression vectors pMF156 (P_{PIR}-SEAP-pA) and pWW36 (P_{ETR}-SEAP-pA) resulted in (i) maximum SEAP expression when both transactivators are bound to their promoters (absence of small molecule inputs), (ii) half-maximum reporter gene expression if either one of the two transactivators promotes transcription (+EM/−PI or −EM/+PI), or (iii) full repression of SEAP transcription in the presence of both antibiotics (Fig. 3C).

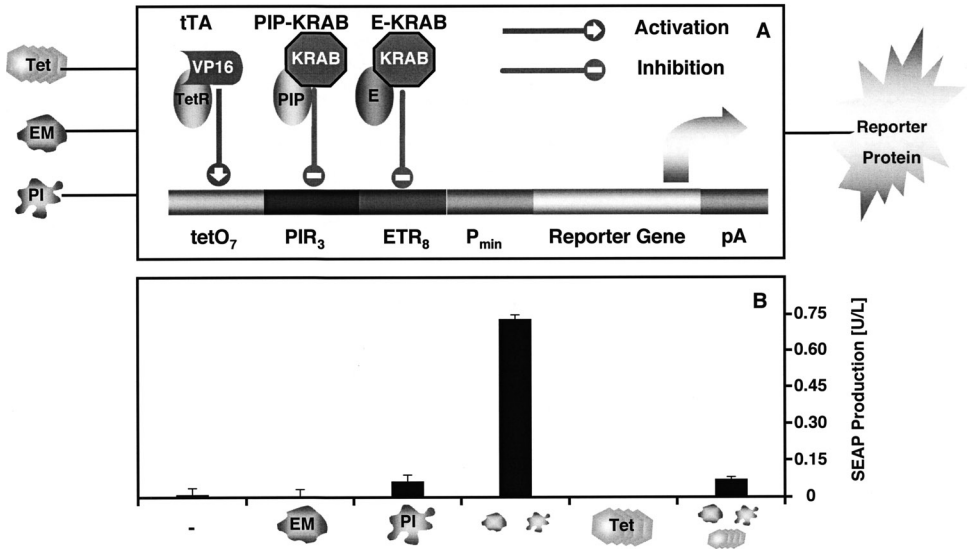

Figure 2. Diagram and expression profile of a logic gate integrating three independent input signals. (**A**) Tetracycline-dependent transactivator (tTA) as well as the macrolide- and streptogramin-dependent transrepressors E-KRAB and PIP-KRAB are constitutively expressed and modulate SEAP expression, driven by a chimeric promoter harboring tTA ($tetO_7$), PIP-KRAB (PIR_3), and E-KRAB (ETR_8) specific operator modules, in response to tetracycline (Tet), pristinamycin (PI), or erythromycin (EM). (**B**) SEAP production profiles were assessed in CHO-K1 48 h after transfection of pSAM200 (PhCMV-tTA-pA), pMF207 (P_{hCMV}-PIP-KRAB-pA), pWW43 (P_{hCMV}-E-KRAB-pA), and pBP215 ($tetO_7$-PIR_3-ETR_8-$P_{hCMVmin}$-SEAP-pA).

NAND BioLogic gates are able to detect co-presence of two external signals and could thus serve as network modules that integrate two external signals into one. NAND logic of transcriptional expression control requires the presence of multiprotein corepressor assemblies (Knoepfler and Eisenman, 1999).

Capitalizing on the NAND design concept an OR-type BioLogic gate could be constructed by parallel configuration of two IF gates consisting for example of the two antibiotic-inducible gene regulation systems PIP_{ON} (Fussenegger et al., 2000) and E_{ON} (Weber et al., 2002) (Fig. 4A). Co-transfection of the PIP_{ON} [pMF208 (P_{PIR}ON-SEAP-pA)/pMF207 (P_{SV40}-PIP-KRAB-pA)] and E_{ON} [pWW56 (P_{ETR}ON-SEAP-pA)/pWW43 (P_{SV40}-E-KRAB-pA)] expression components into CHO-K1 cells resulted in SEAP expression whenever at least one of the two signals is

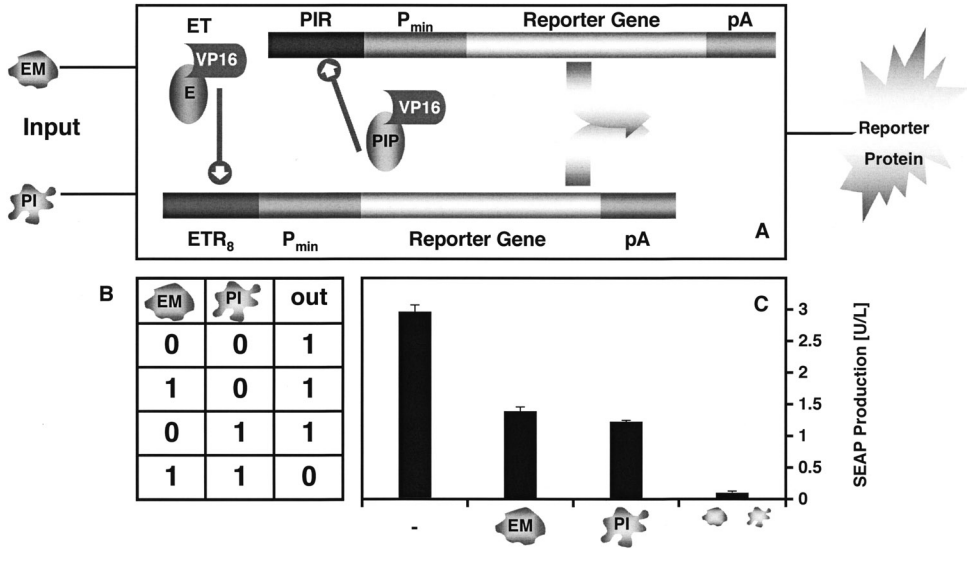

Figure 3. Schematic molecular and switch board-type representation of the NAND gate including its validation in mammalian cells. (**A**) Transactivators ET (E-VP16) and PIT (PIP-VP16) bind and activate their cognate promoters P_{ETR} and P_{PIR} which drive separate SEAP expression units in response to erythromycin (EM) and pristinamycin (PI) input signals. (**B**) Boolean approximation of the NAND gate. (**C**) SEAP production levels originating from CHO-K1 cells co-transfected with pWW35 (P_{hCMV}-ET-pA), pMF156 (P_{hCMV}-PIT-pA), pWW36 (P_{ETR}-SEAP-pA), and pBP134 (P_{PIR}-SEAP-pA) and cultured in the presence and absence of different EM/PI cocktails for 48 h.

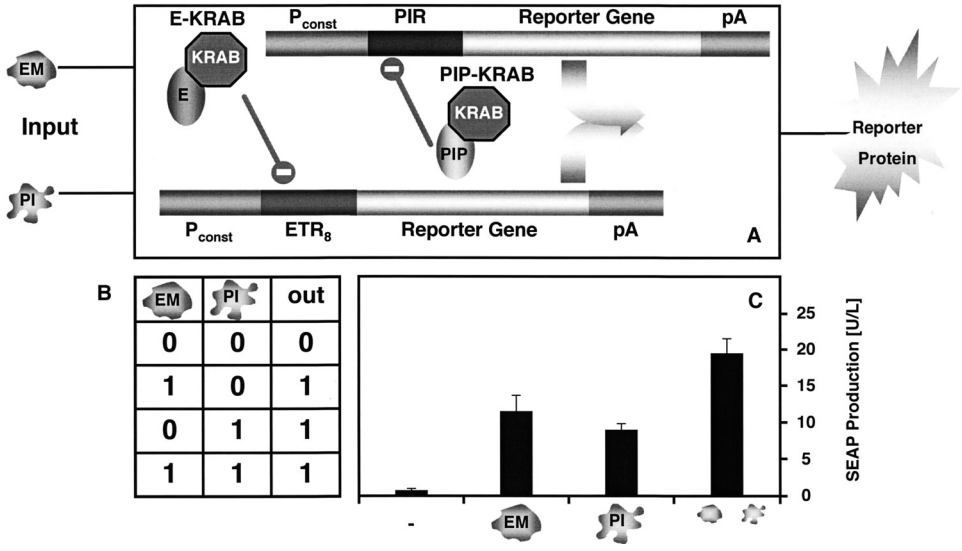

Figure 4. Characterization of the OR gate. (**A**) Constitutive expression of the macrolide- and streptogramin-dependent transrepressors E-KRAB and PIP-KRAB modulate SEAP expression of their cognate promoters (P_{ETR}ON, P_{hCMV}-ETR; P_{PIR}ON, P_{hCMV}-PIR) in an erythromycin- or pristinamycin-inducible manner. (**B**) Boolean conversion of the OR gate. (**C**) Validation of OR-type regulation in CHO-K1 cells co-transfected with pWW43 (P_{SV40}-E-KRAB-pA), pWW56 (P_{ETR}ON-SEAP-pA), pMF207 (P_{SV40}-PIP-KRAB-pA), and pMF208 (P_{PIR}ON-SEAP-pA). Following integration of different antibiotic input signals SEAP production was assessed 48 h post transfection.

entered into the artificial regulatory network (Fig. 4C). Maximum transgene expression is achieved when both expression units (P_{PIR}ON-SEAP-pA and P_{ETR}ON-SEAP-pA are fully induced by pristinamycin and erythromycin (Fig. 4C). Such OR-gates are at work, whenever several signals impinge on a single effector function. Highly redundant multichannel networks have evolved, wherever one function is of paramount importance for survival and must be activated under a variety of environmental circumstances (Fambrough et al., 1999; Dixit and Mak, 2002; Miller et al., 2002). In all previous examples, BioLogic gates were controlled by transcription control systems whose transactivators and transrepressors were constitutively expressed and acted on a single or two parallel-assembled expression units. In-line configuration of several BioLogic gates in which one gate modulates the activity of the following one(s) provide unmatched complexity in the design of artificial regulatory networks, thereby

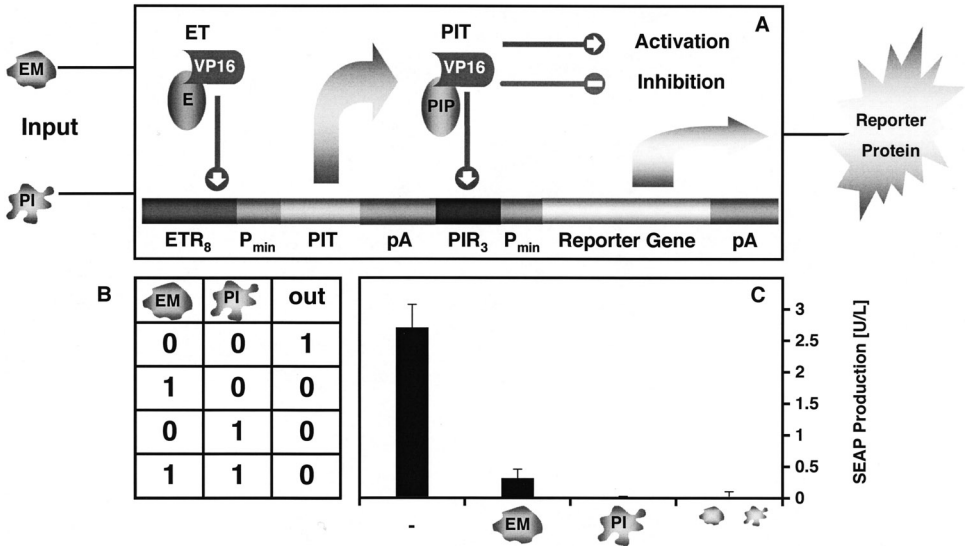

Figure 5. Artificial regulatory cascades with NOR gate signal integration: molecular and Boolean diagrams and in vivo validation. (**A**) A sequential transcription cascade is triggered by abundant macrolide-dependent transactivator (ET; E-VP16) which binds and induces P_{ETR}-driven transcription of the streptogramin-dependent transactivator (PIT; PIP-VP16) in the absence of erythromycin (EM). PIT continues to induce desired target gene expression in a pristinamycin (PI)-repressible manner. (**B**) Switchboard-like representation of NOR-type signal integration. (**C**) SEAP production levels of CHO-K1 engineered for NOR-controlled SEAP expression grown for 48 h under different signal input configurations (presence or absence of EM/PI).

approaching well-orchestrated circuit integrations evolved in mammalian cells. As a non-exclusive example, we designed a NOR-type regulation circuit by arranging two independent NOT gates in consecutive configuration (Fig. 5A). The NOR regulation network is triggered by a constitutively expressed macrolide-dependent transactivator ET which modulates P_{ETR}-driven expression of the streptogramin-dependent transactivator PIT that then adjusts P_{PIR}-mediated expression of the desired transgene. This NOR-type regulatory cascade enables two levels of signal interventions: (i) macrolide-responsive PIT production and (ii) pristinamycin-controlled transgene expression. Leaky PIT transcripts accumulating in the presence of macrolide antibiotics result in low but significant transgene expression. Exclusive addition of pristinamycin represses the target gene to the detection levels as does provision of both antibiotics. The closer a transcription block is set at the target gene the tighter a regulatory cascade will be. We have validated these considerations by co-transfecting a constitutive ET expression vector (pWW35 [Weber et al., 2002]) together with a two-transcript target plasmid (P_{ETR}-PIT-pA-P_{PIR}-SEAP-pA; pBP138) encoding PIT driven by the macrolide-responsive (P_{ETR}) and SEAP by the streptogramin-responsive promoters (P_{PIR}) into CHO-K1 cells (Fig. 5C). NOR-like expression control mimics translational signal transduction cascades, which can be interrupted or reversed at every stage. Such networks are involved in the control of hepatocyte-specific gene expression (Duncan et al., 1998). The inverse NOT IF gate or INVERTER regulatory network is a logic function the Boolean output of which is 1 except if the two signals are entered in a 1/0 mode into the system (Fig. 6A). A basic INVERTER configuration is achieved by in-series connection of two independent IF gates (Fig. 6A). Constitutive expression of the macrolide-dependent transrepressor E-KRAB modulates expression of PIP-KRAB driven by the macrolide-inducible promoter (P_{ETR}ON) in a macrolide-responsive manner. Subsequently, PIP-KRAB adjusts reporter gene transcription via streptogramin-modulated interaction with the cognate streptogramin-inducible promoter (P_{PIR}ON). The generic INVERTER configuration tested in CHO-K1 cells following cotransfection of pWW43 (P_{hCMV}-E-KRAB-pA), pBP232 (P_{ETR} ON-PIP-KRAB-pA), and pBP103 (P_{PIR}ON-SAMY-pA; SAMY, secreted α-amylase) produced two different readout scenarios based on different two-signal inputs: full SAMY expression (i) in the absence of antibiotics (E-KRAB-mediated repression of PIP-KRAB, P_{PIR}ON remains active), (ii) in the presence of pristinamycin (PIP-KRAB is produced but cannot bind and repress P_{PIR}ON) as well as (iii) in the presence of erythromycin and pristinamycin (both transcription units are fully derepressed) while (iv) addition of erythromycin alone resulted in PIP-KRAB-mediated transgene repression (PIP-KRAB is produced and silences P_{PIR}ON) (Fig. 6C).

The tremendous potential associated with pharmacologic control of artificial regulatory networks in rational reprogramming of mammalian cell phenotypes for tissue engineering or multiregulated multigene therapeutic interventions in advanced gene therapy scenarios has often been recognized (Hasty et al., 2002; Kitano, 2002). Yet, the pioneering design of gene networks remained restricted to implementation in prokaryotes due to limited availability of different human-compatible gene control systems responsive to clinically licensed small-molecule drugs. Capitalizing on recent advances in the design of compatible gene

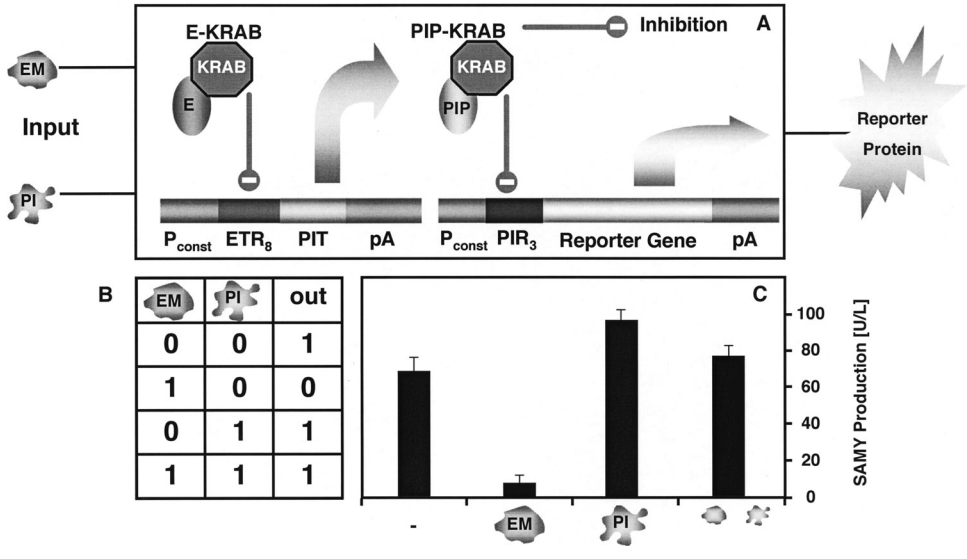

Figure 6. Inverter-type signal integration shown at the genetic, Boolean, and cellular levels. (**A**) Macrolide-dependent transrepressor (E-KRAB) fine-tunes expression of the streptogramin-dependent transrepressor (PIP-KRAB) in an erythromycin (EM)-repressible manner. PIP-KRAB may carry the transcription signal forward and modulate reporter gene expression in response to pristinamycin (PI). (**B**) INVERTER-type signal integration scheme in a digital mode. (**C**) INVERTER-controlled SEAP production profiles following integration of EM/PI input signals in CHO-K1 cells harboring respective control units shown in (**A**).

control systems multiregulated multigene interventions had become a scientific reality (Weber et al., 2002). For optimal molecular interventions in complex regulatory networks orchestrating the cellular transcriptome in space and time (Ibarra et al., 2002; Aebersold and Mann, 2003), we have constructed a variety of switchboard-type transgene control units. These artificial regulatory networks follow natural design principles, which have been implemented in electronic circuits. Increased compatibility of gene and electronic regulatory networks may revolutionize the machine–patient interface in the not-too-distant future.

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