Engineering Molecular Circuits Using Synthetic Biology in Mammalian Cells

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Abstract

Synthetic biology has made significant leaps over the past decade, and it now enables rational and predictable reprogramming of cells to conduct complex physiological activities. The bases for cellular reprogramming are mainly genetic control components affecting gene expression. A huge variety of these modules, ranging from engineered fusion proteins regulating transcription to artificial RNA devices affecting translation, is available, and they often feature a highly modular scaffold. First endeavors to combine these modules have led to autoregulated expression systems and genetic cascades. Analogous to the rational engineering of electronic circuits, the existing repertoire of artificial regulatory elements has further enabled the ambitious reprogramming of cells to perform Boolean calculations or to mimic the oscillation of circadian clocks. Cells harboring synthetic gene circuits are not limited to cell culture, as they have been successfully implanted in animals to obtain tailor-made therapeutics that have made it possible to restore urea or glucose homeostasis as well as to offer an innovative approach to artificial insemination.

INTRODUCTION

Genetic switch: an intracellular encoded component that affects target gene expression on a transcriptional or posttranscriptional level

Nature has evolved multitudinous gene circuits that enable cells to live and to adapt to environmental changes (1, 2). Although the bases of these networks are often simple genetic switches, complex circuitry has resulted in versatile gene expression patterns that range from straightforward ON/OFF states to advanced oscillating behavior (3). Synthetic biology has aimed to redesign existing gene circuits to gain detailed knowledge about the components involved. Beyond that, novel gene networks have reprogrammed cells to accomplish sophisticated tasks in the areas of biotechnology and biomedicine. This review provides a comprehensive description of the advances and milestones in the engineering of versatile gene circuits in mammalian cells. For readers interested in the progress made in bacterial synthetic biology, we recommend a recent review focused on engineered prokaryotic gene regulation systems (4).

INDIVIDUAL COMPONENTS: GENETIC SWITCHES

As is the case in most complex electronic circuits, in which simple electric ON/OFF gates sum together, genetic circuits are composed of basic genetic switches that affect gene expression. Owing to their high modularity, these switches are the basis for higher-order control elements that accomplish complex tasks. Awareness of the available basic elements, their characteristics, and their compatibility is a prerequisite for engineering advanced networks. Accordingly, the main genetic switches are introduced briefly here. In general, genetic control elements consist of an input domain that senses the presence of a trigger and an output domain that eventually affects target gene expression. On the basis of this universal buildup, several types of switches that act on the transcriptional or posttranscriptional level have been developed over the past few decades (**Figure 1**).

Transcriptional Regulators

Transcriptional regulators are gene control elements widely used in biotechnology and synthetic biology. The underlying mechanism of these elements is often site-specific recruitment or blockage of RNA polymerase at a defined gene. Control components share a highly modular scaffold and usually comprise a DNA-binding domain specific for a DNA operator sequence proximal a promoter region as well as a transcriptional activator or repressor domain. Transcriptional activator domains are often used in combination with minimal or weak promoters, whereas repressors can downregulate strong promoters. Therefore, the virus-derived vp16 (5), human p65 (6), and E2F4 (7) domains are typical activator domains and the Krüppel-associated box (KRAB) (8) a typical repressor domain. The protein domains mediating site-specific DNA binding may be ligand-independent TALEs (transcription activator-like effectors) and zinc-finger domains or ligand-responsive bacterial repressor domains.

Programmable DNA binding: transcription activator-like effectors and zinc-finger domains. Although an inducer cannot externally control the DNA binding of TALEs, they have received a great deal of attention recently because their DNA recognition motif can be genetically programmed in a rational fashion (9). The structure of TALE proteins, which naturally originate from *Xanthomonas* sp., includes central repeat domains that mediate sequence-specific DNA binding. Within this structure, two adjacent amino acids were identified that determine the base specificity of binding; this eventually led to the discovery of the code that correlates these amino acids to DNA nucleobase binding (10, 11). Through application of this code, TALE variants have

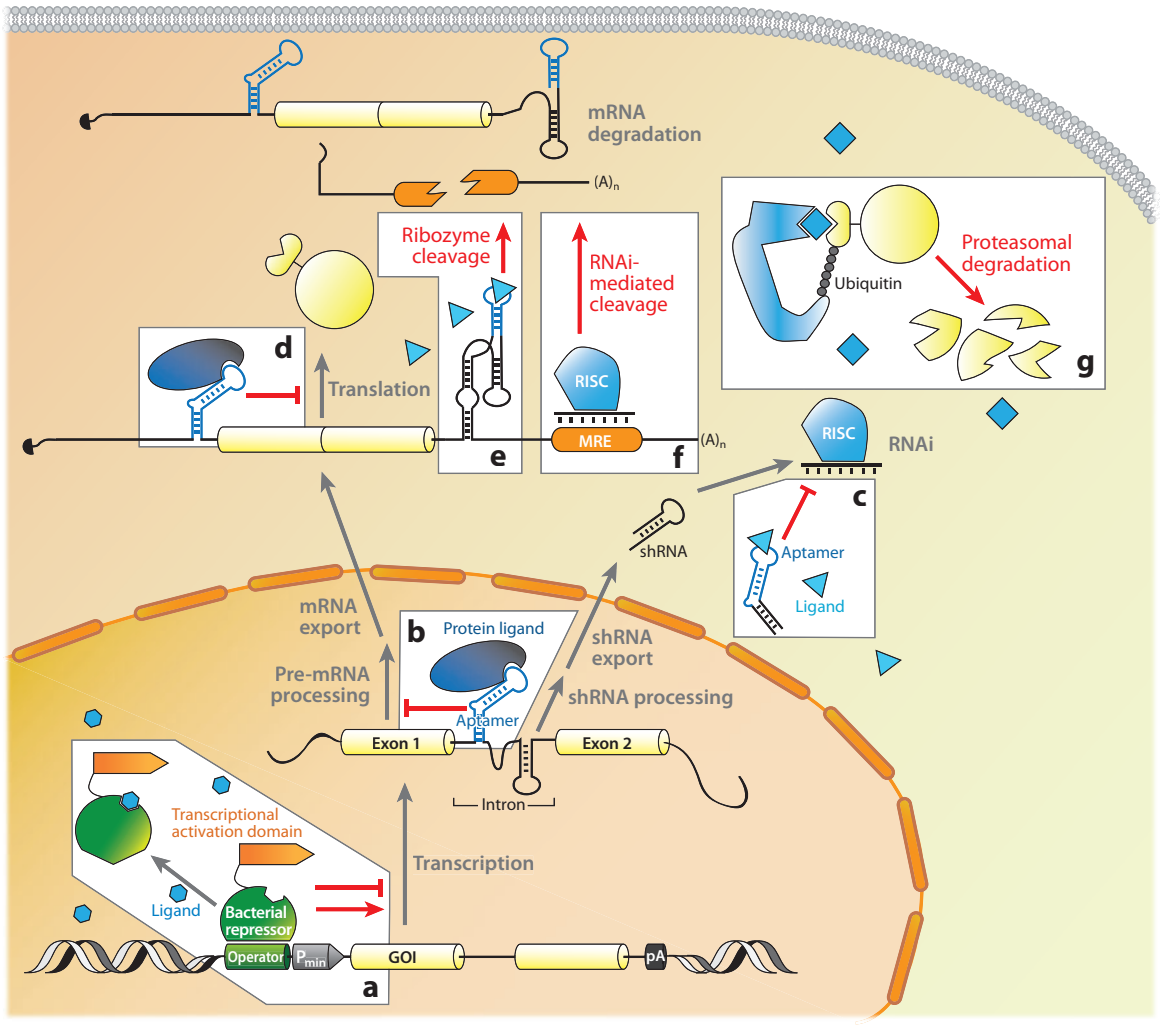


Figure 1

A schematic of some existing gene regulation systems that act on different levels of expression. (*a*) Transcription can be efficiently regulated using the modular assembly of a bacterial repressor fused to a transcriptional activation or repression domain. Upon ligand addition, the DNA affinity of the fusion protein can be reversed to enable transcriptional control (25, 27, 35–45). (*b*) Pre-mRNA processing can involve splicing, which is controllable by aptamer insertion close to defined splice sites. In the presence of a protein ligand, the splice site is masked and the splicing event at this position inhibited (75, 81). (*c*) Short hairpin RNAs (shRNAs), which also can be intronically encoded, require Dicer processing. Again, ligand binding to aptamers located in the hairpin region of the shRNA was used to mask Dicer recognition sites (63, 69, 70). Moreover, ligand-bound aptamers (*d*) can block ribosome scanning if placed into the 5′ untranslated region (77, 78) or (*e*) can control *cis*-ribozyme cleavage kinetics that eventually affect mRNA stability (74, 75). (*f*) RNA interference (RNAi)-mediated gene silencing is targeted to a gene of interest by inserting microRNA recognition elements (MREs) into the mRNA (93). (*g*) Finally, protein levels are also adjustable by regulating protein stability. For example, ligand-induced dimerization can be used to render the protein a substrate for an ubiquitin ligase, which designates the dimer for eventual proteasomal degradation (84, 85). Gray arrows denote the event described, red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: $(A)_n$, poly(A)-tail; GOI, gene of interest; P_{min}, minimal promoter; RISC, RNA-induced silencing complex.

been designed to recognize predefined operator sites on the DNA (9). These were then fused to the transcriptional activator vp16, and this construct was able to induce the transcription of genes in human (12) and plant cells (13). Zinc-finger proteins have also been used to target transcriptional activators to predefined DNA regions. Similar to TALEs, zinc-finger domains can be programmed to recognize defined DNA sequences (14) and, by attaching effector domains, used to control gene expression (15–17). However, zinc-finger proteins seem to be more difficult to reprogram and also exhibit more off-target binding sites compared with TALEs (18). Transcriptional regulators with programmable target operator sites have been used mainly in genome research to artificially induce expression of specific genes; they have not yet found their way into the field of synthetic biology. Ligand-inducible transcriptional regulators have been used instead because external control of the activity of TALEs and zinc-finger proteins is not possible.

Transactivator/transsilencer: a fusion protein consisting of a ligand-responsive DNA binding site and an effector domain controlling transcription

Dimerization-dependent transcriptional activation. Ligand dependency can be engineered into systems that do not initially respond to external triggers. Yeast-two-hybrid-like systems for mammalian cells were developed to capitalize on ligand-inducible protein dimerization in which conditional gene expression relies on the trigger-induced dimerization of two protein domains. By fusing a protein dimerization domain to a transcriptional regulator or a DNA operator binding protein, ligand-dependent dimerization eventually results in recruitment of the effector domain to a desired minimal promoter, thereby enabling transcriptional control. For example, the compound rapamycin (17) as well as some analogs (20, 21) successfully demonstrated efficient dimerization induction of the FK506-binding protein (FKBP12) with the FKBP12-rapamycin-binding (FRB) domain.

Remarkably, because the modularity of dimerization-dependent transcriptional activators only requires appropriate dimerization domains, it was possible to expand the repertoire of triggers to electromagnetic waves. After implementing the plant proteins FKF1 (22) and GIGANTEA (23), which form heterodimers in the presence of blue light, into the system described above, reporter gene expression could be controlled by the intensity of appropriate light irradiation (24). Transcription of the luciferase reporter gene was induced more than fourfold in the presence of light with a wavelength of 450 nm (24).

Bacterial repressors as ligand-dependent regulators in mammalian cells. The tetracycline-dependent transactivator (tTA) system consists of the tTA protein and a gene of interest under control of a minimal or weak promoter proximal to a tTA binding motif. It was first described by Gossen & Bujard (25) in 1992 and is one of the most-characterized ligand-responsive gene regulation systems for mammalian cells. The tTA protein is composed of the above-mentioned viral vp16 domain fused with the bacterial tetracycline-dependent repressor protein TetR. As a homodimer, TetR binds with high affinity to its operator site tetO. However, adding the ligand tetracycline results in a structural change in the DNA-binding helix-turn-helix motif that releases TetR from the tetO operator site (26). In eukaryotes, gene of interest expression can be controlled by placing its coding region downstream of an oligomeric tetO repeat proximal to a minimal or weak promoter and then coexpressing the tTA fusion protein (27). In the absence of tetracycline or its stabilized derivative doxycycline, gene expression is turned ON, but it is efficiently inhibited when the ligand is added (**Figure 1*a***) (27).

In addition, mutation of TetR allowed the engineering of a tTA variant with reversed activity. Unlike the original, this reverse tTA (rtTA) induced gene expression only in the presence of the ligand (28). Furthermore, tTA has been genetically altered to exclusively bind to other operator sites, which allows for the concomitant usage of different tTA variants in a single cell (29–31). The efficacy of these systems can be further improved by genetically merging two different tTA

or rTA monomers to create a defined single-chain variant. This prevents the formation of inactive tTA heterodimers from different transactivator variants (32, 33). Finally, the bacterial TetR and rTetR proteins have also been fused to the KRAB domain to result in the tetracycline-dependent transsilencers tTS (8) and rtTS (34), respectively.

However, the modularity of this system is not limited to the effector domain; the bacterial repressor is also easily interchangeable. Replacing TetR with other ligand-dependent bacterial repressors featuring a similar helix-turn-helix architecture (26) has led to the development of novel transcriptional regulators for mammalian cells. This has allowed the construction of gene expression systems regulated by a diverse range of ligands, from endogenously available metabolites such as L-arginine (35), biotin (36), and urate (37) to antibiotics such as macrolides (e.g., erythromycin) (38) and streptogramins (e.g., pristinamycin I) (39), to the compounds phloretin (40), 6-hydroxy-nicotine (41, 42), and 2-phenylethyl-butyrate (43). In addition to these soluble compounds, gaseous acetaldehyde was also used successfully as a ligand to control target gene expression (44, 45).

Rewiring of internal pathways. Rewiring of cellular pathways is a promising alternative to engineering orthogonal chimeric transcriptional regulators. A prominent example is the heterologous expression of a G protein–coupled receptor (GPCR) in a mammalian target cell. Understanding the downstream signaling cascade facilitates the control of a desired gene of interest if its expression is placed under the control of the corresponding transcription factor. Gene reporter assays for determining GPCR activation have been developed for all signaling cascades, including CREB (46), NFAT, NF-κB, c-Fos, and c-Jun (47), by placing the transcription factor binding site proximal to a minimal promoter. Therefore, gene regulation systems based on ligand-triggered GPCR activation can be engineered by putting the gene of interest under control of the respective endogenous transcription factor. Examples for GPCR-controlled gene circuits are described in more detail below.

Posttranscriptional Regulators

Posttranscriptional regulation has received a lot of attention recently, especially with the discovery of RNA interference (RNAi) (48, 49). Scientists have begun capitalizing on the potential of posttranscriptional regulation to engineer novel genetic control elements.

RNA interference. RNAi mainly designates the downregulation of gene expression via sequence-targeted mRNA degradation mediated by the RNA-induced silencing complex (RISC) (50). Shortly summarized, a double-stranded RNA structure is recognized and processed by the Drosha-DGCR8 complex and subsequently by Dicer (51). The guide strand from the RNA is then loaded on the RISC and serves as a sequence template for mRNAs, which are cleaved by the protein-RNA complex and eventually degraded (50). For a detailed description of RNAi mechanisms, please see other reviews (52–54). Because the protein machinery for RNAi is encoded in mammalian cells, this mechanism can be used to knock down the expression of specific genes by introducing the appropriate double-stranded RNA (**Figure 1*f***). However, optimization may be required when establishing a suitable target sequence to prevent unspecific gene inhibition.

To obtain control over RNAi efficacy, expression of the corresponding double-stranded RNA, which is usually encoded as a hairpin-forming structure [short hairpin RNA (shRNA)], can be placed under the control of ligand-dependent transcriptional effectors that activate RNA polymerase II (55, 56) and III (57). On one hand, the shRNA can be integrated into the mRNA of any gene if placed into intronic regions (58, 59). Mimicking naturally found genetic arrangements

(60), this allows for the simultaneous expression of a gene and an shRNA because RNA splicing occurs temporally prior to shRNA processing (58). On the other hand, placing the shRNA in the 5′ untranslated region (5′-UTR) still enables efficient RNAi gene knockdown, but it inhibits translation of the gene that is part of the shRNA-mRNA because processing severely reduces mRNA stability. Such a setup is a useful way to monitor the efficacy of shRNA processing (61).

Aptamer: a short nucleic acid structure that binds a ligand with high specificity and affinity

A different approach to obtaining ligand-dependent RNAi involves regulation of processing efficacy through combination of ligand-responsive RNA elements [so-called aptamers (62)] with shRNAs (63). Aptamers are short nucleic acid stretches that, by folding in distinct 3D structures, bind protein or small-molecule ligands with high affinity and specificity (64–66). Ligand binding often induces a structural change in aptamers, which can influence larger RNA structures surrounding the aptamer both in vitro and in vivo (67). Indeed, although there is a wide assortment of small compound–recognizing aptamers, most mammalian cell experiments use the theophylline-dependent aptamer (68). Theophylline is slightly toxic at the concentrations needed to enable structural changes, but the aptamer is highly specific, for example, discriminating the methylated analog of theophylline, caffeine, with a thousand-fold diminished binding affinity (68).

Replacement of the shRNA loop region with the theophylline aptamer facilitated efficient target gene inhibition only in the absence of the inducer; adding theophylline masked the Dicer recognition region (**Figure 1*c***) (63, 69). The same mechanism worked for the L7Ae protein-dependent aptamer (70). The theophylline aptamer was also integrated in a more indirect setup in which it controlled the activity of a Hammerhead ribozyme (HHR) attached to an shRNA. Only theophylline-regulated self-cleavage of the HHR, which replaced the loop region of the shRNA, results in correct shRNA folding. This shRNA then is recognized and eventually processed by Dicer (71).

mRNA stability. In addition to RNAi-mediated posttranscriptional regulation, mRNA stability can be controlled directly. Therefore, an HHR was introduced into the UTRs as a *cis*-acting element. Self-cleavage-induced removal of essential mRNA elements, such as the 5′-cap or the poly-(A)-tail, leads to a reduced mRNA half-life, which eventually inhibits target gene expression (72, 73). Again, ligand-responsive aptamers were used to regulate the catalytic activity of the HHR to obtain controllable gene expression systems (**Figure 1*e***) (74, 75).

Translation efficiency. In addition to HHRs, aptamers inserted into the 5′-UTR can also influence protein level. Stabilization of RNA tertiary structures by small molecules or steric hindrance by proteins binding to aptamers contributes to inhibition of ribosomal scanning for a translation start site (76). In a groundbreaking experiment, Green & Werstruck (77) downregulated reporter gene expression upon addition of Hoechst dyes that specifically bind to their corresponding 5′-UTR-located aptamer repeats. Likewise, using proteins as aptamer ligands can significantly improve the inhibition rate, as shown recently with the archaeal ribosomal protein L7Ae (**Figure 1*d***) (78).

Splicing efficacy. The accessibility of splicing sites for the spliceosome can be hindered by proximal RNA aptamers binding sterically demanding proteins. This makes it possible to gain control over alternative splicing decisions. In this way, Pumilio protein domains, which possess rationally programmable RNA-binding properties comparable with those of TALEs (79, 80), represent a versatile scaffold for targeting predefined RNA sites (81). Available aptamers that bind intracellular proteins were also successfully integrated and used for splice control (**Figure 1*b***) (75).

Protein stability. Cellular protein levels are defined by the efficiency of gene expression as well as by protein stability. Protein levels are therefore affected directly by degradation of the respective protein. Ligand-dependent protein stability systems have been developed in consideration of this point. In one approach, a mutated human FKBP12 variant gene is fused with the gene of interest. Although the added domain reveals the protein for degradation, supplementing the system with the specific orthogonal ligand Shld1 inhibits destabilization and eventually increases protein levels (82).

Likewise, proteins can be destabilized by targeted ubiquitinylation, which serves as a degradation signal for the proteasome (83). For example, the plant hormone auxin can be used to induce dimerization of the AID (auxin-inducible degron) domain with an ubiquitin ligase. Ubiquitinylated AID is then subjected to proteasomal degradation (84, 85). Target proteins fused to AID thus became trigger-induced degradable upon auxin addition (**Figure 1*c***) (86).

Autoregulation: regulation of a genetic switch by its own expression product

Regulatory gene cascade: genetic switches that are serially assembled to result in gene expression regulators controlled by other regulators

CIRCUITS: SYNTHETIC GENE NETWORKS

Higher-Order Control

Genetic switches represent simple ON/OFF gates similar to those in electronic circuits. They provide an output response that is based on an input that can be a ligand or even a process such as transcription. Having established this huge repertoire of genetic switches, the next step is to connect them to obtain higher-order control elements.

Gene expression cascades. The most basic circuits are composed of the serial arrangement of control elements, which eventually produces autoregulated feedback loops and expression cascades. Owing to the high modularity of engineered networks, these elementary circuits are, similar to the basic genetic switches, integral parts of more complex networks.

Autoregulated feedback loops are regulatory elements that influence their own efficacy. Both negative and positive feedback loops are possible. The latter, which are also known as feedforward loops, can be engineered by placing the tTA gene under the control of its own regulatory capacity. In this way, the leakiness of the system results in low levels of tTA, which then amplifies its own transcription until tTA is expressed strongly (**Figure 2*a***) (87, 88). Remarkably, ligand-induced repression of this system revealed a significantly slower intracellular reporter gene reduction when compared with an expression system that lacks the feedforward loop (87). A negative feedback loop was constructed by encoding a transcriptional repressor under its own transcriptional control (89). Moreover, posttranscriptionally acting RNAi was also used to construct negative feedback loops that are part of the advanced circuits described below (90).

Regulatory gene cascades are based on the serial connection of several regulatory elements such that each component controls the expression of the following component. The first regulatory gene cascade in mammalian cells used three layers of transcriptional activators. First, tTA transcription was placed into a feedforward loop that amplified its own expression levels in the absence of tetracycline. Second, the erythromycin-dependent activator ET1 (E-vp16) was encoded on the same transcript, connected by an internal ribosome entry site (IRES), which enables effective translation of polycistronic mRNAs in mammalian cells. Third, ET1 activated the streptogramin-dependent transactivator PIT (Pip-vp16), which triggered reporter gene expression. Because all transcriptional activators used in this study were ligand responsive, the final reporter level could be adjusted gradually by adding a particular or multiple trigger molecules (**Figure 2*a*,*b***). As anticipated, the presence of all ligands inhibited reporter expression completely (88).

Thus, not only do gene cascades offer higher levels of complexity and control compared with a single genetic switch, but they also require the timing of expression regulation to be considered. For example, in a model experiment, a simplified cascade consisted of the transcriptional activator tTA, which controlled the pristinamycin-responsive repressor Pip-KRAB; this, in turn, inhibited reporter gene expression. On one hand, reporter gene expression could be reconstituted quickly by adding the ligand pristinamycin I, which released the transcriptional repressor from the reporter gene promoter. Addition of tetracycline, on the other hand, caused Pip-KRAB expression to stop. Until its eventual degradation, the remaining Pip-KRAB protein continued to repress expression of the reporter secreted embryonic alkaline phosphatase (SEAP). Therefore, SEAP expression

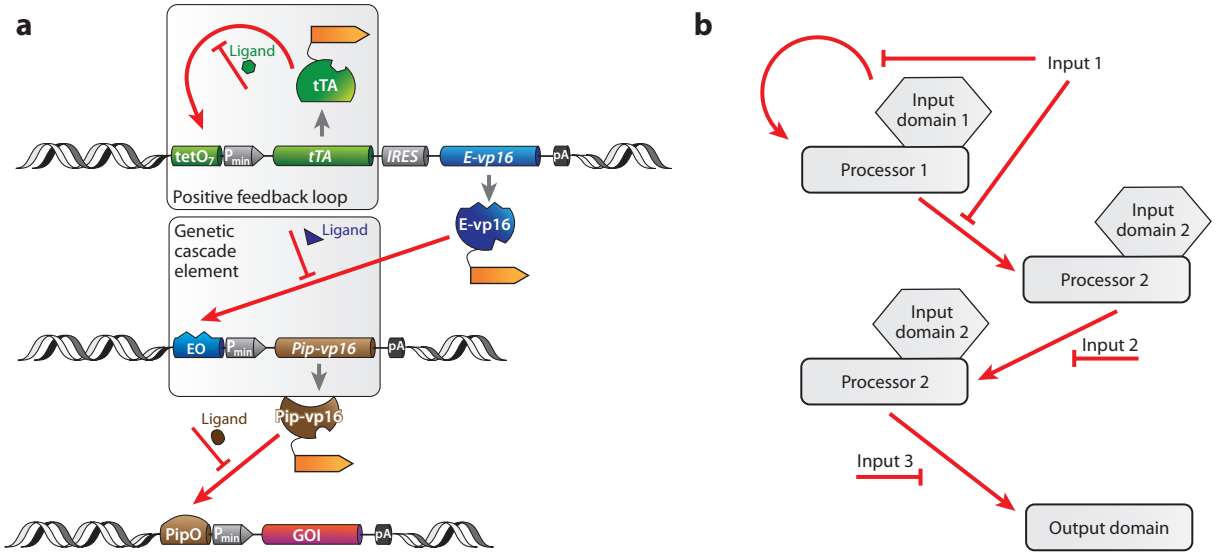


Figure 2

Gene circuits based on serial and parallel connection of transcriptional regulators. (*a*) A genetic cascade consisting of three levels of transcriptional regulators: Tetracycline-dependent transactivator (tTA), controlled by a feedforward loop, drives expression of E-vp16, which induces Pip-vp16. Finally, Pip-vp16 starts transcription of the gene of interest (GOI). The GOI output level can be fine-tuned by addition of one or two of the ligands, whereas complete inhibition is obtained in the presence of all three ligands (88). (*b*) Schematic model of the network underlying the genetic cascade described in panel *a*. (*c–g*) Design of NOT IF and NAND logic gates based on parallel connection of transcriptional regulators (92). (*c*) The NOT IF logic gate, which exhibits output gene expression only if pristinamycin I is present, can be constructed in mammalian cells by consecutively placing the operator sites for scbR-vp16 and Pip-KRAB (Krüppel-associated box) upstream of a minimal promoter. As soon as Pip-KRAB is bound or if scbR-vp16 is not bound to its operator site, GOI expression is inhibited (92). (*d*) Realization of the NOT IF gate using posttranscriptional regulators: Two short hairpin RNAs (shRNAs) were programmed to target reporter mRNA that contains the microRNA recognition elements (MREs) A and B in the 3′-untranslated region (3′-UTR). Transcription of the shRNA A was induced by the presence of reverse tTA (rtTA), whereas addition of LacI-KRAB inhibited shRNA B. Considering the transcriptional regulator rtTA and LacI-KRAB as input signals, this setup provides output gene expression exclusively in the presence of rtTA (93). (*e*) A schematic model of the network underlying the NOT IF gate and the corresponding truth table. (*f*) The NAND gate was implemented in mammalian cells using a rationally designed gene network. E-vp16 and Pipvp16 each regulated a separate copy of the GOI. In this setup, output gene expression is repressed only in the presence of both ligands (92). (*g*) Schematic model of the network underlying the NAND gate and the corresponding truth table. Red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: E, erythromycin-responsive transcriptional repressor domain; ET1, erythromycin-responsive transcriptional activator; ET1O and EO, operator site of E; IRES, internal ribosome entry site; pA, polyadenylation signal; P_{const}, constitutive promoter; Pip, pristinamycin-responsive transcriptional repressor domain; PIT, pristinamycin I–responsive transcriptional activator; PITO and PipO, operator site of Pip; P_{min}, minimal promoter; RISC, RNA-induced silencing complex; scbO, operator site of scbR; scbR, 2-(1V-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide-responsive transcriptional repressor domain; $tetO_7$, heptameric operator site for tTA.

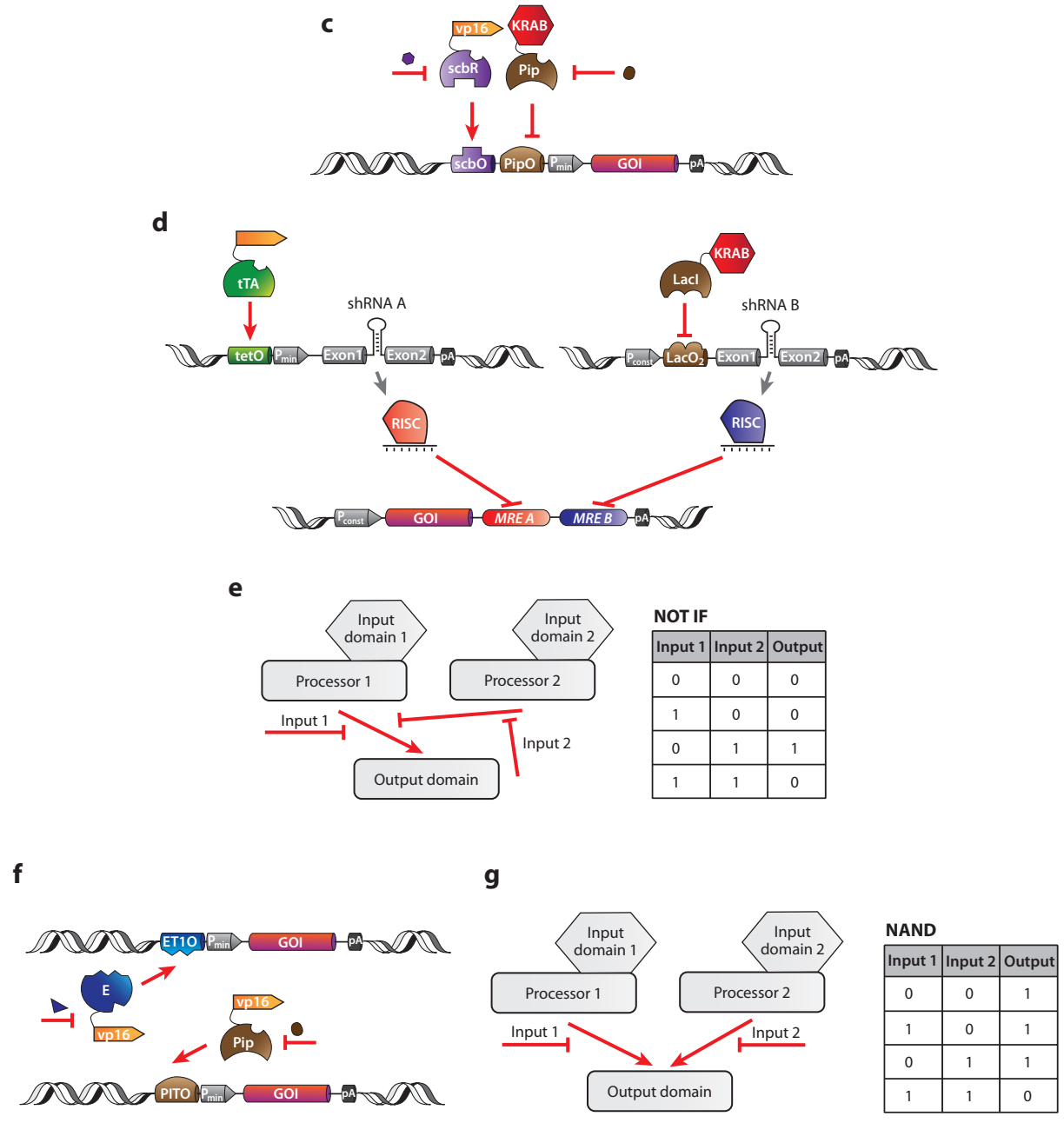


Figure 2

(*Continued*)

was induced with a time delay that could be further fine-tuned by adjusting the protein stability of Pip-KRAB (91).

Logic gate: conversion of two input signals into one output signal that follows Boolean logic

Logic gates: biocomputing. A parallel arrangement of regulatory elements that have a mutual effect on gene expression makes it possible to design and construct logic gates. By performing Boolean calculations, logic gates convert information from two inputs into one output. Because logic gates are the basis of any electronic device, establishment of efficient logic gates in living cells is a promising method to construct biocomputers with great parallel calculation power, particularly when reprogrammed cells are arranged in multicellular tissue-like assemblies.

Transcriptional regulators are powerful building blocks for engineering logic gates in mammalian cells. That these transcriptional regulators possess orthogonal DNA recognition sites predisposes them for simultaneous usage in a single cellular system. For example, the Boolean NOT IF gate that exhibits reporter expression output only in the presence of one input alone was engineered by using the pristinamycin I–responsive transsilencer Pip-KRAB and the 2-(1V-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide (SCB1)-responsive transactivator scbR-vp16 (**Figure 2*c*,*e***). The Pip- and scbR-binding operator sites were arranged consecutively upstream of a minimal promoter running the SEAP reporter gene. In this setup, the reporter is efficiently expressed only if scbR-vp16 is bound to the operator site, which requires the addition of pristinamycin I. As soon as scbR-vp16 is released or Pip-KRAB is bound to its operator site on this construct, RNA recruitment and thus SEAP expression are inhibited. In summary, this NOT IF gate setup generated reporter output in the presence of pristinamycin I alone, but not in the absence of either ligand or after erythromycin addition (92).

In contrast to such a consecutive assembly of operator sites, the NAND gate, which exhibits repressed reporter output only in the presence of both ligand inputs, was implemented by engineering the two transactivators E-vp16 and Pip-vp16 to control SEAP reporter expression from two distinct constructs (**Figure 2*f*,*g***). The first SEAP gene was put under control of an E-vp16-responsive promoter and the second under a Pip-vp16-responsive promoter. Cells harboring these gene circuits expressed SEAP as long as at least one of the transactivators was bound to its operator's site; only upon simultaneous addition of both ligands, erythromycin and pristinamycin I, was SEAP expression inhibited (92).

Furthermore, a NOR gate that gives reporter output only in the absence of either ligand was successfully engineered by using the same transactivators, E-vp16 and Pip-vp16, in a different circuit. In this case, the transactivators were arranged in a regulatory cascade in which an E-vp16-responsive promoter drove Pip-vp16 expression. In turn, implementation of the Pip-vp16 operator site upstream of a minimal promoter controlled reporter expression. Reporter expression is hereby promoted only if both transactivators are bound to their respective operator sites. Upon addition of either ligand, the regulatory cascade is interrupted and reporter output inhibited (92).

Recently, posttranscriptionally acting regulators also have been used in logic gates: Specifically, shRNAs were intronically encoded under the control of RNA polymerase II–dependent promoters that transcriptional regulators controlled externally. The shRNAs were programmed to recognize defined regions on the reporter mRNA known as microRNA (miRNA) recognition elements (MREs), which eventually resulted in reduced expression levels. Placement of several of these orthogonal recognition elements in the same mRNA enabled the circuitry of the single inputs to generate a Boolean calculation-based output (93). As an example, the above-described NOT IF gate that enables reporter output in the presence of only one input also could be engineered by applying such shRNA-based circuitry: The reporter mRNA contained the MREs of two distinct shRNAs, A and B, which led to inhibition of reporter expression in the presence of either shRNA. Transcription of shRNA A was put under control of a LacI-KRAB-dependent promoter, whereas

shRNA B transcription was rtTA dependent. Thereby, the reporter gene is expressed only if LacI-KRAB is bound and rtTA is not bound to their respective operator sites, thus preventing transcription of shRNA A and B. Importantly, in this setup the signal input is defined by the presence or absence of the transcriptional regulators and not by the ligands affecting their DNA-binding properties (see **Figure 2*d***) (93).

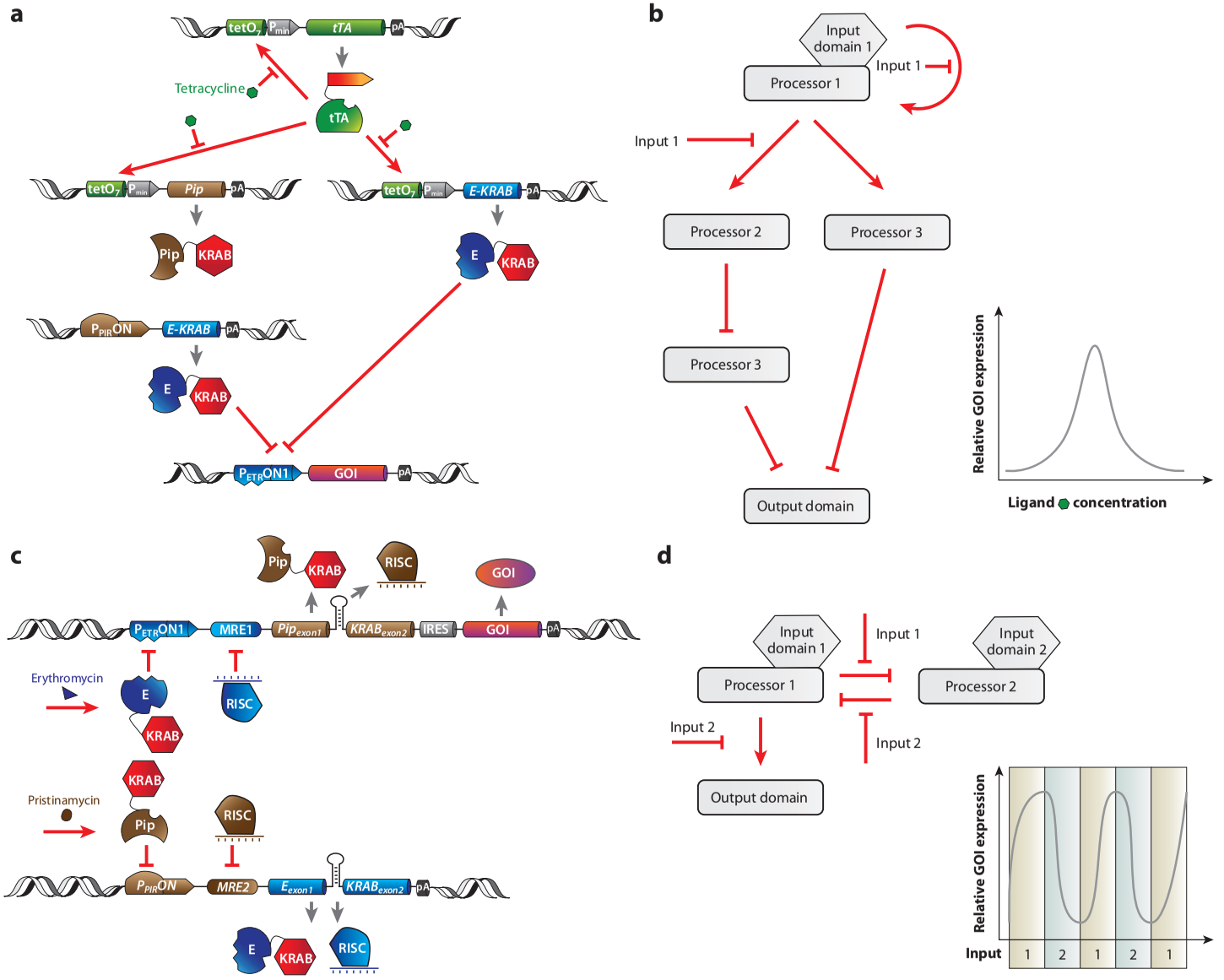
Bandpass filter. Similar to many of the circuits described here, bandpass filters originate from electronics and optics. They allow only a defined signal frequency to pass and filter out higher and lower bands. To establish a bandpass filter in mammalian cells, the electronics terminology had to be translated into biology. Hence, a biological bandpass filter controls gene output by filtering for a specific inducer concentration and blocking deviant concentrations. Such an element allows for controlled gene expression that is based not only on the presence of a specific ligand but also on a predefined concentration of that ligand.

Circuits for bandpass filters in mammalian cells consist of two major opposing components, one that induces gene expression upon ligand addition and one that results in repression. Combination of these components yields peak reporter expression at medium ligand concentration, at which point neither the inducing nor repressing component is fully activated (94).

This circuit was realized using the Tet system as the superordinate control element. Regulated itself by a feedforward loop, tTA indirectly controlled reporter protein levels by regulating the efficacy of both the inductive and repressive components of the circuit. On one hand, inductive component regulation was achieved by placing the expression of the erythromycin-dependent transsilencer under the control of tTA. Once expressed, the transsilencer binds to its operator site on the reporter construct, which inhibits gene expression in the absence of tetracycline. Addition of tetracycline, however, resulted in diminished levels of the transsilencer and eventually induced reporter expression. Tetracycline-dependent repression of the reporter, on the other hand, was achieved by introducing a genetic inverter into the circuit. Therefore, tTA runs the transcription of a third transcriptional regulator, which inhibits the expression of a second copy of the erythromycin-dependent transsilencer that also blocks reporter gene transcription (**Figure 3*a***). Owing to this genetic inverter, addition of tetracycline resulted in elevated transsilencer levels and inhibited the final reporter expression (94). When these two circuit components were combined into one cellular system, increasing the tetracycline concentration initially led to elevated reporter levels. Once the ligand concentration exceeded a certain level, the repressive components predominated and inhibited reporter gene transcription. In summary, this biological bandpass filter was successfully engineered by combining a gene cascade and a genetic inverter to obtain an inducible and repressible signal conversion for the tetracycline input (**Figure 3*a*,*b***) (94).

Toggle switch. A toggle switch is a circuit that contains an output domain possessing two states that can be taken alternately upon input. The input is not required to maintain the states but rather to interchange between them. In a biological context, repeated ligand input causes reporter output to change between high and low levels, whereas ligand removal should keep the system in the most recent state. This contrasts with common gene regulation systems, in which the presence of ligands is essential to maintain a defined expression level and ligand removal results in reversion to the ground state.

The first genetic toggle switch for mammalian cells was engineered on the basis of two transcriptional silencers, E-KRAB and Pip-KRAB, which are macrolide and streptogramin responsive, respectively (95). Pip-KRAB was followed by an IRES that facilitated efficient translation of a subsequent reporter gene, and this construct was placed under the control of an E-KRAB-dependent promoter. At the same time, E-KRAB transcription was put under control of Pip-KRAB by placing



the Pip operator site after the promoter for E-KRAB expression. This setup allowed the preferential transcription of only one construct while inhibiting expression of the other. Administration of erythromycin resulted in the release of the transsilencer from its operator site and, therefore, in the expression of Pip-KRAB and the reporter gene. Subsequent replacement of erythromycin with the streptogramin pristinamycin I reversed the transcription states by inhibiting Pip-KRAB and inducing E-KRAB expression. Withdrawal of any ligand did not influence the state of the biological toggle switch (95). Optimization of individual parts of the circuit allowed for even better performance of this toggle switch. Therefore, insertion of intronically encoded shRNAs that targeted the other construct into each transsilencer enhanced repression efficacy (**Figure 3*c,d***) (58).

Oscillator. Generally, an oscillator interchanges repeatedly and autonomously between two separate states. Although oscillation is common in electronics and physics, it is also of prime importance in biology. In mammals, for example, the circadian clock facilitates oscillatory changes in gene expression that account for day and night periods. Using a complex network of transcriptional activators and repressors that involves mutual feedback loops, the circadian clock allows for temporally controlled expression of gene clusters on the basis of a 24-h rhythm (96–98). Interestingly, this endogenous oscillator can be intracellularly hijacked by placing a gene of interest under the control of an appropriate transcription factor (96).

Such a genetic oscillator was reconstructed artificially by following the building principle of the endogenous oscillator (99). The aim was to achieve self-sustaining oscillation of reporter expression based on transcriptional activators and repressors. Therefore, a destabilized green fluorescent protein (GFP) variant that offered advanced temporal resolution of gene expression was placed under the control of the tTA transcriptional activator. Because tTA directly controlled GFP transcription, tTA is also required to exhibit an oscillating expression pattern. To this end, tTA transcription was put under autoregulated control to construct a tTA-dependent feedforward loop that was responsible for the transition from the OFF to the ON state. Additionally, tTA expression was repressed by a time-delayed negative feedback loop. The pristinamycin I–dependent transactivator (PIT), the transcription of which was also under tTA control, initiated the negative feedback loop, which consisted of a tTA-targeting antisense RNA. This gene cascade enables

Figure 3

Complex gene networks. (*a*) Setup for a genetic bandpass filter that enables the expression of a gene of interest (GOI) only at a defined concentration; higher and lower inducer levels repress transcription. The GOI is negatively regulated by E-KRAB (Krüppel-associated box), which is encoded by two gene copies, each representing a component of the circuit. The first was directly controlled by tetracycline-dependent transactivator (tTA), therefore resulting in an OFF switch upon addition of tetracycline. The other E-KRAB copy is under control of Pip-KRAB, which is tTA dependent. Therefore, addition of tetracycline to the second circuit component eventually repressed E-KRAB and induced expression of the GOI. By combining these components into one circuit, a bandpass-like output with peak GOI expression levels at a defined concentration is obtained (94). (*b*) Schematic model of the network underlying the construction of a bandpass filter and the expected output pattern. (*c*) Exemplary circuit design for a genetic toggle switch, which maintains gene expression output level when ligand is removed but changes upon the addition of a second ligand. The GOI is expressed together with Pip-KRAB under the control of E-KRAB. Pip-KRAB is used to control E-KRAB transcription (95). System performance can be further improved by intronically encoding a short hairpin RNA targeting the microRNA recognition element (MRE) located on the corresponding construct (58). (*d*) Schematic model of the network underlying the construction of a toggle switch and the expected output pattern. Red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: E, erythromycin-responsive transcriptional repressor domain; MRE, miRNA recognition element; pA, polyadenylation signal; $P_{ETR}ON1$, constitutive promoter with proximal E operator site; Pip, pristinamycin-responsive transcriptional repressor domain; P_{min}, minimal promoter; $P_{Pir}ON$, constitutive promoter with proximal Pip operator site; RISC, RNA-induced silencing complex; $tetO_7$, heptameric operator site for tTA.

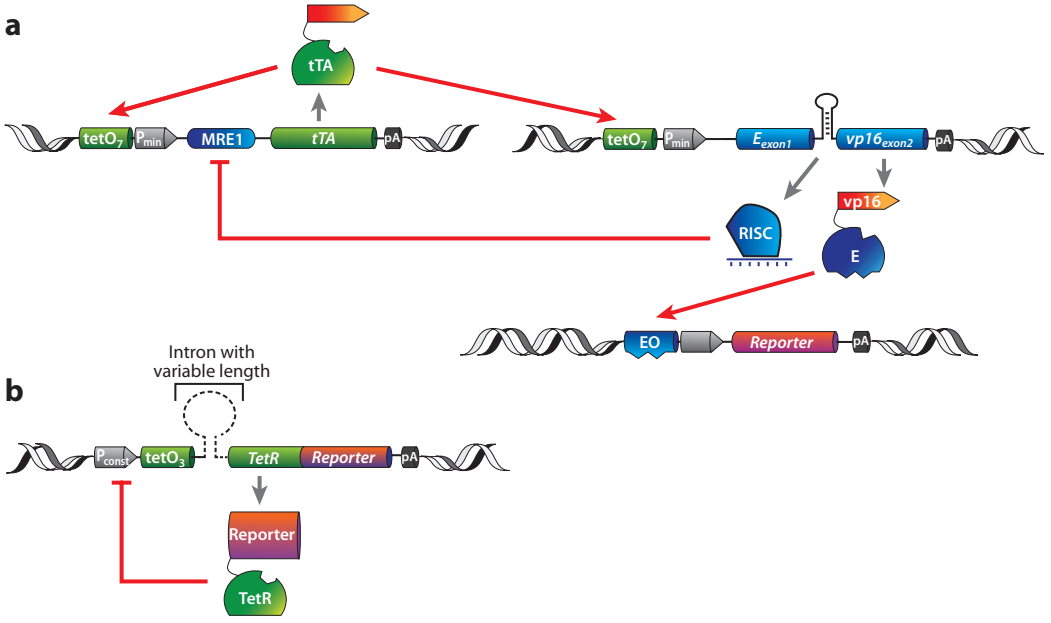


Figure 4

Genetic networks enabling self-sustaining gene expression oscillation. (*a*) A reporter gene was put under the control of E-vp16. Both the reporter and E-vp16 exhibited self-sustained oscillating expression levels through application of a time-delayed negative feedback loop consisting of an intronically encoded short hairpin RNA (shRNA) located in the E-vp16 transcript that inhibited tetracycline-dependent transactivator (tTA) expression. tTA was required to induce E-vp16 transcription (90). (*b*) In another oscillator setup, a TetR-reporter fusion was used to repress its own transcription. Introduction of intronic spaces with variable length upstream of the coding region enabled the essential time delay because transcription time is dependent on intron length, which enables fine-tuning of the properties of the negative feedback (100). Red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: E, erythromycin-responsive transcriptional repressor domain; EO, operator site of E; MRE1, microRNA recognition element 1; pA, polyadenylation signal; P_{const}, constitutive promoter; P_{min}, minimal promoter; RISC, RNA-induced silencing complex.

time-delayed repression of tTA in a tTA-dependent manner, which in turn enables the autonomous repetition of tTA induction and repression. Rational adjustment of the number of gene templates introduced into the cells could fine-tune the frequency of this genetic oscillator (99).

Several methods exist to engineer the main feature of a genetic oscillator, namely, the time-delayed negative feedback loop. In a second setup, the GFP reporter was driven by the macrolide-dependent transactivator ET1 (also known as E-vp16), which contained an intronically encoded shRNA-targeting tTA that was, consequently, inhibiting. tTA induced its transcription in an autoregulated manner but also induced expression of the ET1 construct. Therefore, the time-delayed negative feedback loop is based on the RNAi-dependent tTA inhibition (**Figure 4*a***). Interestingly, this gene circuit resulted in a low-frequency oscillator with a 26-h period, which almost mimics the timing of the natural circadian clock (90).

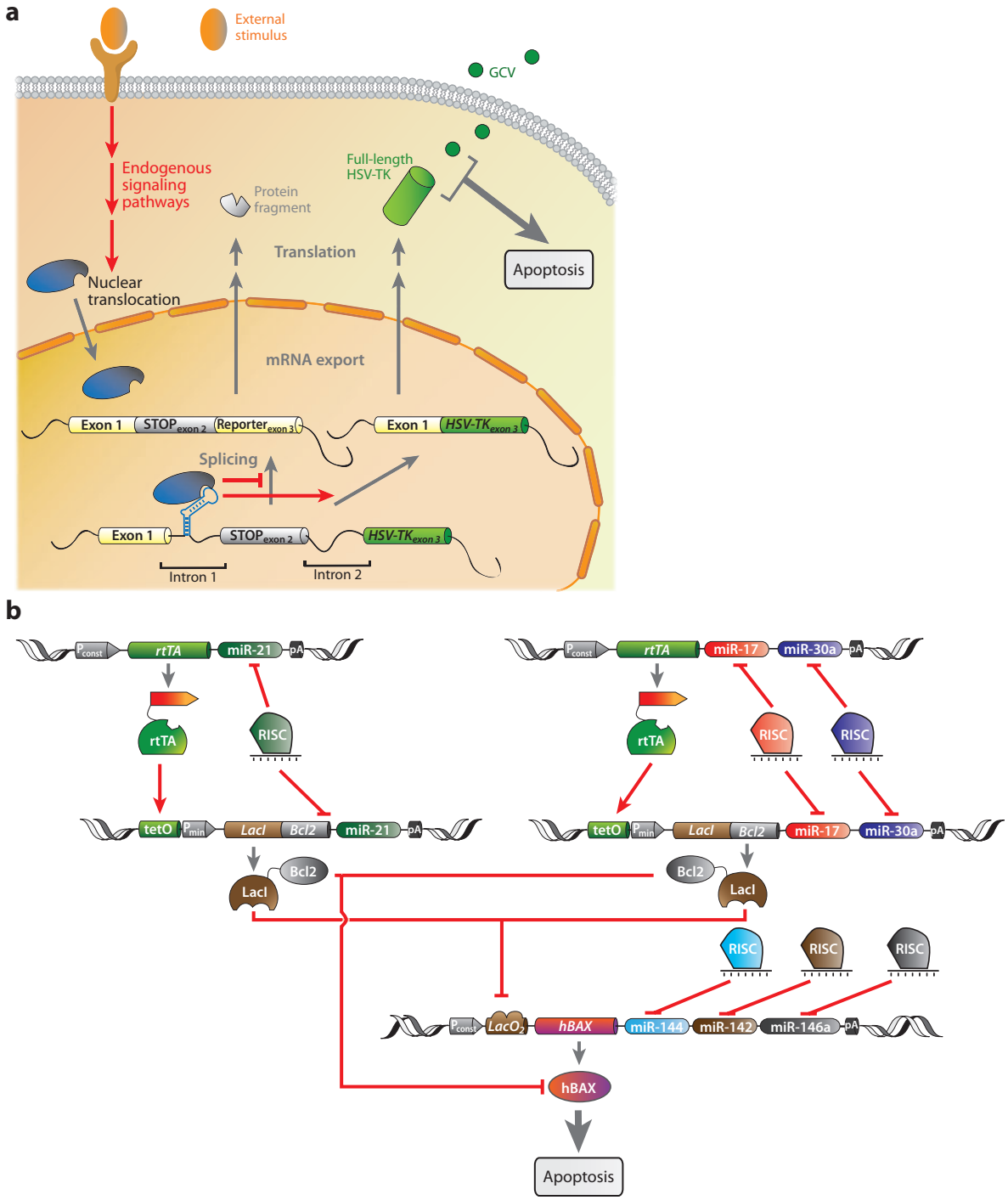
Furthermore, a time-delayed negative feedback loop also can be generated by influencing the timing of repressor transcription. In one example, a TetR-repressible promoter controlled transcription of a reporter and the Tet repressor. The time delay essential for oscillation was introduced into this circuit by implementing into the transcript long intronic spaces that were located upstream of the two genes (**Figure 4*b***). Owing to the increased transcript length, RNA polymerase requires more time for complete transcription. This eventually resulted in a delayed

expression depending on the intron length, which therefore also represented a way to adjust oscillation frequency (100).

Rewiring internal pathways with functional RNAs. Rewiring of endogenous cellular signaling pathways has gained increasing attention recently. Although such networks are not orthogonal to possible cellular influences, they do offer a promising alternative to the transcriptional regulators described above because existing pathways are often quite robust and highly optimized for signal transduction. Hijacking these pathways would make it possible to implement artificial circuits based on a limited number of additional constructs that supplement, and therefore rewire, existing networks.

In a pioneering experiment, Smolke et al. (75) capitalized on RNA aptamers that had been introduced proximal to intron splicing sites. As shown previously (81), proteins that recognize these aptamer sites prevent the binding of essential splice factors to the pre-mRNA, which inhibits splicing. Thus, Smolke and colleagues engineered a transcript that contained three exons and two introns. The first exon encoded a common 5′-UTR, the second a stop codon, and the third the gene of interest. In this splicing setup, inclusion of the middle exon in the processed mRNA led to no gene expression. However, exclusion resulted in efficient full-length translation of the gene of interest (75). The protein-aptamer interaction directed splicing to exclude the middle exon by masking the corresponding splice site. Because multiple aptamers specific for endogenous proteins are available, this setup represents a versatile platform for tapping cellular conditions. This was demonstrated by using the NF-κB-binding (101) or the β-catenin-binding (102) aptamers to control expression of the Herpes simplex virus thymidine kinase (HSV-TK). Receptor-induced nuclear translocation of both NF-κB and β-catenin led to a significant increase in HSV-TK production, which triggered apoptosis only in combination with administration of the prodrug gancivlovir (**Figure 5*a***). Therefore, this system offers a sophisticated cell-fate decision-making circuit that features a safety latch that requires not only elevated nuclear protein levels but also the second input gancivlovir (75).

Considering the important role that RNAi plays in endogenous gene regulation, rewiring RNA input, in addition to the protein signal cascades, is a promising approach for the engineering of novel gene circuits. In particular, the upregulation of specific endogenous miRNAs was shown to trigger cancer formation (103), which makes such upregulation a reliable biomarker (104) as well as an interesting target for cancer therapy (103). In a pioneering approach, a gene circuit was constructed that could not only measure cellular miRNA levels but also react accordingly (105). Benenson and colleagues (93) used the basic concept of shRNAs that regulate gene expression in a sequence-specific manner and introduced MREs of oncogenic miRNAs into the 3′-UTR of a gene of interest. Hereupon, its expression is inhibited when the corresponding miRNA is upregulated: The cells were reprogrammed to trigger apoptosis by expressing the proapoptotic human Bcl-2-associated X protein (hBax) only in the case of elevated levels of the oncogenic miRNAs miR-17, -21, and -30a (105), which are specific to HeLa cancer cell lines (106). hBax expression was thereby the last step in a regulatory cascade starting with the rtTA-dependent transcription of a LacI-Bcl2 fusion protein. Because the hBax expression construct harbored a LacI operator site, the LacI-Bcl2 protein could inhibit the apoptotic effect of hBax on the transcriptional level (LacI) as well as on the protein level based on antiapoptotic Bcl2-hBax interactions (105). MREs of miRNA-17, -21, and -30a were introduced into the noncoding regions of both rtTA and LacI-Bcl2 mRNAs. Therefore, elevated miRNA-17, -21 and -30a levels resulted in diminished rtTA and LacI-Bcl2 expression, thus increasing intracellular hBax levels and eventually leading to apoptosis. One key advantage of using miRNAs as intracellular biomarkers is their cancer-specific expression pattern (106), which ultimately makes it possible to engineer cell-line-specific gene circuits that are mediated



by a fail-safe circuit to ensure activation in defined cells only. As an example, Benenson et al. (93) rendered this apoptosis trigger active only in HeLa cells. Therefore, the hBax expression construct received an additional safety latch: The 3′-UTR of hBax was complemented with MREs specific for miRNA-142, -144 and -146a, which are present at low levels in HeLa cells but elevated in other cell lines (**Figure 5*b***). This setup enabled that hBax expression is possible only in HeLa cells and is inhibited in the other cell lines tested (105).

Prosthetic gene networks: engineered gene circuits that complement or treat a predefined physiological or metabolic disorder

Microencapsulated cells: mammalian cells that are surrounded by an artificial semipermeable membrane

Prosthetic Gene Networks

In addition to gene circuits that sense endogenous factors and then make cell-fate decisions in culture, mammalian cells have been reprogrammed to tackle diseases in whole animals through prosthetic gene networks. Because systemic gene therapy is not yet possible, scientists have focused on two ways of introducing reprogrammed cells into organisms: The first is reprogramming and return of previously isolated host cells, and the second is implantation of xenogeneic microencapsulated cells harboring engineered gene circuits.

Control of T cell proliferation in mice. Following the first approach, T cell proliferation in mice was engineered to be small-molecule dependent. The underlying artificial gene circuits are based on rewiring the signal pathway of the paracrine cytokine interleukin 2 (IL-2) (107). In the CTLL-2 mice used in this experiment, T cells constitutively express the IL-2 receptor; therefore, IL-2 is the sole mediator of T cell proliferation (108). Consequently, administration of IL-2 induces growth of the T cell population, whereas removing IL-2 results in apoptosis. A gene regulation system capitalizing on this existing cell model allowed for the exogenous control of the T cell population. Once aptamer-dependent ribozymes were placed in the 3′-UTR of the IL-2 gene, its expression became responsive to theophylline. Addition of theophylline inhibited ribozyme self-cleavage, which eventually led to efficient IL-2 translation. In the absence of theophylline, however, ribozyme-mediated mRNA destabilization resulted in significantly reduced target gene expression. Interestingly, this system performed best when three copies of the small molecule–responsive ribozyme were incorporated into the 3′-UTR (107). As anticipated, these engineered cells transferred quite well into mice, which exhibited an increased T cell population in the presence of theophylline compared with when it was absent (107).

Figure 5

Gene circuits that make cell-fate decisions. (*a*) Endogenous protein levels involved in intracellular signaling were measured to control gene expression. Capitalizing on signal-dependent nuclear translocation of proteins, intron splicing was inhibited once these proteins bound to their corresponding aptamers, thus masking essential splice sites. In the setup depicted here, masking of splice sites resulted in exclusion of exon 2, which harbors a stop signal that would lead to preliminary protein translation abortion. Full-length HSV-TK protein is produced only if the endogenous protein is translocated into the nucleus upon external triggering. As a safety latch, the final cell-fate decision is made only in the presence of the drug gancivlovir (GCV), which induced cell apoptosis in combination with HSV-TK (75). (*b*) A cell-fate circuit that detects high levels of the microRNAs (miRNAs) miR-17, -21 and -30a in HeLa cancer cells. A genetic cascade was engineered that consists of a reverse tetracycline-dependent transactivator (rtTA), which induces the LacI-Bcl2 fusion protein; LacI eventually blocks transcription of the apoptotic human Bcl-2-associated X protein (hBax), and Bcl2 inhibits its molecular function. In this setup, miRNA recognition elements (MREs) for the corresponding miRNAs were introduced into the rtTA and LacI mRNAs. At elevated levels of the miRNAs, rtTA and LacI-Bcl2 expression was repressed and apoptosis induced. As a safety latch, hBax expression was put under control of the miRNAs miR-144, miR-142, and miR-146, which are elevated in other cell lines. This means that the cell-fate decision is made only in HeLa cancer cells and not in other cells exhibiting elevated levels of the other miRNAs (105). Gray arrows denote the event described, red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: LacI, lactose-dependent transcriptional inhibitor; $LacO_2$, operator site of LacI; pA, polyadenylation signal; P_{const}, constitutive promoter; P_{min}, minimal promoter; RISC, RNA-induced silencing complex.

Microencapsulation: surrounding implanted cells with a semipermeable membrane that shields the cells from the host immune system but enables nutrition supply

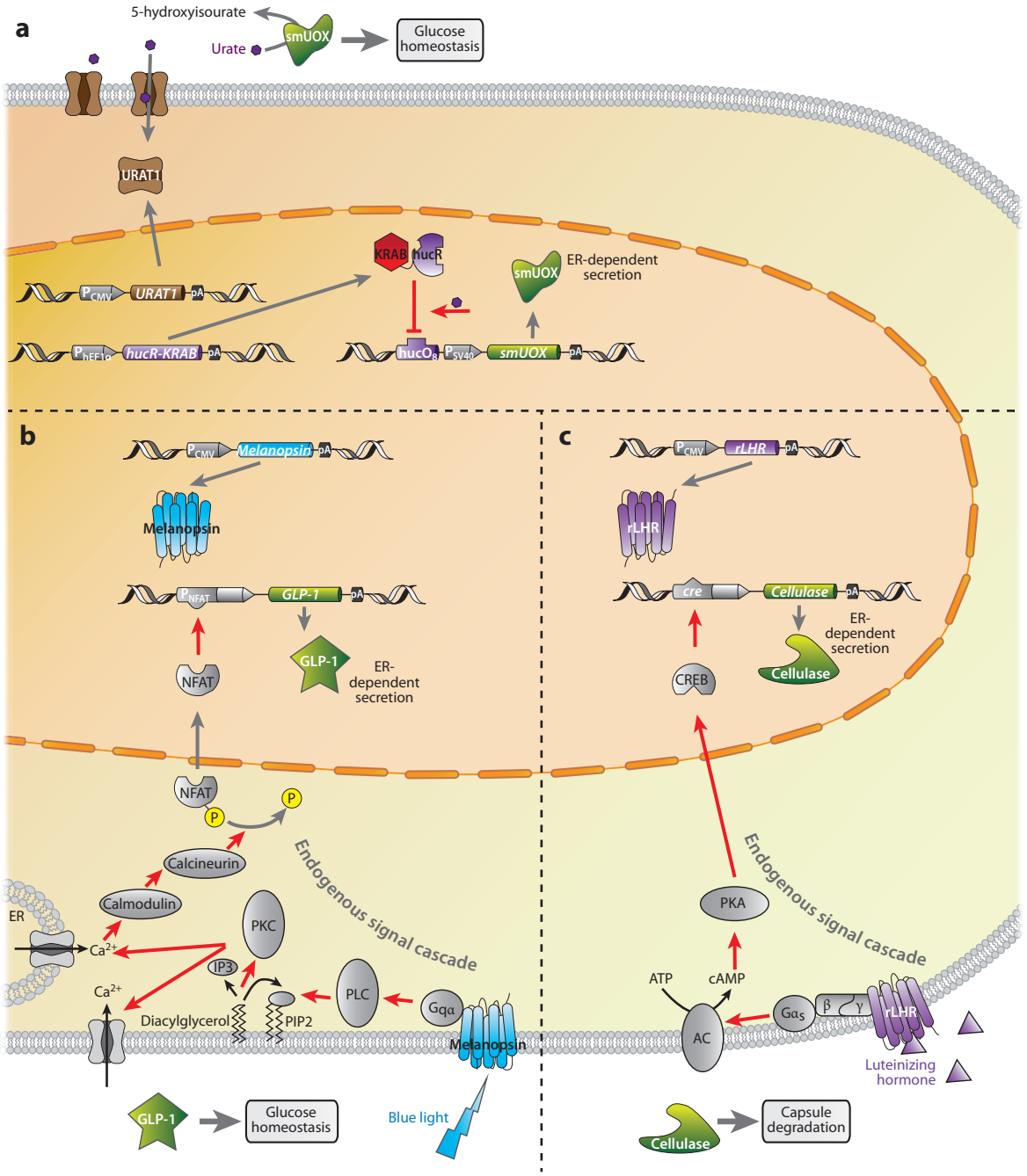
In the second microencapsulation approach, the choice of cell lines is not limited to the repertoire of allogeneic cells. The capsule, which often consists of alginate-poly-L-lysine or cellulose sulfate, protects the cells from an immediate immune response but facilitates essential nutrition supply and the export of secreted therapeutic proteins and metabolites. In particular, use of immortalized cell lines simplifies handling and circuit engineering, which creates the potential to construct gene networks featuring complexity comparable with those already used in cell culture (109).

Taking a shot of light. Encapsulated cells were recently reprogrammed to enhance blood glucose homeostasis in a diabetic mouse model upon irradiation with blue light of a defined wavelength. A glucagon-like peptide-1 variant (shGLP-1) that controls blood glucose homeostasis via stimulation of insulin and inhibition of glucagon production (110) was put under the transcriptional control of the heterologous G protein–coupled receptor (GPCR) melanopsin (111). Once illuminated, this protein, which was originally expressed in intrinsically photosensitive retinal ganglion cells and which harbored the chromophore 11-*cis*-retinal (112), triggered a defined endogenous signaling cascade. The activated $G_{q\alpha}$ subunits stimulated phospholipase C activity, which eventually led to cytosolic Ca^{2+} influx. This triggered the calmodulin-dependent activation of calcineurin, which enables translocation of NFAT (nuclear factor of activated T cell), an endogenous transcription factor, into the nucleus by dephosphorylation. NFAT, which bound to the operator sites, finally induced transcription of the target genes (113). Because this pathway is conserved in other mammalian cells, heterologous expression of melanopsin in HEK-293 cells was expected to enable controlled NFAT-dependent gene expression. Indeed, combined expression of both melanopsin and the NFAT-driven reporter gene (shGLP-1) led to blue light–responsive gene regulation (**Figure 6*b***) (111). The robustness of this hijacked circuit even enabled the cells to be microencapsulated and subsequently implanted into mice. Blue light irradiation was used to

Figure 6

Prosthetic engineered networks in encapsulated cells implanted into mice. (*a*) A gene circuit that restores urate homeostasis in host mice. Elevated blood urate levels are detected by the intracellular sensor protein hucR-KRAB (Krüppel-associated box), which is released from its operator site $hucO_8$ in the presence of urate. Hereupon, expression of smUOX, a secreted variant of a urate oxidase, is induced that eventually restores urate homeostasis in the blood by oxidizing urate to 5-hydroxyisourate. Coexpressing the urate importer URAT1 increased the sensitivity of the network (37). (*b*) A gene circuit that restores blood glucose homeostasis in a type-II diabetes mouse model upon blue light irradiation. Cells were reprogrammed by heterologous expression of melanopsin, a G protein–coupled receptor (GPCR) that is triggered by blue light. Hereupon, an endogenous signal cascade begins in which $G_{q\alpha}$ activates phospholipase C (PLC), which eventually leads to Ca^{2+} influx. This induced a calmodulin- and calcineurin-dependent dephosphorylation and nuclear translocation of the transcription factor NFAT (nuclear factor of activated T cell), which started transcription of the heterologous secreted variant of glucagon-like peptide-1 (GLP-1). GLP-1 restored blood glucose homeostasis by stimulation of host insulin and inhibition of glucagon production (111). (*c*) An artificial bovine insemination device implanted into a cow uterus. In addition to cells harboring the depicted gene circuit, bull sperm were implemented into microcapsules consisting of cellulose. Bovine insemination can be successful only during ovulation. Therefore, measurement of endogenous luteinizing hormone (LH) levels through expression of the heterologous LH-dependent GPCR triggered sperm release. Elevated LH levels during ovulation resulted in GPCR-mediated intracellular signaling, in which $G_{\alpha s}$ activated the adenylate cyclase (AC)-dependent formation of cAMP that, in turn, activated CREB (cAMP response element binding)-dependent transcription of a heterologous secreted variant of a cellulase. This cellulase molecularly degraded the capsule, releasing the bull sperm into the cow uterus (124). Red arrows indicate activation (*arrowhead*) or inhibition (*blocked line*) of the respective event. Abbreviations: cre, cAMP response element; ER, endoplasmic reticulum; IP3, inositol trisphosphate; P, promoter; pA, poly adenylation signal; P_{CMV}, cytomegalovirus promoter; $P_{hEF1\alpha}$, human elongation factor 1α promoter; PIP2, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; P_{NFAT}, nuclear of activated T cells promoter; P_{SV40}, simian vacuolating 40 virus promoter; RISC, RNA-induced silencing complex; rLHR, rat luteinizing hormone receptor.

trigger shGLP-1 expression, which elevated insulin levels and successfully prevented spiking of blood glucose in type-II diabetic mice. The blue light could be delivered transdermally or by fiber optics that enable intracorporeal placement at a defined site, which increases the potential of this approach (111).



Artificial urate homeostasis. Many of the gene regulation systems developed in vitro have worked efficiently in encapsulated cells in vivo. The diversity of these systems means that genes of interest can be induced or repressed with a great variety of ligands, ranging from externally administrable molecules (40, 114–116) to endogenous metabolites (117). The latest generation of encapsulated cells has been engineered to measure defined parameters of the patient's physiology and even take appropriate action if distortion of host homeostasis is detected. In general, gene networks that fulfill these high demands for detection followed by appropriate therapeutic response must consist of two complementary circuit components. The sensory component, which is responsible for measuring a predefined physiological parameter, controls the output component that must restore the original state, thereby tackling the disease in question.

This principle was successfully implemented by construction of a sophisticated gene circuit that restored disturbed urate homeostasis. Elevated blood levels of urate cause several pathologies, including gout and tumor lysis syndrome (118), and result in monosodium urate and uric acid crystals, which form painful accumulations in joints. However, basal levels of urate are thought to mediate protection from oxidative stress (119). Therefore, an effective treatment should take into account the protective effect of a low level of urate, thereby precisely restoring physiological homeostasis instead of completely depleting blood urate.

As described above, the engineered gene circuit consisted of a sensory component and an output component implemented in HeLa cells. The sensory component was constructed by fusing the bacterial urate-responsive repressor HucR (120) with the KRAB domain, which resulted in the urate-dependent transsilencer HucR-KRAB. Sensor sensitivity was adjusted to physiological conditions by coexpressing the urate-importing transporter URAT1 (121). Upon urate binding, HucR-KRAB was released from its operator site, which initiated expression of the secreted urate oxidase (UOX) (**Figure 6*a***) (122). UOX, the output component, oxidizes urate to the nonhazardous 5-hydroxyisourate. At low urate levels, however, UOX expression is repressed to ensure oxidative protection. Symptoms of gout were significantly reduced when microencapsulated cells harboring this sophisticated gene network were implanted into mice that usually exhibit pathological elevated blood urate levels (37).

Artificial cow insemination. Rewiring of endogenous pathways has provided a powerful platform for development of high-performance engineering circuits. Endogenous signaling has even been used to engineer microencapsulated cells that enabled improved artificial insemination of cows. Thus far, efficient agricultural cow insemination has been based primarily on the farmer's ability to recognize the precise time of ovulation, but ovulation time could be determined easily by measuring endogenous luteinizing hormone (LH) levels. The output component in this particular case is the release of sperm into the cow uterus. By integrating these essential input/output components, a system was designed based on the implantation of microencapsulated sensory cells, together with sperm, into the cow uterus. The sensory cells that harbored the heterologously expressed LH-GPCR (123) measured endogenous LH levels. Increasing LH concentration induced GPCR-mediated activation of adenylyl cyclase, which triggered cAMP formation and eventually led to CREB1-dependent transcriptional activation of the target genes. By encoding a secreted variant of a cellulase under the control of CREB1, receptor activation finally resulted in high extracellular cellulase concentrations (**Figure 6*c***). At this point, the cellulose capsule was degraded and the enclosed sperm was released into the cow uterus. Therefore, this advanced gene circuit enabled the precise spatiotemporal release of a predefined cargo into living animals (124)

SUMMARY

Despite being a relatively young scientific discipline, synthetic biology has achieved some impressive milestones. First, many basic genetic control elements have been established and characterized that act on both the transcriptional and posttranscriptional levels. This repertoire has enabled control of gene expression with a variety of inducers including exogenous small molecules, metabolites, proteins, and even electromagnetic waves. Higher-order gene networks have been successfully engineered by capitalizing on the modularity of genetic elements. Inspired by electronics, complex biological circuits were achieved through the combination of basic elements such as positive and negative feedback loops, genetic inverters, and logic gates. This made it possible to engineer reprogrammed cells that mimic bandpass filters, toggle switches, and oscillators. The available genetic elements and circuits also act as a valuable toolbox for the development of novel and innovative therapeutic approaches. Microencapsulated cells that tackle physiological disorders, along with the reprogramming of T cells and advanced cell-fate decision networks, have impressively demonstrated the potential of synthetic biology in personalized medicine. Looking ahead, synthetic biology must keep its promise by demonstrating that it is possible to rationally engineer circuits that are capable of performing most complex calculations and yielding most gene expression patterns. Although the existing genetic toolbox enables the engineering of such complex circuits, the search for more optimized and advantageous components is still ongoing. The development of chemically traceless and inexpensive gene regulation systems based on physical inducers such as electromagnetic waves is an especially promising approach. With the available genetic components and circuits, it will be possible to promote biocomputing from basic Boolean calculations in cell culture to more complex mathematical calculations in multicellular tissue-like assemblies. Also, focus will be put on further therapeutic innovations by engineering cells harboring more complex circuits that enable measurements of several host physiological parameters and that eventually act accordingly.

FUTURE ISSUES

1. Novel gene expression systems based on electromagnetic waves will be developed. This cost-effective and traceless method for regulating target gene expression will be a central element in future gene circuits because it enables precise spatiotemporal control over inducer administration.
2. The progress in reprogramming mammalian cells to perform more complex Boolean calculations will result in advanced biocomputers capable of performing mathematical calculations.
3. Advanced gene circuits that are implemented into single mammalian cells will be connected with each other by cell-to-cell communication systems to obtain more complex networks.
4. In the future, prosthetic networks should be engineered to measure several physiological or metabolic parameters simultaneously and act accordingly.
5. Novel gene circuits should be engineered that allow for a bidirectional interface with electronic devices: Establishment of a functional and programmable communication between mammalian cells and electronic chips would offer great opportunities for synthetic biology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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105. Described cell type–specific intracellular detection of elevated miRNA levels.

107. Demonstrated the application of aptamer-controlled ribozyme cleavage mediation control over T cell proliferation in mice.

111. Determined that rewiring of endogenous GPCR pathways to engineer a light-responsive expression system restores glucose homeostasis in mice.

124. Showed that artificial cow insemination is mediated by microencapsulation of cells harboring a sensory circuit that determines ovulation time.

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Errata

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