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Review

Mammalian synthetic biology: Engineering of sophisticated gene networks

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Abstract

With the recent development of a wide range of inducible mammalian transgene control systems it has now become possible to create functional synthetic gene networks by linking and connecting systems into various configurations. The past 5 years has thus seen the design and construction of the first synthetic mammalian gene regulatory networks. These networks have built upon pioneering advances in prokaryotic synthetic networks and possess an impressive range of functionalities that will some day enable the engineering of sophisticated inter- and intra-cellular functions to become a reality. At a relatively simple level, the modular linking of transcriptional components has enabled the creation of genetic networks that are strongly analogous to the architectural design and functionality of electronic circuits. Thus, by combining components in different serial or parallel configurations it is possible to produce networks that follow strict logic in integrating multiple independent signals (logic gates and transcriptional cascades) or which temporally modify input signals (time-delay circuits). Progressing in terms of sophistication, synthetic transcriptional networks have also been constructed which emulate naturally occurring genetic properties, such as bistability or dynamic instability. Toggle switches which possess "memory" so as to remember transient administered inputs, hysteric switches which are resistant to stochastic fluctuations in inputs, and oscillatory networks which produce regularly timed expression outputs, are all examples of networks that have been constructed using such properties. Initial steps have also been made in designing the above networks to respond not only to exogenous signals, but also endogenous signals that may be associated with aberrant cellular function or physiology thereby providing a means for tightly controlled gene therapy applications. Moving beyond pure transcriptional control, synthetic networks have also been created which utilize phenomena, such as post-transcriptional silencing, translational control, or inter-cellular signaling to produce novel network-based control both within and between cells. It is envisaged in the not-too-distant future that these networks will provide the basis for highly sophisticated genetic manipulations in biopharmaceutical manufacturing, gene therapy and tissue engineering applications.
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Contents

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1. Introduction

The rapid accumulation of genetic information, together with advances in molecular manipulation techniques, have opened new possibilities for gene therapy and biomedical engineering. The ability to combine naturally occurring genetic components in unique ways has provided the opportunity to manipulate cellular behavior using *de novo* synthetic genetic networks (Hasty et al., 2002; Kaern et al., 2003; McDaniel and Weiss, 2005). Many of these synthetic networks have their origination in prokaryotic systems where they have enabled the creation of a range of sophisticated functionalities as well as the testing and validation of many phenomena observed in natural genetic networks (Hasty et al., 2002; Sprinzak and Elowitz, 2005). With the development of a wide range of eukaryotic-compatible gene control systems, it has now become possible to develop equivalent networks in mammalian cells and organisms (Weber and Fussenegger, 2002, 2004a, 2006). This development paves the way for a new generation of therapeutic interventions in which genes affecting cellular and organ physiology are manipulated in highly orchestrated and controlled ways. Within only a few years, an already impressive range of mammalian compatible genetic devices have been constructed in which the expression of a target gene can be customized to a range of sensing parameters and expression behaviors (Kramer et al., 2004a; Kramer et al., 2005; Kramer and Fussenegger, 2005a; Kramer et al., 2004b; Kramer et al., 2003). These networks have not only improved our understanding of gene network behavior in mammalian cells but have already resulted in devices that could be useful for research and/or therapeutic applications.

This review describes the advances that have been made in the engineering of *de novo* mammalian genetic networks with a focus upon describing networks that have been experimentally tested and validated. Advances in network functionality include the creation of stable autofeedback motifs, in which expression variation across a population is minimized (Becskei and Serrano, 2000); the engineering of logical information "gates," where a range of input combinations produce highly defined outputs in a manner directly analogous to electrical circuits (Kramer et al., 2004a); the development of transcriptional cascades, which have enabled the range of inputs to a network to be greatly increased as well as providing an opportunity to accurately and precisely titrate transgene expression (Kramer et al., 2003); and the creation of bistable toggle and hysteric switches, akin to the development of network "memory" (Kramer and Fussenegger, 2005a; Kramer et al., 2004b). In addition, steps have been taken to extend network design beyond the sole use of transcriptional control systems to incorporate other elements of gene control, such as gene silencing and translation control (Malphettes and Fussenegger, 2006b; Schlatter and Fussenegger, 2003; Schlatter et al., 2003). The first advances have also been made in the creation of host-integrated networks; that is, prosthetic genetic networks which are capable of responding to physiological cues so that they are effectively integrated into the host-cell's biology (Kramer et al., 2005). Such networks, in response to acute or pathological cues, hold great promise for the controlled manipulation of cellular processes, such as protein synthesis, metabolism, cell growth and differentiation. Finally, to fully exploit the benefits of genetic networks it will be necessary to develop systems in which behavior is not just coordinated within a cell but across a population of cells within or across different tissues and organisms. To this end, initial attempts have also been made to demonstrate the feasibility of inter-cell communication networks amongst mammalian cell populations engineered with different synthetic network configurations (Weber et al., in press).

In nature, any step of gene expression can be controlled, from DNA transcription to RNA post-transcriptional modification, transport and decay, through to protein translation and post-translational modification and transport of the protein itself. While in theory this means synthetic gene networks could also be constructed at any level of this hierarchy the prevalence of transcriptional control systems means that most synthetic mammalian networks have utilized gene control systems that operate at the transcriptional level.

2. Transcriptional gene networks

2.1. Control elements

Initial higher-order and mammalian gene networks have all been constructed using heterologous transcriptional control elements. Simple ON/OFF mammalian transcriptional control systems typically utilize bacterial response regulators which, in their native setting, are capable of specifically binding a target promoter and either inhibiting or activating transcription. Binding of the response regulator to its cognate promoter is dependent upon an appropriate inducer molecule whose presence or absence, depending upon the system, causes either association or disassociation of the regulator from its target promoter (Jacob and Monod, 1961). By placing the regulator's DNA binding site (the "operator") adjacent to an eukaryotic promoter, the response regulator can function as a heterologous DNA binding protein (DBP) which, when appropriately modified, inducibly or repressibly associates with its hybrid promoter

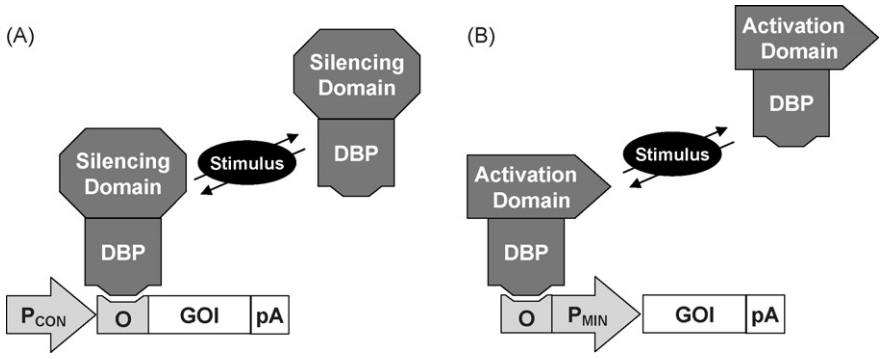

Fig. 1. Typical molecular configurations for synthetic transactivator and transrepressor control systems. (A) For a transrepressor control system, the DNA binding protein (DBP) – typically a bacterial transcriptional repressor – binds one or several specific operator sites (O) placed immediately downstream of a strong constitutive eukaryotic promoter (P_{CON}). Either by itself through steric hindrance, or when fused to a eukaryotic transcriptional silencing domain, binding of the DBP represses transcription of the gene of interest (GOI). (B) For a transactivation control system the same operator site is placed immediately upstream of a minimally active eukaryotic promoter (P_{MIN}). In this case the DBP is fused to an eukaryotic activation domain such that upon binding transcription is initiated. In either case, the presence or absence of a stimulus affects DBP binding thereby enabling control of transcription.

(Kramer and Fussenegger, 2005b) (Fig. 1). In one type of configuration, the operator is placed immediately downstream of a strong constitutive eukaryotic promoter which upon DBP binding causes steric interference of the cells' RNA polymerase II machinery thereby preventing initiation of transcription. To further silence gene activity a eukaryotic transcriptional silencer, such as the Kruppel-associated box protein (KRAB), can be fused to the DBP resulting in active repression of eukaryotic transcription (Bellefroid et al., 1991). In an alternate configuration, the operator is placed immediately adjacent to a minimally active eukaryotic promoter which by itself is transcriptionally inactive. By fusing an eukaryotic transcriptional activation domain, such as the *Herpes simplex* virus VP16, to the DBP (Triezenberg et al., 1988), a DBP-transactivator can be engineered which upon association with the operator-minimal promoter hybrid results in transcriptional activation. In either configuration, repression or activation of transcription is dependent upon the DBP binding its cognate promoter which can be controlled through the presence or absence of the DBP's relevant inducer. By convention, systems in which the presence of an inducer switches on transcription are referred to as ON-type systems whereas the opposite, where the presence of an inducer switches off transcription, are referred to as OFF-type systems (Kramer and Fussenegger, 2005b). The induction characteristics of such systems depend upon many features, such as the eukaryotic promoter used, the number and placement of operator sites, the selected transcriptional activation or repression domain, and finally the host-cell-type. By altering these features it is possible to rationally design gene control systems with differing characteristics (Malphettes et al., 2006, 2005; Weber et al., 2002b, 2002c).

To date, a variety of eukaryotic gene control systems responsive to different inducers have been developed. The first systems were based on well studied bacterial antibiotic response regulators sensitive to common antibiotics, such as tetracycline (Gossen and Bujard, 1992), streptogramins (Fussenegger et al., 2000), macrolides (Weber et al., 2002a) and coumermycin/novobiocin (Zhao et al., 2003). Subsequent systems have exploited response regulators sensitive to other signaling molecules, metabolic compounds and drugs, such as butyrolactones (quorum-sensing) (Weber et al., 2003), rapamycin (immunosuppressive drug) (Rivera et al., 1996), estrogen (hormone) (Braselmann et al., 1993), gaseous acetaldehyde (Weber et al., 2004), and 6-hydroxy-nicotine (Malphettes et al., 2005) amongst others. A comprehensive overview of these and other gene control systems can be found in several recent reviews (Weber and Fussenegger, 2004a, 2004b, 2006). However, given the low interference of many antibiotics with eukaryotic biology, and that different antibiotic-responsive systems can be readily combined with minimal cross-interference, it is these systems that have correspondingly formed the molecular building blocks of most mammalian synthetic gene networks.

2.2. Autofeedback

Apart from simple binary ON/OFF transcriptional control systems, where a hetereologously expressed transcriptional controller activates or represses transcription of a gene of interest from a chimeric promoter, the simplest genetic networks consist of autofeedback motifs in which the transcriptional controller itself is expressed from its cognate promoter. Autofeedback describes the mechanism in which a protein, directly or indirectly, influences its own production. Through differences in genetic architecture, autofeedback mechanisms can either be positive, in which a protein stimulates its own production, or negative, in which a protein inhibits its own production. In mammalian systems, autofeedback mechanisms have been used to engineer simple one-step regulatable expression constructs responsive to one or more inducers, thus minimizing the need for multi-engineering approaches (Fussenegger et al., 1997; Moser et al., 2001). For example, in one such system a single genetic construct containing a tetracycline-regulatable promoter ($P_{hCMV*-1}$) drives expression of a multicistronic expression unit containing the desired product genes followed by a gene encoding the tetracycline-responsive transactivator (TetR-VP16). In the presence of tetracycline, the system is switched off and exhibits minimal product gene and TetR-VP16 expression. However, upon tetracycline removal the positive feedback loop is

activated which leads to higher TetR-VP16 expression levels with concomitant higher expression of all other cistrons encoded on the same multicistronic construct (Fussenegger et al., 1997). Unfortunately, compared to two-component systems, autoregulated systems generally exhibit slightly poorer inductions characteristics with generally lower maximum expression levels and higher basal leakiness. For some applications this poorer performance may be critical to their operation, yet for others the convenience of a single-step genetic manipulation may more than offset any losses in induction characteristics.

The importance of autofeedback mechanisms in gene control is supported by evidence that approximately 40% of known transcription factors in *E. coli* utilize negative autoregulation to control their own production (Thieffry et al., 1998), while eukaryotic transcription factors use both positive and negative autoregulation to control expression levels (Bateman, 1998). In early work on synthetic genetic networks it was established that a practical function for such feedback networks is the creation of expression stability (Becskei and Serrano, 2000; Savageau, 1974). Thus, expression systems harboring an autofeedback loop for the expression of a transcriptional controller exhibit up to a three-fold reduction in expression variation across a population of cells compared to isogenic systems lacking feedback control (Becskei and Serrano, 2000). Stability is an intrinsic property of many gene networks and stands in contrast to a "classical" gene regulation system where even modest fluctuations in regulatory components can significantly impact expression levels (Becskei et al., 2001; Becskei and Serrano, 2000). Systems that are capable of remaining close to a steady-state, despite the influence of considerable variation and random perturbation, are said to be stable. A wide range of biological processes, from metabolic homeostasis to cellular growth and development, must be capable of withstanding a certain degree of variation and difference in order to produce a unified and consistent outcome (Alon et al., 1999; Barkai and Leibler, 1997; Little et al., 1999). Hence, it is not surprising to find that the synthetic networks constructed to date, that have required some element of expression stability, have all involved either a direct or indirect feedback mechanism (Gardner et al., 2000; Kramer and Fussenegger, 2005a; Kramer et al., 2004b).

2.3. Logic gates

In integrating multiple signals to generate either ON or OFF outputs it is easier to conceptualize a gene regulatory network as analogous to electronic circuitry (McAdams and Shapiro, 1995). From an architecture perspective, genetic networks possess a modular structure that can be interconnected in varying ways to produce different outcomes each of which follows a strict logic. This has been demonstrated by combining either in series or in parallel several compatible ON-type or OFF-type transcription control systems responsive to either macrolide, streptogramin or butyrolactone input signals (Fig. 2) (Kramer et al., 2004a). It is possible to describe the resulting logic of these network combinations, which integrate two independent signals, using boolean operator truth tables. Thus, a network in which expression (i.e. output) only occurs IF input 1 is present and input 2 is NOT present is classified as a NOTIF gate. In a NAND gate, output always occurs unless both inputs are present. An OR gate describes an operation whereby expression occurs if either input is present. The opposite, where expression is repressed if either input is present reflects a NOR gate. Finally, an INVERTER gate, which is the opposite of a NOT gate, occurs when expression is only repressed if input 1 is present and input 2 is absent. In each of these cases, different outcomes to the same two input signals have been engineered by selection and rearrangement of simple transcriptional control units (Kramer et al., 2004a). Such engineered mechanisms would clearly benefit any application that requires a particular response to highly specific inputs. While the above networks were rationally designed to produce the required logic patterns, it is conceivable that other novel and potentially useful logic patterns could be created by varying the connectivity of transcription control units in hitherto unknown ways. To this end, either random recombination or directed evolutionary approaches could be used to generate novel networks as has been demonstrated for synthetic prokaryotic networks (Guet et al., 2002; Yokobayashi et al., 2002).

2.4. Time-delay circuit

To continue the electronic circuit analogy, the above logic gates with their trigger-inducible transcriptional controls can be considered the equivalent of simple transistor-like switches. In electronics, the creation of a wide variety of *in silico* devices with highly complex information processing capabilities have been enabled by the sequential wiring of transistors with other electronic circuit components, such as diodes, capacitors and resistors. By using a combination of a trigger-inducible transcriptional device together with protein-modification mechanism it has been possible to emulate such electronic devices to engineer a synthetic time-delay circuit (Weber et al., 2007). In nature, time-delay circuitries at a transcriptional level have been identified in a range of processes including quorum-sensing cross-talk (Basu et al., 2005; Viretta and Fussenegger, 2004), NF-κB activation (Covert et al., 2005) and circadian clock rhythms (Bratsun et al., 2005; Forger and Peskin, 2003).

The synthetic time-delay circuit (Fig. 3) consists of a separated and modified tetracycline transactivator (TetR and VP16) in which BirA-mediated ligation of biotin (vitamin H) is required for the two components of the transactivator to dimerize and activate transcription (Weber et al., 2007). As the biotinylation reaction is irreversible, the presence of biotin leads to an accumulation of biotinylated and dimerized transactivator which retains its activity after the removal of biotin from the culture medium, until it is ultimately degraded by proteolysis. In this manner, the accumulation of biotinylated and dimerized transactivator and its subsequent degradation represent the electronic equivalents of a capacitor and resistor, respectively. When the capacitor was fully charged, through pre-incubation with biotin, reporter expression levels remain relatively unchanged for approximately 30 h following biotin removal. At this point however, the capacitor became effectively discharged after which reporter production

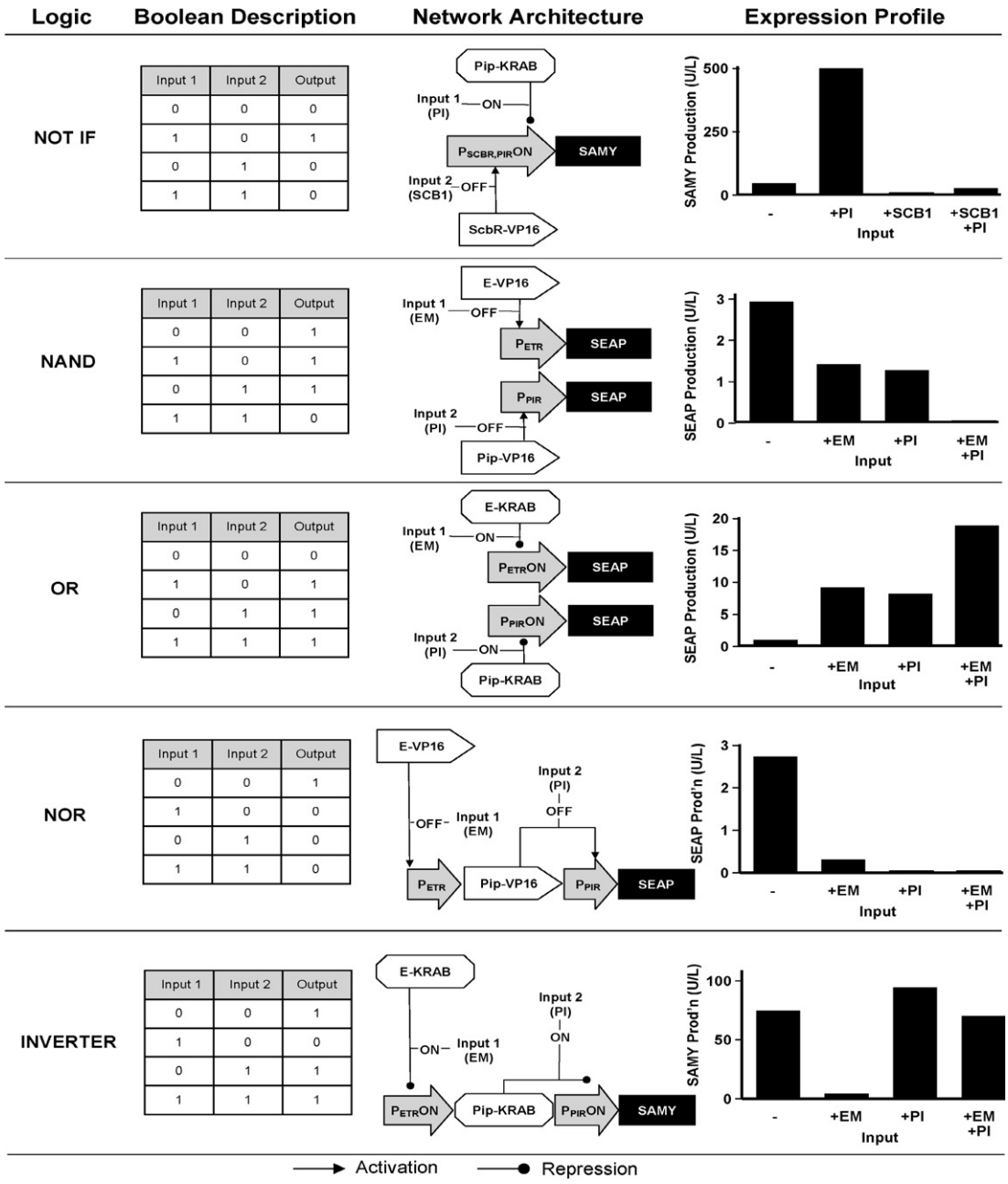

Fig. 2. Transcription-based logic gates in which modular transcriptional units are re-arranged to yield expression profiles that follow strict Boolean logic. For each gate, the expression profiles of each synthetic system reflect the input and output characteristics of the boolean description. Unless otherwise indicated, all of the depicted transactivators and transrepressors were constitutively expressed from P_{SV40} promoters. In the NOT IF gate, reporter gene expression (SAMY; *Bacillus stearothermophilus*-derived secreted α-amylase) is controlled from a chimeric promoter ($P_{SCBR,PIR}$) that contains operator sites for the butyrolactone-dependent transactivator (ScbR-VP16) and the streptogramin-responsive transrepressor (Pip-KRAB). Expression only occurs when ScbR-VP16 is bound to the chimeric promoter, which occurs in the absence of 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide (SCB1), and when Pip-KRAB is disassociated from the promoter which occurs in presence of pristinamycin (PI). For the NAND gate, reporter expression (SEAP; human placental-secreted alkaline phosphatase) is modulated from two promoters (P_{ETR} and P_{PIR}) that are separately modulated by a macrolide-dependent transactivator (E-VP16) and a streptogramin-dependent transactivator (Pip-VP16). Expression occurs when either or both transactivators are associated with their cognate promoters. Disassociation of E-VP16, which occurs in the presence of erythromycin (EM), and PIP-VP16, which occurs in the presence of pristinamycin (PI), results in no expression. The OR gate is similar in architecture to the NAND gate but uses the transrepressor versions (i.e. E-KRAB and Pip-KRAB) of the macrolide- and streptogramin-responsive transcription control systems. Expression occurs when either or both transrepressors are disassociated from their cognate promoters (P_{ETR}ON and P_{PIR}ON, respectively) which occurs in the presence of either or both of EM and PI. In the NOR gate, reporter expression is driven by a Pip-VP16-responsive promoter (P_{PIR}), with Pip-VP16 expression in turn under the control of a E-VP16-responsive promoter (P_{ETR}). In this short linear cascade, expression only results when both E-VP16 and Pip-VP16 are associated with their respective cognate promoters which necessitates the absence of both EM and PI, respectively. The INVERTER gate is identical in architecture to the NOR gate, but uses the E-KRAB and Pip-KRAB transrepressors and their respective cognate promoters, P_{ETR}ON and P_{PIR}ON, also arranged into a linear cascade. In this instance, expression always occurs unless both E-KRAB is disassociated from, and Pip-KRAB associated with, its respective cognate promoters. This only occurs in the presence of EM and absence of PI (Kramer et al., 2004a).

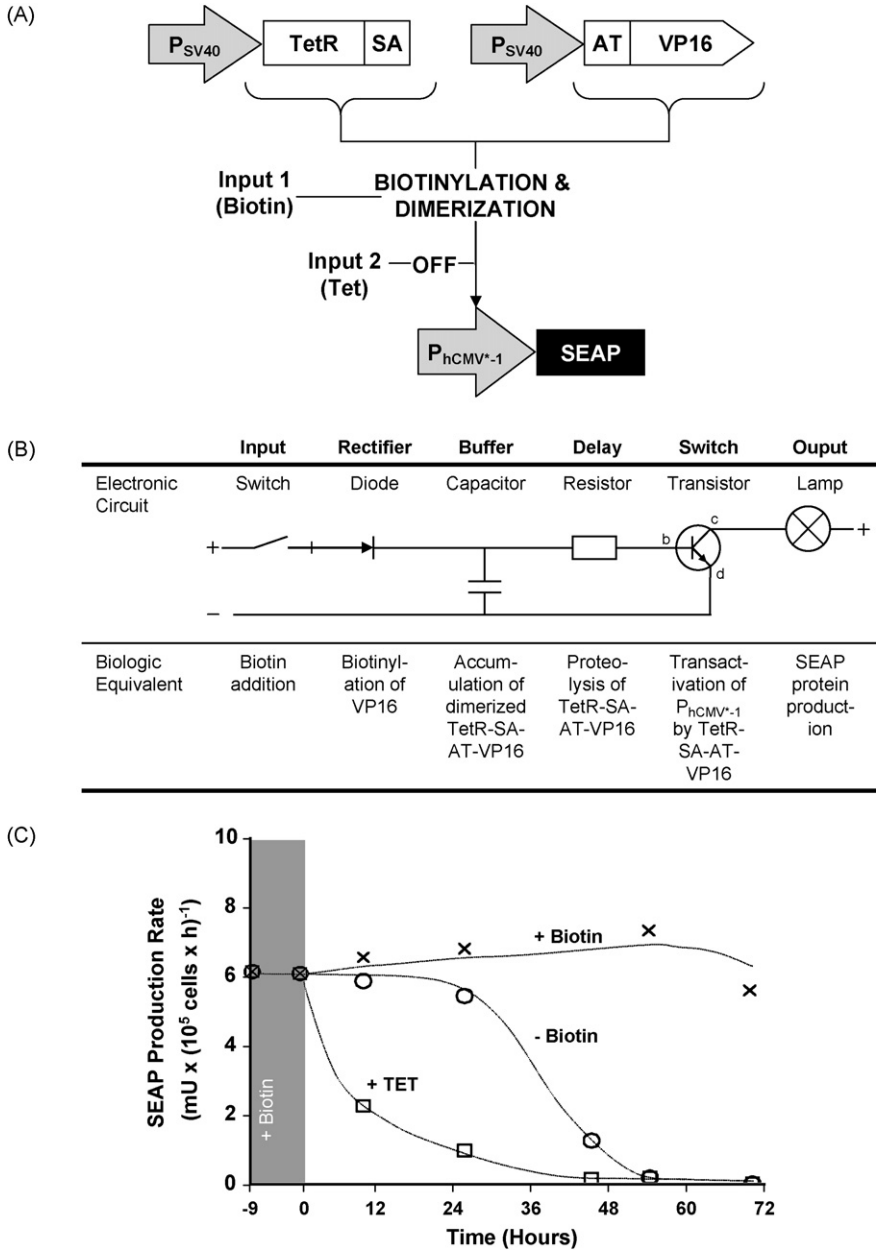

Fig. 3. Genetic and molecular design (A), electronic circuit depiction (B) and expression rates (C) of a synthetic time-delay network. The network consists of a modified chimeric TetR-VP16 transactivator and a TetR-VP16-responsive promoter ($P_{hCMV*-1}$) which drives expression of a reporter gene (SEAP; human placental-secreted alkaline phosphatase). In this case the two components of the transactivator (TetR and VP16) are separately and constitutively expressed from P_{SV40} promoters as fusion proteins with streptavidin (SA) and an AVITAG biotinylation signal (AT), respectively. Administration of biotin, together with ectopic expression of a heterologous *E. coli* BirA biotin ligase (not shown), causes irreversible biotinylation of AT-VP16 thereby enabling it to dimerize with TetR-SA which results in a functional TetR-VP16 transactivator that can be controlled with tetracycline (Tet). In an electronic time-delay circuit, activating a switch enables current flow through a diode into a capacitor that becomes charged. The capacitor's charge slowly dissipates to the base of the resistor (b) where it causes current to flow from collector (c) to emitter (e) where it ultimately results in activation of the output lamp. In the biological equivalent depiction, addition of biotin represents the initial input (switch) which drives VP16 biotinylation (diode) resulting in the accumulation of biotinylated VP16 which dimerizes with TetR-SA (capacitor). Biotinylated and dimerized TetR-SA-AV-VP16 is eventually degraded by proteolysis (resistor), but before which it binds $P_{hCMV*-1}$ resulting in promoter activation (transistor) and subsequent reporter expression (output). In the absence of biotin, VP16 is not biotinylated and the network remains silent (not shown). In the presence of biotin (+Biotin), the circuit's capacitor remains fully charged with SEAP expression rate maintained at a relatively constant level. Upon biotin removal however (−Biotin), the SEAP expression rate is initially maintained after which expression sharply declines. The delay in expression reduction represents the time required for the capacitor (biotinylated and dimerized TetR-SA-AV-VP16) to fully discharge (through proteolysis) In contrast, if TetR-SA-AV-VP16 activation of $P_{hCMV*-1}$ is switched off through Tet addition, the time-delay circuitry is effectively over-ridden with a corresponding immediate and acute reduction in SEAP expression levels (Weber et al., 2007).

markedly decreased. In addition, it was shown that the duration of the time delay could be modified by altering the capacitor or resistor characteristics. Hence, the delay could be shortened by either reducing the biotin pre-incubation time, thereby reducing the accumulation of functionalized TetR-VP16 and the capacitors charge, or by increasing proteolysis of the TetR-VP16 through attachment of an ubiquitinylation signal (PEST sequence) thereby increasing the capacitor's discharge rate through its resistor (Weber et al., 2007). Time-delay circuitries could potentially play a useful role in therapeutic applications requiring multiple sequentional interventions where a secondary or follow-up intervention is required at a precise point after an appropriate signal or event has occurred.

2.5. Transcriptional cascades

Both the NOR and INVERTER gates described above involved the serial linking of transcriptional units into regulatory cascades whereby the gene product of one regulatable promoter controls expression from a second regulatable promoter. Obviously it is possible to repeat this process and devise cascades involving multiple serially connected transcriptional units. This has been achieved by several groups using different combinations of transcriptional control units. Often the aim has been to reduce the residual leakiness of a regulatable promoter, thereby improving the degree of transcriptional control and opening the window of transgene regulation (Aubrecht et al., 1996; Imhof et al., 2000). Other approaches have sought to use a transcriptional cascade to engineer gene control systems in which target genes can be accurately and reliably titrated to multiple levels (Kramer et al., 2003; Krueger et al., 2006). The transcriptional co-operativity of many transcriptional control systems typically results in sigmoid-shaped dose-response characteristics where the range within which it is possible to find an intermediate level of expression (between ON and OFF) is relatively narrow. Much as dosing of pharmaceuticals is critical to their efficacy, it is conceivable that gene therapy applications will also require precise titration of a transgene into a therapeutic window. All or nothing control mechanisms, or at least systems which are not reliably capable of intermediate levels of adjustment, may accordingly be of limited use.

In one engineered system, the residual leakiness of three antibiotic-inducible regulation systems was exploited to create a three-level regulatory cascade capable of intermediate transgene expression (Fig. 4) (Kramer et al., 2003). The serial linking of the tetracycline, macrolide and streptogramin transactivators together with respective responsive promoters resulted in a network whereby each transactivator acted as the activator of the next system. Hence, addition of each respective antibiotic prevented transcription of the next component in the cascade. Control of the first transactivator in the cascade was achieved using an autofeedback loop. As each system and inducer operates at a different level in the cascade, the impact of transcription leakiness upon overall expression varies depending at which point in the cascade it occurs. Thus, at high-level points within the cascade, transcriptional leakiness is amplified by latter stages thereby limiting the extent of expression reduction. Closing the

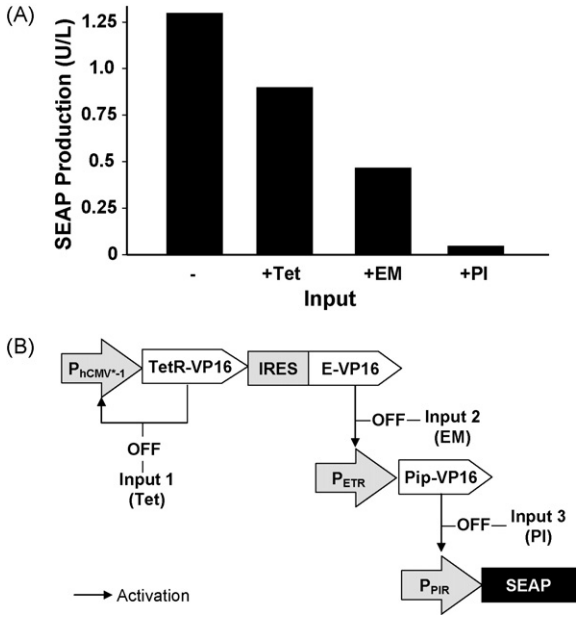

Fig. 4. Regulation performance (A) and genetic design (B) of a synthetic three-level regulatory cascade which consists of three linearly connected transactivator control systems. Reporter expression (SEAP; human placental-secreted alkaline phosphatase) is directly controlled by a Pip-VP16–responsive promoter (P_{PIR}), with Pip-VP16 expression itself under the control of an E-VP16-responsive promoter (P_{ETR}). E-VP16 is di-cistronically expressed, via an internal ribosome entry site (IRES), together with TetR-VP16, from a TetR-VP16-responsive promoter ($P_{hCMV*-1}$). TetR-VP16 thus controls its own expression via a positive autofeedback loop. The cascade can be controlled at each of the three levels by administration of the relevant inducer molecule; tetracycline (Tet) for TetR-VP16, erythromycin (EM) for E-VP16 and pristinamycin (PI) for Pip-VP16. Administration of Tet closes the autofeedback loop at the top of the cascade. However, residual leakiness in the system ensures that the cascade is not completely shut-off with overall expression still at approximately 70% of maximum expression. Closing the cascade at subsequent levels, either through EM or PI administration, has a progressively greater impact upon overall expression as the scope for amplification of residual leakiness is progressively reduced. Thus, EM administration reduces expression to approximately 40% whereas PI administration reduces expression within the system to almost baseline levels (Kramer et al., 2003).

cascade at lower levels has a progressively greater impact upon overall expression as the scope for amplification of residual leakiness is progressively reduced. As each intervention utilizes a different inducer it was possible to select the desired intervention point. Thus, expression levels of 100% (no cascade intervention), 70% (intervention at first level of cascade), 40% (intervention at second level) and close to 0% (intervention at third and final level) of transgene were possible (Kramer et al., 2003).

2.6. Bistable expression networks

Logic gates, time-delay circuits and transcriptional cascades show that engineered genetic circuits can process information or cues received from the environment in different ways to produce specific outcomes. A major further step forward in the evolution of synthetic engineering has been the engineering of circuits which not only process information currently available in the environment, but which can store information on the past.

To do so, a network needs to possess "memory" which necessitates that the network is capable of binary expression, that it exhibits bistability and that it is self-sustaining (Hasty et al., 2002).

In a typical graded transcriptional response there is no clear separation of expression states (Fig. 5A) Input changes in a classed graded system, for example through an increase in the concentration of an inducer, typically results in a continuous (or graded) sigmoid-shaped transcriptional response. This pattern reflects an effect known as co-operativity and can be due to either response regulator multimerization and/or co-operative binding, where initial binding enhances subsequent binding, of responsive regulators to multiple operator sites in the promoter (Kringstein et al., 1998). Importantly, when viewed across a population of cells, increasing the level of inducer does not alter the unimodal distribution pattern of expressing cells, but simply shifts the distribution upwards reflecting an overall increase in expression across the entire cell population. Using a simple autofeedback mechanism, in which a transactivator is transcribed from its own cognate promoter, it has been demonstrated that feedback loops can convert a graded transcriptional response into a binary expression read-out (Becskei et al., 2001). Feedback prevents cells from adopting an intermediary expression status such that they can only reside in an ON or OFF state. In this case, addition of an inducer does not shift the distribution of a population of cells, but rather it changes the proportion of cells residing in either state. While expression has been delineated into two or more states, such a system is still not capable of memory as cells can randomly flip between states. A further requirement is a property referred to as bistability where cells remain in the state they are being induced to. Finally, a network can only possess memory where it remains in an expression state long after the stimulus used to force it into that state has been removed. Such a self-sustaining mechanism is analogous to a typical light switch or toggle. Switching a light ON or OFF only requires a single transient, rather than a persistent, input.

2.6.1. Toggle switch

Employing the same network architecture used in the synthetic *E. coli* toggle switch (Gardner et al., 2000), a synthetic mammalian toggle switch capable of self-sustaining bistable expression has been created (Fig. 5B) (Kramer et al., 2004b). The switch was constructed from two inducible transrepressors; the streptogramin-responsive Pip-KRAB system (Fussenegger et al., 2000) and the macrolide-responsive E-KRAB system (Weber et al., 2002a), which are inducible by pristinamycin and erythromycin, respectively. Each transrepressor was transcribed from the other transrepressor's cognate promoter thereby establishing a mutually inhibitory arrangement where each repressor inhibited the promoter of the opposing repressor. By co-cistronically encoding a reporter gene downstream of the Pip-KRAB repressor it was possible to monitor which repressor was currently active, and accordingly determine the expression status of the network. Owing to the mutually inhibitory arrangement of the two repressor genes the network was capable of one of two binary states. A HIGH response in which Pip-KRAB and the downstream reporter gene are transcribed from the P_{ETR}ON promoter, and a LOW response in which E-KRAB is transcribed from the P_{PIR}ON promoter. In the absence of either inducer molecule, any low or leaky expression from one control system ensures repression of the other system. The network is balanced as long as both systems exhibit the same (low) expression levels with neither expression system able to predominate over the other. However, addition of either inducer molecule effectively tips the balance such that expression from one system is increased while expression from the other system is concomitantly further repressed. For example, the addition of erythromycin results in disassociation of E-KRAB from the P_{ETR}ON promoter thereby de-repressing the promoter and causing active transcription of Pip-KRAB. Once, Pip-KRAB expression reaches a sufficient level, it represses expression of E-KRAB from the P_{PIR}ON promoter. As the expression of Pip-KRAB over E-KRAB becomes self-perpetuating, erythromycin can be withdrawn and the network continues indefinitely in its altered state. In this manner, the network behaves as a bistable "toggle" switch in which the maintenance of either LOW or HIGH expression state does not require the on-going presence of pristinamycin or erythromycin, respectively. In addition, it was also demonstrated that the status of the toggle could be maintained across cell-generations thus indicating that network memory could be passed to progeny cells, and that the expression profile could be repeatedly switched between expression states over a 2-week period thus also indicating that the system was fully reversible (Kramer et al., 2004b).

Sustained expression stability and reversible switching are two key features required for epigenetic memory. Such memory involves sustained imprinting of differential expression levels, including to subsequent cell-generations, as well as switching between expression levels in response to appropriate stimuli. A number of natural epigenetic switches have been described in different organisms and play a role in coordinating diverse processes, such as cell fate and memory (Orlando, 2003), plant development (Kohler and Grossniklaus, 2002) and lysogeny (Angeli et al., 2004; Casadesus and D'Ari, 2002). Apart from establishing a model for how epigenetic imprinting may occur in multicellular organisms, the synthetic mammalian switch may also have important therapeutic applications. Classical transcription control systems operate in a dose-dependent manner and require the on-going presence of regulating molecules to sustain transgene expression levels. Prolonged exposure to regulating molecules (e.g. antibiotics) can be associated with clinical ramifications, such as the selection of pathogen resistance (Wegener et al., 1997) and the accumulation of antibiotics in bone and teeth (Kapunisk-Uner et al., 1996). A self-sustaining, yet reversible, genetic network which requires only a transient stimulus to establish a steady-state may provide an attractive means of overcoming such considerations. The toggle described above represents an important first step towards generating an epigenetic "memory" device. However, to reach its potential and be of therapeutic use, more work to improve the induction performance (i.e. lower OFF leakiness and/or higher ON expression) is required.

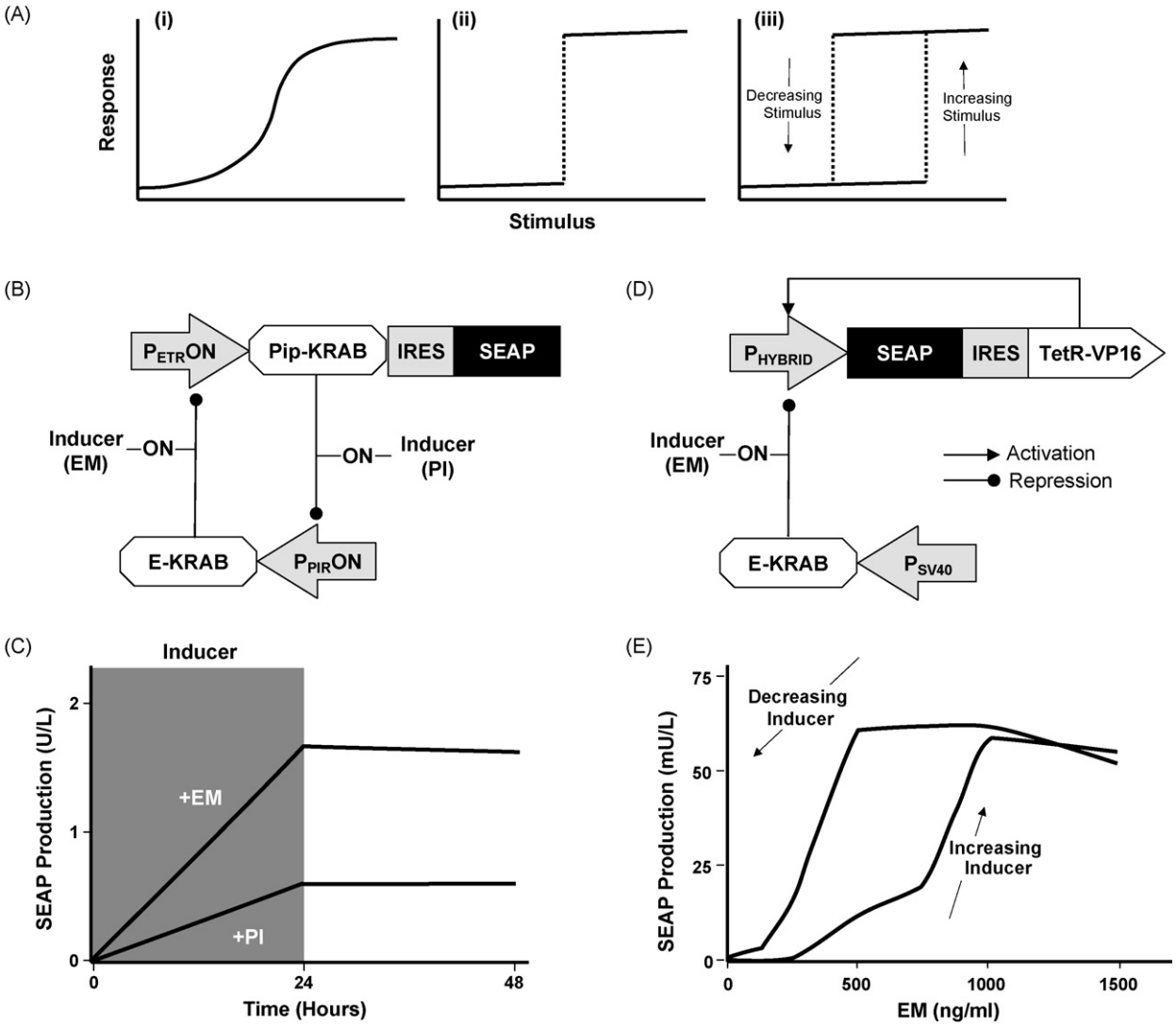

Fig. 5. Engineering expression stability. (A) Stimulus-response profiles for graded (i), generic bistable (ii), and hysteretic bistable (iii) genetic networks. In a graded genetic system an increasing stimuli is progressively converted into an increasing response which often exhibits a sigmoidal character due to activator or repressor co-operativity. In a generic bistable network the system exhibits quasi-discontinuous behavior whereby it only resides in one of two alternative steady-states. Changes in stimuli beyond a threshold point trigger a switch from one state to the other. Where a network not only possesses bistability, but remains in an expression state after the stimulus used to move into that state has been removed, it can then be said to possess memory and behaves analogously to a "toggle" switch. (B) Genetic architecture and (C) response profile of an engineered self-sustaining toggle. The toggle switch was assembled using two transrepressor control systems arranged to repress each other's expression. In the depicted configuration, the streptogramin-dependent transrepressor, Pip-KRAB, inhibits transcription via a Pip-KRAB-responsive promoter (P_{PIR}ON) of the erythromycin-dependent transrepressor E-KRAB. E-KRAB in turn inhibits transcription from an E-KRAB-responsive promoter (P_{ETR}ON) of a di-cistronic construct encoding Pip-KRAB and a downstream reporter gene (SEAP; human placental-secreted alkaline phosphatase) translated via an internal ribosome entry site (IRES). Administration of each transrepressors respective inducer, pristinamycin (PI) for Pip-KRAB and erythromycin (EM) for E-KRAB, inhibits the repressive effect of the responsive transrepressor. Thus, addition of EM results in co-expression of Pip-KRAB and the reporter gene with concomitant repression of E-KRAB (a HIGH response), whereas addition of PI results in expression of E-KRAB with concomitant co-repression of Pip-KRAB and the reporter gene (a LOW response). In each case only a transient pulse of inducer is required to enable the opposing repressor to be maximally transcribed until, in a self-perpetuating manner, it stably represses the originally active promoter. Both the HIGH and LOW responses are maintained in a steady-state following removal of relevant inducer molecules (non-shaded region) (Kramer et al., 2004b). A hysteretic bistable network is one in which the threshold switching point from one state to the other is not constant but differs according to which state the system is moving from. (D) Genetic architecture and (E) response profile of an engineered hysteretic switch. The switch utilizes a chimeric promoter (P_{hybrid}) that is responsive to both a tetracycline-dependent transactivator (TetR-VP16) and an erythromycin-dependent transrepressor (E-KRAB). P_{hybrid} di-cistronically encodes a reporter gene (SEAP) and, via an internal ribosome entry site (IRES), TetR-VP16 thereby creating a positive autofeedback loop. E-KRAB is expressed from an independent constitutive promoter (P_{SV40}) and inhibits P_{hybrid} in a erythromycin (EM) dose-dependent manner. The response of the network to different EM concentrations is dependent upon the network's EM cultivation history. Thus, a higher concentration of EM is required to switch the system from OFF to ON than is required to return the system from an ON to OFF state (Kramer and Fussenegger, 2005a).

2.6.2. *Hysteretic switch*

Bistable switches can be characterized by their quasi-discontinuous movement between expression states once a controlling stimulus has reached a specific threshold. However, an alternate switch behavior found in natural biology is the hysteretic switch (Ozbudak et al., 2004). Hysteresis is a phenomenon that occurs at both the molecular and macroscopic levels and essential describes behavior in which the threshold

required to switch from one state to another varies depending upon the starting state. To use a non-biological example, traffic jams often exhibit hysteresis because the car density required to alleviate the traffic jam is less than the density which initially caused the jam. In a genetic network, a switch exhibits hysteresis when a different concentration of inducer is required to shift a system from one state to another than is required for the reverse shift (Angeli et al., 2004; Atkinson et al., 2003; Becskei et al., 2001; Becskei and Serrano, 2000; Gardner et al., 2000; Xiong and Ferrell, 2003). Hysteretic behavior has been observed in several natural examples including the control of lactose utilization in *E. coli* (Ozbudak et al., 2004), and ensuring unidirectional cell-cycle progression in eukaryotes (Sha et al., 2003). A significant advantage of a hysteretic system is its inherent ability to buffer against modest changes in the inducing molecule. Hence, minor fluctuations in inducer concentration close to the point at which a system in switched on will ultimately result in the system being fully turned on as the system is not able to turn off unless the inducer concentration shifts far enough to enable the reverse switch. Such devices could have broad potential for applications in which the input signal is prone to minor fluctuations but for which a constant all or nothing expression status is required.

A synthetic hysteretic switch has been constructed in mammalian cells using a positive autofeedback mechanism and competitive transcriptional regulation (Fig. 5B) (Kramer and Fussenegger, 2005a). The system involved the juxtaposition of a tetracycline-dependent transactivator (TetR-VP16), which induces its own transcription via positive feedback together with a reporter gene, against a competing erythromycin (EM)-dependent transrepressor (E-KRAB) which was capable of inhibiting the TetR-VP16-mediated positive feedback. The hysteretic behavior of the synthetic network results from the competitive interaction of TetR-VP16 and E-KRAB for the same promoter (P_{hybrid}; promoter containing operator sites for both TetR and E binding). At high EM concentrations, disassociation of E-KRAB from P_{hybrid} enables TetR-VP16 to prevail resulting in positive feedback and high reporter expression (i.e. an ON configuration). At low EM concentrations E-KRAB binds P_{hybrid} and inhibits both TetR-VP16 positive feedback and reporter expression (i.e. an OFF configuration). It is only at intermediate EM concentrations that the prevalence of E-KRAB mediated inhibition versus TetR-VP16 mediated positive feedback depends upon historical EM concentration. High historical EM concentration ensures a high level of TetR-VP16 is present which therefore requires greater E-KRAB activity, and correspondingly lower EM concentration, to drive the expression state from ON to OFF. The converse applies for low historical EM concentrations where minimal to no TetR-VP16 is present. For TetR-VP16 to out-compete E-KRAB, full de-repression of all E-KRAB activity is required which is achieved through a relatively much higher EM concentration. In this process the level of active TetR-VP16, and therefore the extent of positive feedback, acts a molecular "memory" of the historical EM concentration of the system, and is the basis for the observed hysteretic behavior. Indeed, if the level of active TetR-P16 in the system is altered and the extent of positive feedback reduced, for example through tetracycline addition, then the EM concentration required to switch the system between ON and OFF configurations gradually changes from hysteretic to a simple graded profile (Kramer and Fussenegger, 2005a).

The synthetic networks described above show that expression stability and memory can be generated from either a double negative feedback loop or a single positive feedback loop. The importance of feedback loops have long been recognized as essential for many cellular processes and is increasingly being identified in natural biological systems, including signaling pathways (Angeli et al., 2004). The maturation of *Xenopus* oocytes, for example, involves the p42 mitogen-activated protein kinase (MAPK) and the cell-division cycle protein kinase Cdc2 which form positive autofeedback loops. Both mediators generate an irreversible switch-like response following transient stimulation with the steroid hormone progesterone. If the feedback loops are selectively disrupted using specific inhibitors, progesterone-induced maturation can still occur, however, the presence of progesterone must be actively maintained. Thus, following disruption of positive feedback the ability of the system to "remember" a transient signal is compromised (Ferrell, 2002; Xiong and Ferrell, 2003).

2.7. Oscillator

The aforementioned networks all generate useful functions by creating expression stability. An alternate, and equally fascinating, behavior can result from dynamic instability. In an oscillatory network, a constant flux of transcriptional components results in periodic, as opposed to stable, expression. Ideally, such periods are of a consistent period and amplitude, and require minimal to no external stimuli. Such network behavior is found in a wide range of natural systems from archaebacteria to eukaryotes with the most prominent example being the circadian rhythm (Schibler and Sassone-Corsi, 2002). In humans, a range of phenomena from body temperature modulation, endocrine production and release, and immune responses exhibit circadian oscillations (Edery, 2000). Circadian clocks have been proposed to consist of autoregulatory loops that use transcriptional feedback and high protein decay rates to maintain 24 h periodicity (Dunlap, 1999; Reppert and Weaver, 2002; Young and Kay, 2001). From an applied or therapeutic use, an oscillatory network could clearly be used in situations requiring recurrent dosing of a therapeutic gene.

A synthetic oscillator (termed the "repressilator") has been constructed in prokaryotes from three common bacterial transcriptional repressor systems that are not part of any natural biological clock mechanism (Elowitz and Leibler, 2000). The three repressor systems were interconnected such that they formed a cyclic negative feedback loop or "daisy-chain." This configuration produced oscillating levels of each repressor protein. A reporter gene, carried on a separate plasmid but also under the control of a promoter induced by one of the repressors, provided a readout of oscillations for that repressor. Unlike other expression functions, the development of a synthetic mammalian oscillator has not yet mirrored the creation of the bacterial equivalent, although given the pattern for these developments, it will

not be surprising to see the emergence of a synthetic eukaryotic network in the near future. However, given the intense interest in understanding the mechanisms responsible for the natural circadian clock, it is also not surprising that attempts have been made to create a synthetic clock from actual clock components. An attempt was made to create a synthetic clock from the core set of positive and negative regulatory elements common to all known circadian mechanisms (Chilov and Fussenegger, 2004). The core set of molecular players are believed to include the *cryptochrome* genes CRY1 and CRY2, the *period* genes PER1, PER2 and PER3, and the positive transcription factors BMAL1 and CLOCK (Gekakis et al., 1998; Sato et al., 2006; van der Horst et al., 1999). BMAL1/CLOCK drive the expression of CRY and PER proteins which accumulate and, upon exceeding a certain threshold level, translocate to the nucleus where they inhibit BMAL1/CLOCK expression as well as their own expression. Thus, CRY and PER proteins act as negative regulators of their own production until BMAL1/CLOCK mediated transcription inhibition is abolished by PER and CRY degradation (Panda et al., 2002). An attempt was made to design a generic oscillator by reproducing the inducible expression of BMAL1 and CLOCK on a "positive" regulation plasmid and the expression of PER and CRY proteins under the control of a BMAL1 and CLOCK inducible promoter on a "negative" regulation plasmid. While not capable of producing sustained oscillations, the system did nevertheless exhibit a single cycle of a clock-like oscillation, and therefore opens the possibility that homologous regulatory components can be used for synthetic constructions. While not yet successful, it can nonetheless be concluded that the design and creation of a successful mammalian clock will necessitate the incorporation of some kind of feedback mechanism. Such a conclusion is supported by recent experimental analysis of the mammalian clock system where directed disruption of CRY-mediated transcriptional autorepression resulted in arrhythmic phenotypes in both single- and multi-cell populations (Sato et al., 2006).

3. Post-transcriptional gene networks

The engineered regulation of gene expression need not only occur at the transcriptional level, and indeed can be engineered at almost any stage in the protein biosynthetic pathway (Fussenegger, 2001). Further downstream of transcription control, but still at the mRNA level, expression can be controlled either through destruction of mRNA template or by impairing translation (Malphettes and Fussenegger, 2004; Schlatter and Fussenegger, 2003; Schlatter et al., 2003).

3.1. Post-transcriptional gene silencing

The classic method for post-transcriptional silencing relies upon the inducible expression of antisense RNAs which lead to translation-inaccessible mRNA duplexes and mRNA degradation (Fux et al., 2001; Singer et al., 2004). However, as RNA duplexes greater than 30 base pairs in length are capable of inducing interferon-based antiviral responses in mammalian organisms which result in global inhibition of mRNA translation, their application is of limited use (Luo et al., 2004). More recent strategies capitalize on the mRNA degradation program known as RNA interference (RNAi) in which double stranded RNA complementary to the transcript being silenced is processed by the nuclease DICER into single-stranded molecules of 21–25 nucleotides which subsequently prime a conserved multi-protein complex known as the RNA-induced silencing complex (RISC). The outcome is the highly specific degradation and or translation inhibition of the targeted transcript (Tuschl, 2001; Tuschl et al., 1999). However, unlike the antisense RNA approach, effective silencing can be mediated through the expression of much smaller, typically 19–21 base pairs in length, short interfering RNA (siRNA) which do not generate an interferon response in mammalian cells (Elbashir et al., 2001). Inducible siRNA expression can be engineered by using classic transcription control systems which utilize appropriately modified promoters (Malphettes and Fussenegger, 2004). In this way, inducible siRNA expression systems have been developed for tetracycline and macrolide control systems, amongst others (Kuninger et al., 2004; Malphettes and Fussenegger, 2004).

In addition to silencing genes in which the siRNA target region is contained within the coding transcript, it is also possible to silence genes with equal effectiveness in which the siRNA target is contained in either the 5′ or 3′ non-translated region of the mRNA transcript (Mangeot et al., 2004). This provides the opportunity to regulate or silence any transgene in which an appropriate siRNA target (designated as a TAG) has been encoded within an associated non-translated region (Malphettes and Fussenegger, 2006a). Exploiting this feature, it has been possible to engineer a combined transcription-translation network that utilizes both transcriptional activation as well as gene silencing (Fig. 6) (Malphettes and Fussenegger, 2006b). In this system, an erythromycin-inducible control system was used to express an siRNA capable of silencing a TAG containing reporter gene that was expressed under the control of the classic tetracycline-responsive promoter ($P_{hCMV*-1}$). Despite its widespread use, $P_{hCMV*-1}$ nonetheless exhibits residual or leaky expression even in the complete absence of its disassociated transactivator. By co-expressing an siRNA against the reporter construct it was possible to reduce any residual expression to basal levels. Importantly, by ensuring siRNA expression was controllable, it was possible to repress siRNA expression and thereby still achieve maximum reporter expression levels. Thus overall, the engineered network creates not only a system with improved regulation performance, but also one in which expression levels can be fine-tuned (Malphettes and Fussenegger, 2006b).

3.2. Modulation of translation initiation

Although much progress has been made in understanding and manipulating protein expression at the transcriptional level, the manipulation of systems operating at the translational level is far less advanced. Indeed, it is only recently that natural systems operating at the translational level – which, for example, play a key role in developmental and cell-cycle regulatory networks – have been described (Pyronnet and Sonenberg, 2001). Several

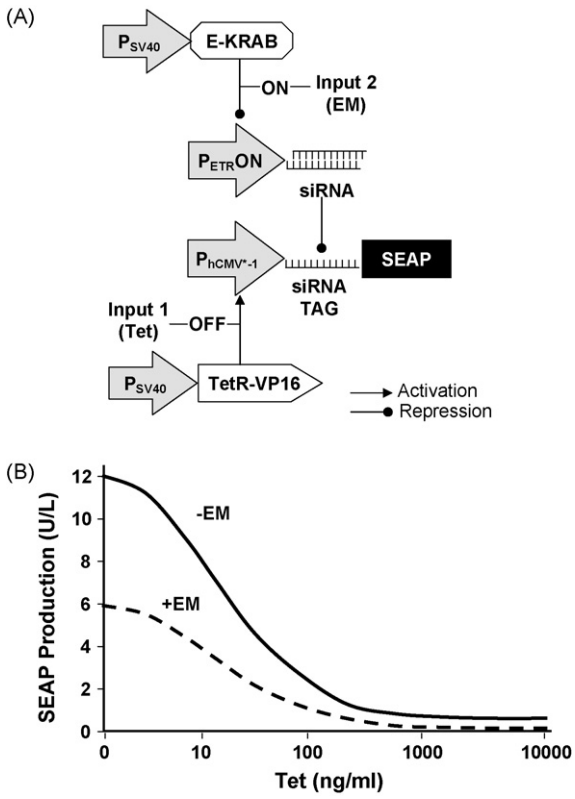

Fig. 6. (A) Genetic design and (B) response profile of a coupled synthetic transcription-translation network. A TetR-VP16-responsive promoter ($P_{hCMV*-1}$) that is responsive to tetracycline (Tet) is used to drive expression of a reporter gene (SEAP; human placental-secreted alkaline phosphatase) that contains, within its 5′ non-translated region, a siRNA specific target sequence. Expression of the corresponding siRNA, from an E-VP16-responsive promoter (P_{ETR}) that is responsive to erythromycin (EM), primes the siRNA specific target, together with SEAP, for RNA interference mediated gene silencing. Both TetR-VP16 and E-VP16 are constitutively expressed. SEAP expression can subsequently be controlled at the transcriptional level via Tet addition, or at the translational level by mRNA reporter silencing through EM withdrawal. Importantly, the residual leakiness of $P_{hCMV*-1}$ in the OFF state (+Tet) can be reduced to baseline levels through inducible siRNA-mediated silencing (Malphettes and Fussenegger, 2006b).

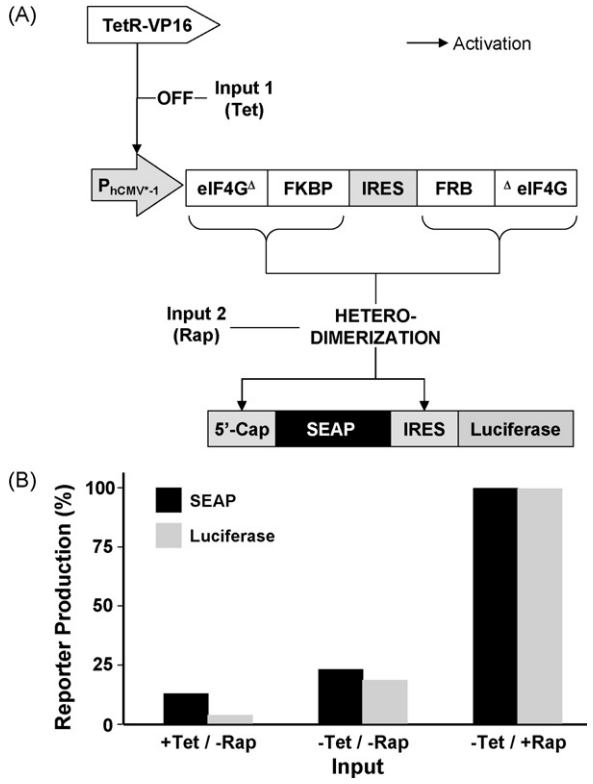

Fig. 7. Genetic and molecular design (A) and expression read-out (B) of a translation control network. The network consists of a tetracycline-responsive transactivator TetR-VP16 (produced constitutively from a P_{SV40} promoter; not shown) which drives expression, via a TetR-VP16-responsive promoter ($P_{hCMV*-1}$), of a discistronic construct encoding truncated versions of translation initiation factor 4G (eIF4G$^{\Delta}$ and $^{\Delta}$eIF4G) which have been fused C- and N-terminally to the FK506-binding protein (FKBP), and FKBP-rapamycin-binding domain (FRB) of the FKBP-rapamycin-associated protein, respectively. In the presence of rapamycin (Rap), eIF4G$^{\Delta}$-FKBP and FRB-$^{\Delta}$eIF4G heterodimerize to form a functional chimeric elongation factor 4G capable of modulating translation initiation of a 5′-cap dependent reporter gene (SEAP; human placental-secreted alkaline phosphatase), as well as a cap-independent (translation initiation by internal ribosome entry site, IRES) reporter gene (*Photinus pyralis* luciferase) both of which are di-cistronically transcribed from a constitutive P_{SV40} promoter (not shown). The network allows reporter gene expression to be controlled at the transcriptional level by the addition of tetracycline (Tet), which switches off expression of the eIF4G truncated fusion proteins, or at the level of translation initiation through the addition of Rap which results in functional eIF4G (Schlatter et al., 2003).

attempts have also been made to engineer synthetic translational networks.

In one such network, the translation initiation factor 4G (eIF4G), which is required for ribosome assembly at either 5′-cap or IRES elements, was engineered to render its functionality rapamycin-dependent thereby creating a mechanism for directly controlling translation initiation (Schlatter et al., 2003) (Fig. 7). The network was based upon the molecular events underlying picornaviral infection in which translation of host-cell proteins is almost completely shut-down resulting in re-direction of protein translation to picornaviral proteins. This shut-down primarily occurs through the proteolytic cleavage of eIF4G into truncated components which no longer possess their translation initiation functionality. By separately expressing the truncated versions of eIF4G as fusions with appropriate rapamycin-inducible heteodimerization domains, it was possible to reconstitute functional eIF4G, and thereby restore translation initiation, through the addition of rapamycin. Furthermore, by placing the expression of the truncated eIF4G fusions under tetracycline-responsive transcription control, a coupled and multi-input transcription-translation network was created (Schlatter et al., 2003).

In an alternative network, the translation control elements responsible for ribosomal protein production during *Xenopus* maturation were manipulated to create a generic mammalian translation control system (Schlatter and Fussenegger, 2003). In *Xenopus*, translation control of ribosomal protein encoding mRNAs is determined by a terminal oligopyrimidine element (TOP) present in the 5′ untranslated region of the mRNA. TOP elements adopt a secondary structure that prevents ribosome binding and initiation of translation. However, this block can be overcome by the binding of various protein factors, such as cellular nucleic acid binding protein (CNBP) and La autoantigen,

to the TOP element, or by interaction with specific anti-TOP oligodeoxynucleotides. By designing a system based on the specific interactions of CNBP, La or anti-TOP oligos, with a TOP-containing reporter gene, it was possible to adjustably and reversibly control translation initiation in engineered mammalian cells (Schlatter and Fussenegger, 2003).

While not yet developed in mammalian systems, an exciting area of post-transcriptional network engineering will take advantage of recently developed riboregulators in which small *trans*-acting RNAs can be used to regulate gene expression in a ligand-dependent manner (Bayer and Smolke, 2005; Isaacs et al., 2004). Riboregulators serve to prevent translation of target mRNA transcripts by binding and forming secondary structures with the mRNA thereby preventing ribosome binding. Addition of an appropriate ligand disrupts this association resulting in a resumption of translation. Importantly, these riboregulators can be custom-engineered to recognize a wide variety of ligands which open the possibility to design networks that sense specific cellular and environmental stimuli.

4. Host-interfaced gene networks

The majority of synthetic genetic networks built and characterized to date have utilized external signals to create a desired function. To reach their therapeutic potential, however, it will be necessary to design networks which are not only capable of responding to external signals, but also to endogenous or physiological signals. Hence, one can imagine sophisticated networks which independently provide a therapeutic outcome in response to pathological signals, but can also be over-ridden or altered through external modulation should the need arise. While still in their infancy, several systems integrating physiological signals – so called "semi-synthetic" systems – have already been developed.

To date, several semi-synthetic systems have been designed which interface various physiological or signaling inputs into synthetic bacterial networks (Farmer and Liao, 2000; Fung et al., 2005; Kobayashi et al., 2004). Progress has also been made in developing mammalian semi-synthetic systems. The mammalian oxygen response system, in which a specific set of endogenous genes is induced in response to low-oxygen levels (e.g. human vascular endothelial growth factor, VEGF), relies upon the translocation of hypoxia-induced factor 1 alpha (HIF-1α) to the nucleus where through a series of interactions it activates expression from promoters containing hypoxia-response elements (HRE). Under normoxic conditions HIF-1α is rapidly degraded thereby preventing the low-oxygen response (Ehrbar et al., 2004; Semenza, 2003). A semi-synthetic network has been created by coupling the HIF-1α response system to a mammalian heterologous regulatory cascade resulting in multi-level gene control that can be influenced by endogenous signals (i.e. oxygen levels) as well as external signals (Fig. 8) (Kramer et al., 2005). By combining three inputs it has been possible to produce six distinct expression states depending upon the combination of signals used.

While representing the first steps towards the therapeutic application of synthetic networks, a major challenge remains

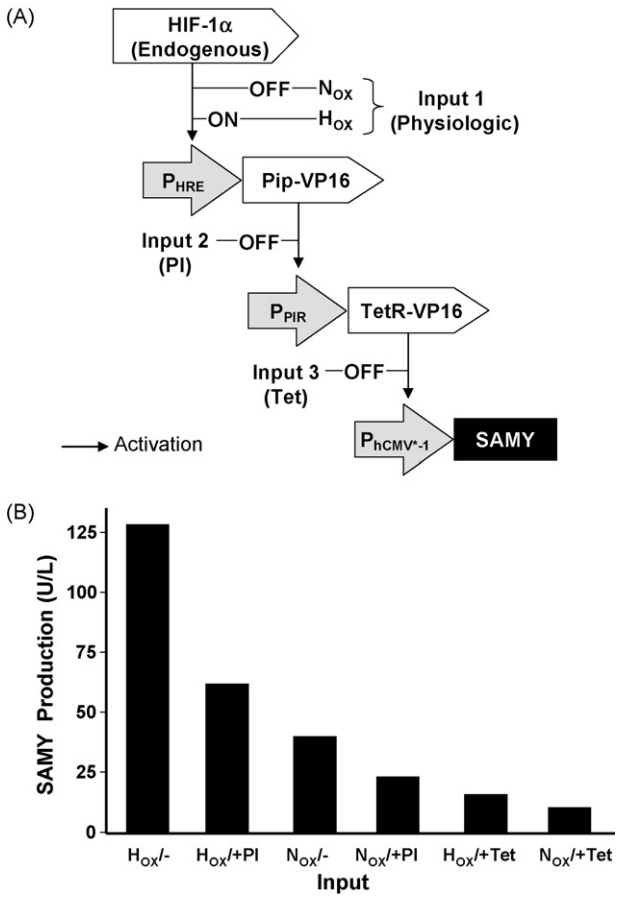

Fig. 8. (A) Response profile and genetic layout (B) of a semi-synthetic regulatory cascade. The semi-synthetic cascade is triggered by endogenous HIF-1α which, under hypoxic conditions (H_{OX}), is mobilized to the nucleus where it binds and activates a synthetic promoter containing hypoxia-response elements (P_{HRE}). Under normoxic conditions (N_{OX}) HIF-1α is rapidly degraded to undetectable levels. Activation of P_{HRE} sets off a transcriptional cascade of two heterologous transcription systems; the streptogramin-dependent transactivator Pip-VP16 which upon expression binds its cognate promoter (P_{PIR}) leading to expression of the tetracycline-responsive transactivator TetR-VP16, which subsequently binds its cognate promoter ($P_{hCMV*-1}$) leading to expression of a reporter gene (SAMY; *Bacillus stearothermophilus*-derived secreted α-amylase). In addition to sensing physiologic oxygen levels via the HIF-1α activator, the system is also responsive to pristinamycin (PI) which interrupts the cascade at Pip-VP16, and tetracycline (Tet) which interrupts the cascade at TetR-VP16. Up to six expression levels can be produced by different combinations of the three inputs (Kramer et al., 2005).

to find and/or preferably design transcription control systems which not only detect changes to a specific endogenous inducer but detect changes within a specified concentration range. The systems constructed to date have largely relied upon serendipity and have sufficed as a proof of concept. Yet to reach their true potential, one will need to find means of detecting and interfacing changes to pathologically relevant molecules.

5. Cross-cellular gene networks

Cell-to-cell communication and intra-cellular signal processing are essential mechanisms for coordinated cell behavior in both single cell populations as well as multicellular organisms. Such mechanisms control not only the development and orches-

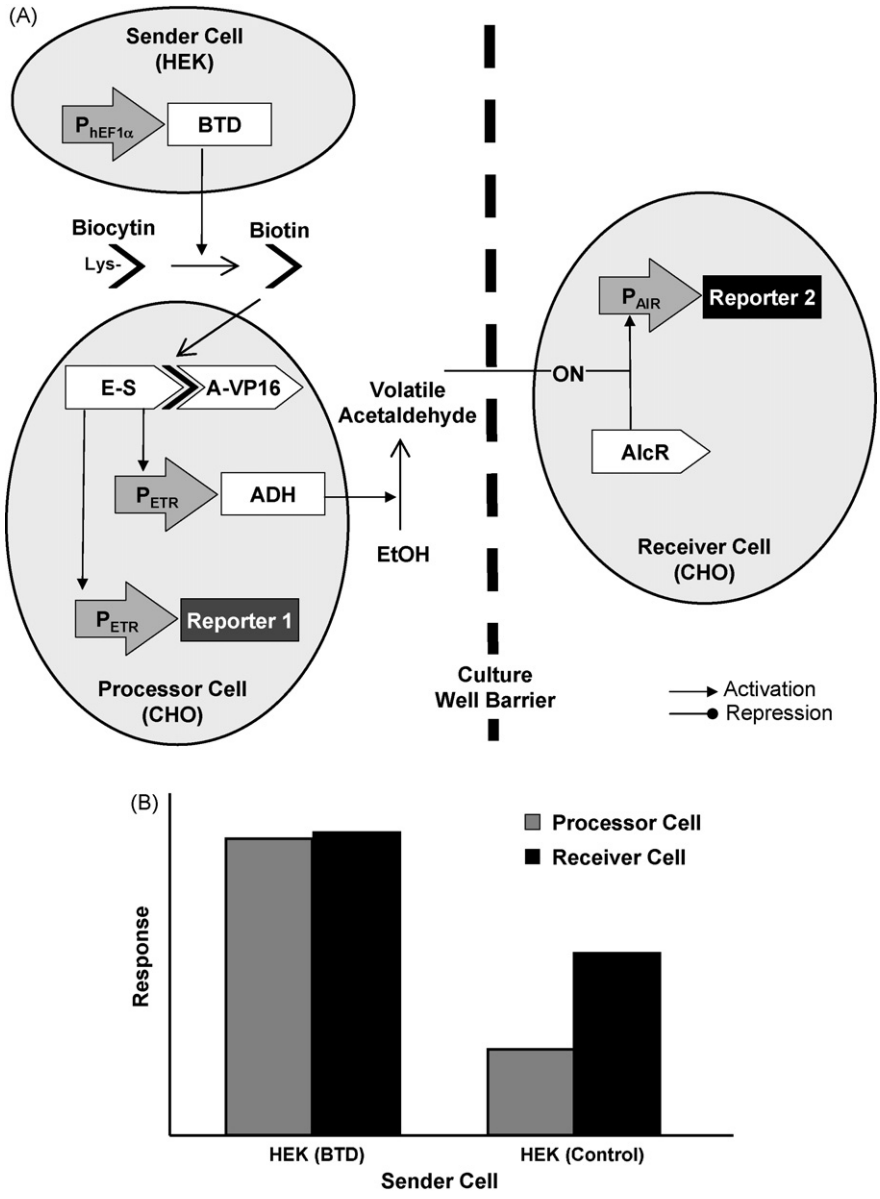

Fig. 9. (A) Genetic and cellular layout and (B) reporter expression profile of a three-step inter-cellular signaling and information processing cascade. The system consists of three engineered cell populations: a human embryonic kidney cell (HEK) "sender" population co-cultivated with a Chinese hamster ovary cell (CHO) "processor" population, and a separately cultured but within the same atmosphere CHO "receiver" population. The sender population constitutively expresses (from $P_{hEF1}\alpha$) human biotinidase (BTD) which catalyses the release of the signal molecule biotin (vitamin H) from biocytin contained within the culture media. Biotin diffuses in liquid phase to the processor cell population which has been engineered to constitutively express BirA ligase (not shown) which ligates biotin to an avitag-VP16 fusion protein (A-VP16) thereby enabling heterodimerization with co-expressed E-streptavidin [E-S] to produce a chimeric E-S-biotin-A-VP16 transactivator capable of inducing P_{ETR}-driven expression of alcohol dehydrogenase (ADH) and a reporter gene. ADH converts ethanol (EtOH) contained within the culture media to volatile acetaldehyde which diffuses via gas phase to the receiver cell population where it activates expression of a second reporter gene which is under the control of a *Aspergillus nidulans*-derived synthetic acetaldehyde-inducible promoter (P_{AIR}). Unless otherwise indicated, all of the depicted transcriptional controllers were constitutively expressed (from P_{SV40} promoters). Both the biotin and volatile acetaldehyde signaling molecules were capable of transmitting information between cell populations which could be processed by engineered transcriptional networks as evidenced by comparison of the expression profile of an equivalent system containing sender cells that lacked BTD (HEK Control) (Weber et al., in press).

tration of complex physiological events in entire organisms, such as cellular differentiation, development and adaptive responses to endogenous and exogenous cues, but also form the basis of many fundamental co-existence patterns between and across different species and kingdoms. It is therefore not surprising that attempts have commenced to replicate these mechanisms using synthetically engineered gene circuits. The band-detection and pulse-generating networks developed by Weiss and colleagues in *E. coli* have been successfully used to generate spatiotemporal differentiation patterns which, much like natural pattern formation, rely upon cell-to-cell communication and signal transduction networks (Basu et al., 2005, 2004; You et al., 2004). By altering parameters within their networks and using different fluorescent reporter genes they have been able to engineer an impressive array of multi-coloured patterns and shapes. In addition to pattern formation, inter-cellular communication via an

artificial quorum-sensing network sensitive to a common cellular metabolite has also be used to synchronize *E. coli* populations (Bulter et al., 2004).

In mammalian cells and higher-order organisms inter- and intra-cellular communication and processing systems have also been recently engineered (Weber et al., in press). In one such system a series of gene networks placed into different cell populations was used to replicate an endocrine signaling system in which small sensor cell populations process physiologic stimuli and convert them into hormones which trigger defined transcriptional responses in specific target cells (Fig. 9). In this case, a multi-step inter-cellular transcriptional cascade involving two separate gene control systems responsive to biotin (vitamin H) and volatile acetaldehyde each of which could diffuse from one cell population to another. Thus, cross-talk between co-cultured sender and processor cells was mediated through liquid diffusion of vitamin H, with subsequent downstream cross-talk between separately cultured processor and receiver cells occurring via gas diffusion of volatile acetaldehyde. The use of volatile acetaldehyde as a signaling message removed the need for two populations to reside in the same liquid environment. This feature was also exploited to engineer inter-kingdom cross-talk between mammalian cells and bacteria, yeast and plants in which precise spatiotemporal control of reporter gene expression was obtained in response to diffusion of the gas signaling molecule. Finally, by engineering the mammalian acetaldehyde responsive gene control system to produce secreted mammalian β-lactamase (sBLA) and neomycin phosphotransferase (NEO) it was possible to create bidirectional cross-talk between mammalian cells and co-cultured *E. coli*. In one such system, engineered mammalian cells were co-cultured with acetaldehyde producing *E. coli* in media containing both ampicillin and neomycin. *E. coli* produces volatile acetaldehyde which diffuses via gas phase to trigger sBLA and NEO expression in the mammalian population which enables the survival of these cells in the presence of neomycin. Concurrently, sBLA hydrolyzes ampicillin thereby enabling survival and growth of *E. coli*. The absence of either cell population from the culture prevents the survival of the other partner thus illustrating the occurrence of bidirectional information flow. Such a system also emulates a fundamental pattern of symbiotic co-existence between differing species; namely mutualism where both partners to an association benefit from the others presence. By altering the molecular configuration and reaction conditions it was also possible to replicate other interspecies co-existence patterns, such as commensalism, amensalism, parasitism, and oscillating predator-prey interactions (Weber et al., in press).

6. Conclusion

All of the engineered mammalian genetic networks thus described have utilized at least some aspect of rational design to produce a behavior that is based upon the modular interaction of DNA and/or RNA sequences, regulatory proteins, and inducing or signaling molecules. By assembling molecular parts not normally associated with each other into different configurations it has been possible to produce an already impressive array of robust network behaviors. As the number of available modules increases, and our ability to understand their interaction continues to improve, it is inevitable that further novel and increasingly more sophisticated synthetic networks will be created. Apart from their use in predicting and testing the properties of naturally occurring genetic circuits, we can expect that the emergence of more sophisticated mammalian genetic networks will enable and stimulate advances in medical applications in the foreseeable future.

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