Chemistry & Biology
Review

Engineering of Synthetic Mammalian Gene Networks

Wilfried Weber[1] and Martin Fussenegger[1,*]
[1]Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, CH-4058 Basel, Switzerland
*Correspondence: fussenegger@bsse.ethz.ch
DOI 10.1016/j.chembiol.2009.02.005

Synthetic biology, the science of engineering complex biological systems with novel functions, is increasingly fascinating researchers across disciplines who gather to design functional biological assemblies in a rational and systematic manner. Although initial success stories were based on reprogramming prokaryotic and lower eukaryotic cells, the design of synthetic mammalian gene circuits is becoming increasingly popular because it promises to foster novel therapeutic opportunities in the not-so-distant future. Here, we discuss the latest generation of mammalian synthetic biology devices assembled to form complex synthetic gene networks, such as regulatory cascades, logic evaluators, hysteretic circuits, epigenetic toggle switches, time-keeping components, drug discovery tools, and "cell phone" units. We further highlight how such circuits could be interconnected to achieve higher-order control networks such as synthetic hormone-like communication systems in animals or synthetic ecosystems with dynamic interspecies crosstalk.

Introduction

Complementary to classic (systems) biology where scientists take biological systems apart to describe or gain insight into functionalities of living systems, synthetic biology takes advantage of rational reassembly of well-characterized biological parts and devices into functional systems with novel or improved characteristics. In recent years, systems biology has provided an impressive wealth of details on the operation dynamics of complex biological systems that had largely been elusive during the genomics era. Because prokaryotes and lower eukaryotes are more accessible to rational and precise genetic engineering, pioneering advances in synthetic biology have mainly been achieved in *Escherichia coli* and the yeast *Saccharomyces cerevisiae* (Benner and Sismour, 2005; Drubin et al., 2007; Sprinzak and Elowitz, 2005). Nonlimiting highlights include the first synthetic bistable toggle switch (Gardner et al., 2000), self-oscillating genetic networks (Elowitz and Leibler, 2000), artificial cell-to-cell communication systems for the construction of pattern-forming band-detection filters (Basu et al., 2005), *E. coli*-based synthetic ecosystems (Balagadde et al., 2008), and the design of complex translation-control networks (Isaacs et al., 2004; Win and Smolke, 2008). Synthetic biology is also beginning to impact translational research as exemplified by engineering of bacteria for improved lycopene production (Alper et al., 2005) or the synthesis of artemisin, the precursor of the antimalaria drug Coartem® (Martin et al., 2003).

Recent advances in heterologous mammalian transcription control technology enabling precise adjustable and reversible gene expression (Weber and Fussenegger, 2006, 2007a) have prompted synthetic biology initiatives in mammalian cells and entire animals (Deans et al., 2007; Greber and Fussenegger, 2007a; Kramer and Fussenegger, 2005; Kramer et al., 2004b; Weber et al., 2007a, 2007c). After functional design of synthetic replicas of natural network topologies (Weber and Fussenegger, 2002) such as regulatory cascades (Kramer et al., 2005, 2004b), epigenetic toggle switches (Kramer et al., 2004b), and hysteretic networks (Kramer and Fussenegger, 2005; May et al., 2008), basic transcription control devices have been assembled and interconnected to achieve higher-order control showing time-delayed expression kinetics (Weber et al., 2007c) and allowing the engineering of synthetic hormone systems in mice as well as the assembly of entire ecosystems with synthetic intra- and interspecies crosstalk (Weber et al., 2007a). Because pioneering strategies to engineer synthetic networks in bacteria and yeast (see references above) are covered elsewhere, we focus on the latest developments in the design of complex mammalian gene networks and discuss future directions of mammalian synthetic biology.

Mammalian Cell-Compatible Biological Parts As Building Blocks For Construction Of Synthetic Gene Networks

A consortium of experts from the Lawrence Berkeley National Laboratory, MIT, and Harvard University have suggested to define synthetic biology as "the design and construction of new biological parts, devices, and systems and the redesign of existing, natural biological systems for useful purposes" (http://syntheticbiology.org). This definition and the approach to construct complex systems in living cells were inspired by information technology and electronic engineering, where complex assemblies like computer networks or the internet are constructed by interconnecting basic modular parts such as transistors, capacitors, or resistors. To transfer this design principle—the construction of higher-order systems by assembling modular parts—to biology, synthetic biologists prefer to use standardized biological parts with well-defined and characterized properties that can be connected to each other in a predictable manner. Next, we provide an overview of today's most prominent mammalian cell-compatible biological parts: synthetic DNA-binding receptors and transcription factors, chemically induced dimerizing proteins, and signal-producing enzymes. Recently developed RNA-based switches are highlighted elsewhere and in this issue (Win and Smolke, 2008; Win et al., 2009).

Synthetic DNA-Binding Receptors and Transcription Factors

Synthetic DNA-binding receptors and transcription factors represent the most prominent family of mammalian cell-compatible

biological parts for use in mammalian synthetic biology related to gene networks. The DNA binding of these chimeric receptors and transcription factors can be modulated by small-molecule compounds or physical conditions and, depending on their allostery, they bind to or are released from their operator in response to the trigger (Weber and Fussenegger, 2007b). Synthetic DNA-binding receptors and transcription factors are typically engineered to modulate the activity of specific target promoters by three distinct mechanisms (Weber and Fussenegger, 2006). (i) DNA-binding proteins which adjustably repress promoters by binding to 3′-placed operator modules. (ii) Synthetic DNA-binding proteins fused to a transsilencing domain (krueppel-associated box protein [KRAB] of human *kox1*) that fine-tune expression of constitutive promoters when bound to operators placed in their vicinity. (iii) Chimeric DNA-binding proteins linked to transcription-activation domains (e.g., herpes simplex virus protein 16 [VP16]) modulate transcription by binding to cognate operators placed 5′ of minimal eukaryotic promoters (e.g., $P_{hCMVmin}$). An impressive repertoire of such synthetic mammalian transcription factors has been designed and validated in mammalian cells and in mice and was shown to be adjustable to a variety of compounds like antibiotics, *Streptomyces*-derived quorum-sensing signals, metabolites, physical conditions, amino acids, vitamins, or food additives (see Weber and Fussenegger, 2007b for a comprehensive overview on transcriptional repressors). Owing to their generic design, synthetic transcription control systems are compatible and can be simultaneously operated in mammalian cells for independent or interconnected control of different (trans)genes (Fux et al., 2004; Weber et al., 2002a). Also, all transcription-control modalities can be optimized for expression performance and regulation window using similar modification principles. (i) The synthetic transcription factor can be expressed using different constitutive (tissue-specific) promoters (Dickins et al., 2007). (ii) The DNA-binding proteins can be fused to various transsilencing or transactivation domains optionally using linker peptides and/or nuclear localization signals (Fussenegger et al., 2000; Weber et al., 2002b). (iii) Different constitutive or minimal promoters could be used to drive target gene expression (Weber et al., 2002b). (iv) Operators can be multimerized to tandem units and placed at different distances from constitutive or minimal promoters (Gossen and Bujard, 1992; Weber et al., 2002b). (v) When flanking inducible expression cassettes with insulator sequences, transgene regulation can be shielded from disturbing transcriptional influence originating from neighboring chromosomal sequences (Pluta et al., 2005). (iv) The DNA-binding proteins can be mutagenized to optimize induction characteristics (Urlinger et al., 2000) or reverse the DNA-binding characteristics in the presence of a specific inducer (Gossen et al., 1995). The tuning flexibility and compatibility among each other have established DNA-binding transcription factors as the most important class of proteins in the construction of synthetic gene networks.

Chemically Induced Dimerizing Proteins

Chemically induced dimerization of engineered proteins has been successfully used for trigger-adjustable induction of membrane-bound receptor activity (Farrar et al., 1996), transcription (Rivera et al., 1996; Zhao et al., 2003), translation (Schlatter et al., 2003), secretion (Rivera et al., 2000), or enzyme activity (Rossi et al., 1997). In contrast to DNA-binding transcription factors, for which highly diverse classes of inducer substances exist, chemically induced dimerization is currently limited to the use of rapamycin-induced dimerization of FK-binding protein (FKBP) with FRB (Rivera et al., 1996), FK506-dependent dissociation of F_M-F_M homodimers (Rivera et al., 2000), dimerization of two FKBP proteins by dimeric FK506 (FK1012, Ho et al., 1996), dimerization of FKBP with cyclophilin C by fusion of FK506 to cyclosporin A (Belshaw et al., 1996), and the aminocoumarin antibiotic-triggered homodimerization of two bacterial gyrase subunits (GyrB, Zhao et al., 2003).

Signal-Producing Enzymes

Signal-producing enzymes can either be used to trigger assembly of different proteins or to produce messenger/inducer substances that activate transgene expression in target cells. Enzyme-triggered assembly of two proteins can be achieved by taking advantage of the *E. coli* biotin ligase BirA. BirA catalyzes biotinylation of proteins containing a specific avitag motif that can then heterodimerize with another protein fused to streptavidin. For example, fusion of avitag to a DNA-binding domain and streptavidin to a transactivation domain enables biotin-inducible BirA-mediated reconstitution of a synthetic transactivator that can activate specific target promoters (Weber et al., 2007c).

Examples for enzyme-generated messenger or inducer compounds include biotinidase, which liberates biotin from biocytin in order to trigger biotin-responsive expression systems (Weber et al., 2007a), or alcohol dehydrogenase, which converts ethanol to acetaldehyde and so activates acetaldehyde-inducible transcription modalities (Weber et al., 2007a).

Synthetic Gene Networks in Mammalian Cells

Heterologous transgene regulation systems described above are minimal control devices that can be used to fine-tune transcription of specific target genes in a dose-dependent manner. They can also be interconnected to provide higher-order regulatory gene networks that enable complex signal processing, optimized regulation performance, and unprecedented control features inaccessible to simple control devices (Greber and Fussenegger, 2007a). Following, we discuss several examples on how individual transgene control devices could be assembled into more complex gene networks with novel regulation characteristics.

Synthetic and Semisynthetic Regulatory Cascades

A prominent example of a synthetic mammalian gene network consisted of an artificial two-step signaling cascade in which a small-molecule-responsive transcription factor controlled expression of a second transcription factor that then modulated transcription of the target gene (Kramer et al., 2003). This daisy-chain interconnection of individual transcription control circuits was then successfully used to design a three-level transcription cascade that provides discrete multilevel control in response to different input signals (Kramer et al., 2003). This synthetic mammalian three-step cascade consisted of the TET-responsive promoter P_{TET} driving dicistronic expression of TET-dependent transactivator (tTA) and erythromycin-dependent transactivator (ET1), whereby tTA was auto-activating P_{TET} in a positive feedback loop together with cocistronically encoded ET1. ET1 was programmed to activate the erythromycin (EM)-responsive

promoter P_{ETR}, which controlled transcription of the streptogramin-dependent transactivator, a PIP-VP16 fusion protein (PIT). PIT was then activating its cognate promoter P_{PIR}, thereby controlling expression of human placental secreted alkaline phosphatase (SEAP) (Figure 1A). The signaling cascade could be interrupted at any level by the addition of TET, EM, or pristinamycin I, specifically inactivating tTA, ET1, and PIT, respectively. Because each antibiotic represses the cascade at a different level in the cascade, the impact of the transcription leakiness on the overall transgene expression level varies depending at which point in the cascade it occurs: expression levels of 100% (no cascade intervention), 66% (intervention at first level of cascade), 33% (intervention at second level), and close to 0% (intervention at third and final level) of transgene were possible (Figure 1A). In this way a precise expression level could be achieved by addition of a particular antibiotic that enables molecule-specific titration of target genes (Kramer et al., 2003).

Capitalizing on the success of the synthetic three-level cascade providing four discrete expression levels in response to three different antibiotics, Kramer and coworkers have also designed a semisynthetic cascade that could process endogenous (e.g., hypoxia) and exogenous (e.g., an antibiotic) signals to provide up to six discrete expression levels defined by the combination of input signals (Figure 1B) (Kramer et al., 2005). The endogenous hypoxia signal was plugged into the cascade via hypoxia-responsive element fused to $P_{SV40min}$. Under hypoxia, the endogenous hypoxia-inducible factor (HIF)-1α translocates into the nucleus, heterodimerizes with HIF-1β and binds to hypoxia-responsive elements, thereby inducing $P_{SV40min}$. At normal oxygen levels (normoxia), hypoxia-inducible factor (HIF)-1α is targeted for proteosomal destruction via its oxygen-dependent degradation domain. The endogenous hypoxia-inducible sensor system was linked to expression of PIT, which was set to control P_{PIR}-driven tTA expression and tTA was arranged to drive P_{TET}-controlled expression of SAMY (Figure 1B). Using different combinations of endogenous (hypoxia) or exogenous (addition of antibiotics) stimuli, SAMY production could be adjusted to six discrete expression levels (Kramer et al., 2005). Semisynthetic cascades provide a first example how synthetic gene networks could be functionally coupled to endogenous control circuits and yet retain responsiveness to external intervention factors.

Coupled Transcription-Translation Networks

Because the flow of genetic information from DNA to protein involves transcription and translation, transgene expression could be adjusted at both levels to enable protein production fine-tuning with unmatched precision and tightness. Although individual transcription (Gossen et al., 1995, see above) and translation (Schlatter et al., 2003) units were successfully used for trigger-inducible heterologous protein production in mammalian cells, their regulation windows (the difference in protein levels reached under fully induced or repressed conditions) seemed to be a system-specific constant: maximum protein production under induced conditions were typically associated with significant leakiness in the repressed state and completely tight control systems only reached mediocre maximum production levels. Coupled transcription-translation networks that suppress leaky transcripts could therefore expand the regulation window and improve overall regulation performance.

In the first coupled transcription-translation network, tetracycline (TET_{OFF}-)-repressible expression of SEAP was functionally linked to EM-inducible expression of a small interfering RNA ($siRNA_{GFP}$) specific for a GFP-derived sequence tag (TAG_{GFP}) engineered into the 5′ untranslated region of SEAP (Figure 1C, Malphettes and Fussenegger, 2006). Although SEAP production was primarily responsive to TET showing typical TET_{OFF} expression profiles, EM-induced kickoff of $siRNA_{GFP}$ transcription during TET-mediated SEAP repression reduced leaky SEAP transcript levels and improved the overall regulation performance of the TET_{OFF} system. The use of siRNA-specific sequence tags unrelated to the target gene enables generic target gene-independent translation control and provides maximum flexibility for use in any coupled transcription-translation network.

In a similar approach, Jim Collins and coworkers (Deans et al., 2007) used the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible Lac switch system to coregulate expression of tetracycline-dependent receptor (TetR) and the gene of interest (GOI) by conditional IPTG-modulated binding LacI to its cognate operator (lacO) placed between the constitutive promoter and TetR or GOI, respectively (Figure 1D). GOI was engineered to contain a sequence tag in the 3′ untranslated region that was specific for a short hairpin RNA (shRNA) transcribed in a TET-specific operator-repressible TET-responsive manner using a TET-specific operator-linked P_{U6} promoter (Figure 1D). In the absence of IPTG, LacI represses transcription of TetR and GOI, which results in derepression of the TET-specific operator-linked P_{U6} promoter and consequent maximum shRNA expression leading to RNA-interference-based elimination of TAGed GOI and remarkable repression of GOI to over 99%.

In order to facilitate the design of such coupled transcription-translation networks, a recent study (Greber and Fussenegger, 2007b) described an efficient strategy for the simultaneous expression of synthetic transcription factors (see above) harboring intronically encoded GOI-specific shRNAs. The potential of this integrated concept was exemplified by the simultaneous expression and knockdown of six genes controlled by a single promoter (Greber and Fussenegger, 2007b). Also, intronic siRNA-based translation control has recently been shown to improve the dynamic range of epigenetic toggle switches (see below; Greber et al., 2008).

Hysteretic Gene Switches

In contrast to the above-described inducible systems with excellent possibilities for adjustment and reversible on/off regulation, many naturally occurring gene switches tend to behave hysteretically (Ozbudak et al., 2004). Classic genetic switches provide a dose-dependent graded expression profile in response to the inducer; however, in hysteretic switches the threshold required to switch from one state to another depends on the expression history, the state of the switch before the change in the inducer concentration. Hysteresis is an important phenomenon, for example in the lactose utilization operon of *E. coli* (Ozbudak et al., 2004) or in ensuring unidirectional cell-cycle progression in eukaryotic cells (Sha et al., 2003). Hysteretic switches make gene expression more robust and noise resistant because the expression state remains unchanged following small fluctuations in inducer levels and only switches in response to significant inducer concentrations changes.

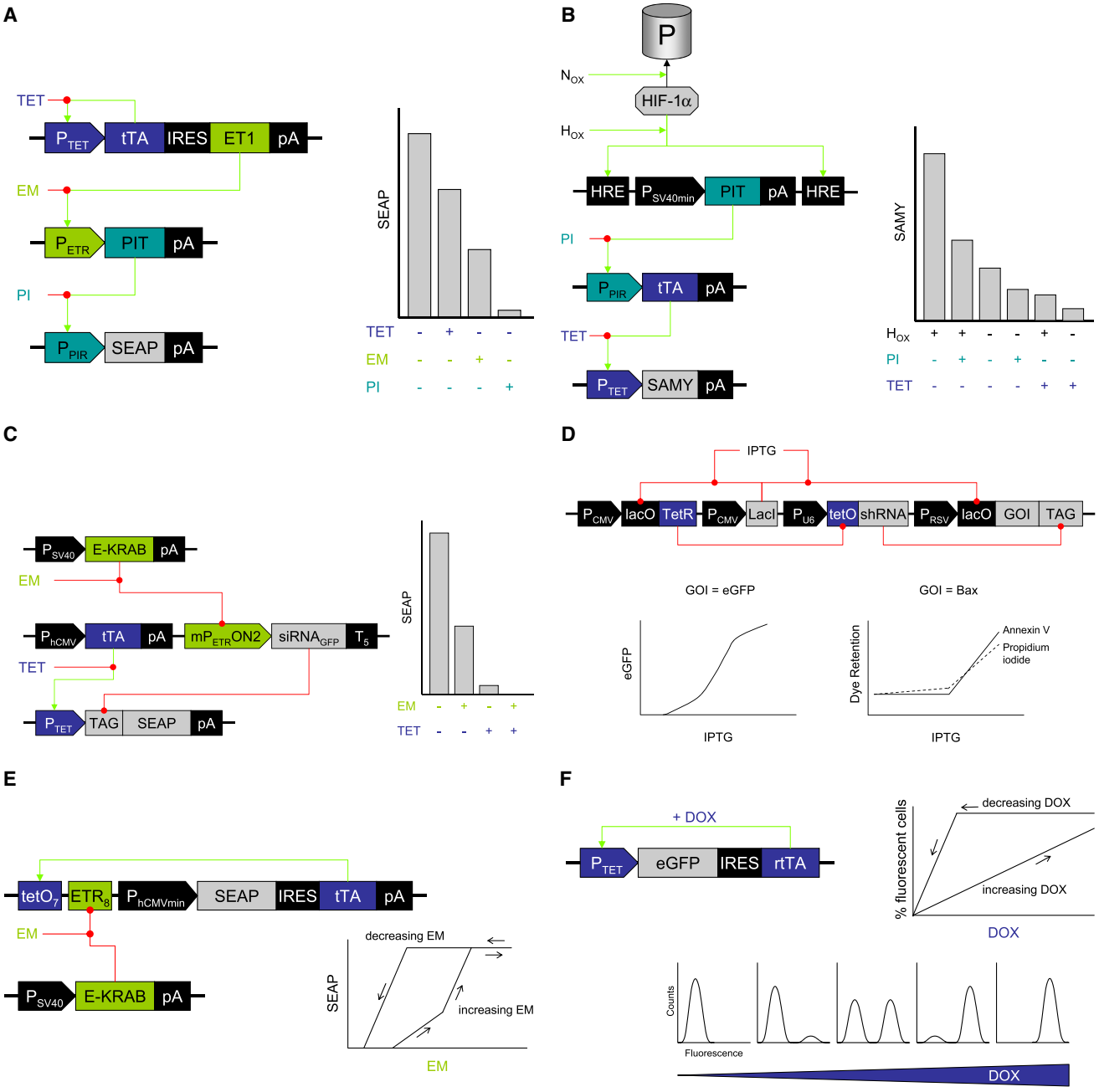

Figure 1. Synthetic gene networks in mammalian cells

(A) Synthetic transcription cascade. The (TET)-responsive promoter P_{TET} triggers expression of the TET- and macrolide-dependent transactivators tTA and ET1. tTA activates P_{TET} in a positive feedback loop while ET1 induces expression of the streptogramin-dependent transactivator PIT under the control of the macrolide-responsive promoter P_{ETR}. PIT finally triggers production of the output gene SEAP (human placental secreted alkaline phosphatase) by binding and activating P_{PIR}. The transcription cascade can be interrupted by specifically inactivating tTA, ET1, or PIT by the addition of TET, erythromycin (EM) or pristinamycin I (PI), respectively, which results in up to four discrete SEAP expression levels.

(B) Semi-synthetic transcription cascade. Under normoxia (N_{OX}), the hypoxia-inducible factor 1α (HIF-1α) is primed for degradation by the proteasome (P). Under hypoxia (H_{OX}), HIF-1α translocates into the nucleus and binds to hypoxia-responsive element (HRE) triggering activation of the minimal simian virus 40 promoter ($P_{SV40min}$) thereby controlling expression of PIT. PIT induces production of tTA by binding and activating P_{PIR}. tTA controls expression of the output gene SAMY (*Bacillus stearothermophilus*-derived secreted α-amylase) via its cognate promoter P_{TET}. The semi-synthetic transcription cascade is interrupted under normoxia or by the addition of PI and TET for inactivating HIF-1α, PIT, and tTA, respectively, which results in up to six discrete SAMY expression levels.

(C) Coupled transcription-translation cascades providing improved regulation performance and tightness. In the absence of EM, E-KRAB represses expression of a small interfering RNA ($siRNA_{GFP}$) specific for a GFP-derived sequence tag (TAG), whereas triggers degradation of SEAP-encoding mRNA harboring the TAG sequence. SEAP expression is activated by tTA in the absence of TET. Therefore, SEAP is fully expressed in the absence of regulating antibiotics, while addition of both, TET and EM represses SEAP below the detection limit. Addition of only one antibiotic results in leaky expression.

(D) One-input-triggered transcription-translation cascade that improves transgene induction characteristics. In the absence of IPTG, LacI represses production of the TET repressor TetR and the gene of interest (GOI). In addition, expression of a short hairpin RNA (shRNA), which is specific for a TAG sequence contained in

Two studies have recently reported design and validation of synthetic hysteretic switches in mammalian cells (Kramer and Fussenegger, 2005; May et al., 2008): (i) (Kramer and Fussenegger, 2005) put tTA under the control of a novel hybrid promoter, which enabled dual TET- and EM-responsive expression. This configuration created a positive feedback loop that rapidly increases tTA expression along with cocistronically encoded SEAP (Figure 1E). In addition, the positive feedback loop can be interrupted by E-KRAB, which, in the absence of EM, binds to the E-specific operator (ETR) operator contained in the hybrid promoter and overrides tTA-based promoter activation. With increasing EM concentrations, SEAP expression increased, depending on the dose, until a plateau was reached. When the EM concentrations were reduced further, SEAP levels decreased, but the decrease occurred at significantly lower EM concentrations than required for triggering the previous activation, thereby demonstrating hysteretic behavior (Kramer and Fussenegger, 2005). (ii) In a subsequent approach, May and coworkers (May et al., 2008) reduced the complexity of the first-generation hysteretic switch by cocistronically expressing the reverse tetracycline-dependent transactivator (rtTA) and enhanced green fluorescent protein (eGFP) in an autoregulated manner (Figure 1F). Administration of the TET analog doxycycline (DOX) triggers binding of rtTA to its promoter P_{TET}, thereby further increasing the expression of rtTA and of cocistronically encoded eGFP. Fluorescence-activated cell sorting analysis of mammalian cells treated with increasing DOX concentrations revealed bimodal eGFP expression: cells are either highly fluorescent or lack any fluorescence, but no intermediate expression levels were detected that would be expected for classic transcription control devoid of any positive feedback loop (Rossi et al., 2000). This bimodal pattern, which is in accordance with a stochastic model, results in stable and uniform levels of expression across the entire cell population, and hardly any clonal variation was observed. The hysteretic characteristics of this system were evaluated by first increasing DOX concentrations until all the cells exhibited eGFP fluorescence, followed by a gradual decrease in DOX, resulting in a decrease in the number of fluorescent cells. This decrease was observed at DOX concentrations that were significantly lower than required to yield comparable fluorescent fractions when ramping up DOX (Figure 1F, May et al., 2008).

Synthetic Gene Networks: Performing Basic Computational Functions

Boolean algebra, a logical calculus of truth values, is at the basis of all devices that process information. Therefore, genetic circuits, which can be designed to perform logical operations of conjunction and disjunction, represent the basic building blocks for cell-based assemblies that could be expected to execute basic computational functions.

Boolean NAND Gate

Boolean operations have been realized in a pilot study in mammalian cells by Kramer et al. (2004a), in which transcriptional control systems were interconnected to operate as NOT, IF, OR, NAND, and NOR gates. For example, the NAND (NOT AND, Figure 2A) gate consisted of ET1, which, in the absence of EM, binds and activates P_{ETR}, resulting in the expression of PIT. In the absence of the streptogramin antibiotic pristinamycin I (PI), PIT binds and activates P_{PIR}, thus triggering the expression of the output gene SEAP. Only in the absence of both antibiotics (EM = 0 AND PI = 0) is SEAP expression active (SEAP = 1); in all other configurations SEAP is inactive (SEAP = 0), reflecting the characteristics of the NAND gate.

Boolean AND Gate

Although the first BioLogic gates (Kramer et al., 2004a) relied on transcriptional control, a study by Rinaudo et al. (2007) described a universal siRNA-based logic evaluator, which operates in mammalian cells. In this configuration, siRNAs are used as input signals to trigger destruction of specific target mRNAs tagged with siRNA-specific sequence motifs (TAGs). Due to the availability of multiple siRNAs and their cognate targets, multiple inputs are possible and enable complex Boolean operations such as (A AND B AND C) OR (D AND E). For the AND gate (Figure 2B), administration of two different input siRNAs ($siRNA_1$ = 1 AND $siRNA_2$ = 1) trigger destruction of two targeted mRNAs harboring respective target sequences (TAG_1 and TAG_2). mRNA destruction prevents translation of coencoded LacI, thereby inducing de-repression of the P_{CAG} promoter fused to LacI-specific lacO operator sites, finally resulting in monomeric red-fluorescent protein (dsRed) expression (dsRed = 1). Administration of only one ($siRNA_1$ = 1 XOR $siRNA_2$ = 1) or no ($siRNA_1$ = 0 AND $siRNA_2$ = 0) input siRNA results in LacI production and subsequent silencing of dsRed (dsRed = 0) (Figure 2B).

An Epigenetic Memory Device

A fundamental characteristic of all data processing devices is the capacity to store information. In order to emulate data storage in a biological system as the basis of potential biocomputing devices, a mammalian memory module was developed with the storage capacity of 1 bit (Kramer et al., 2004b). This bistable toggle switch consists of two promoters driving expression of two different transsilencers that mutually repress these promoters (Figure 2C). The first promoter $P_{SV40}(1)$ triggers expression of SEAP and pristinamycin-inducible protein (PIP)-KRAB, which, in the absence of PI, binds to the PIP-specific operator (PIR) operator 3′ of the second promoter ($P_{SV40}[2]$), thereby repressing $P_{SV40}(2)$. In the presence of PI, PIP-KRAB is released from PIR and $P_{SV40}(2)$ triggers transcription of E-KRAB, which, in

GOI transcripts, reduces GOI expression even further based on a RNA interference-based mechanism eliminating tagged GOI transcripts. In the presence of IPTG, GOI expression is de-repressed and shRNA production is repressed by TetR, resulting in an increase in the activation of GOI expression by more than 100. The system enables dose-dependent expression of eGFP or induction of apoptosis by regulated expression of Bax.
(E) Hysteretic gene switch based on a transcription cascade. Expression of SEAP and the TET-dependent transactivator tTA is activated in a positive feedback loop, where tTA triggers activation of the minimal promoter $P_{hCMVmin}$. The positive feedback loop is interrupted by the binding of the repressor E-KRAB to its operator ETR in the absence of EM. Hysteresis is observed when the EM concentration in the medium is gradually increased and then decreased.
(F) Bimodal and hysteretic gene switch based on a positive feedback loop. In the presence of doxycycline (DOX) P_{TET} is activated in a positive feedback loop by the reverse TET-dependent transactivator (rtTA). eGFP encoded on the same cistron is co-expressed with rtTA. With increasing DOX concentrations, the number of fluorescent cells increases. When the DOX concentration is first increased and then decreased, hysteretic switching behavior of eGFP expression is observed.

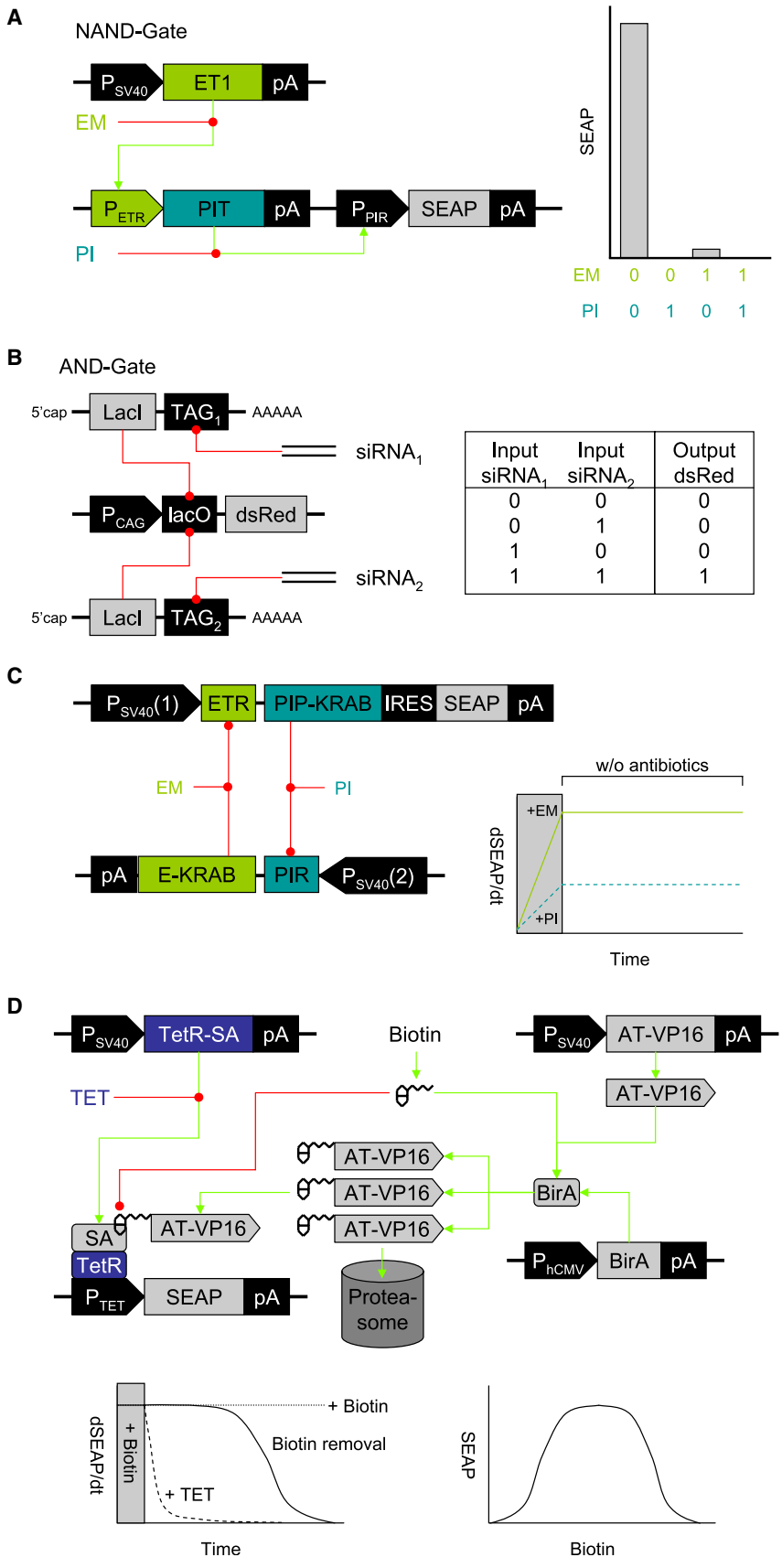

Figure 2. Synthetic gene networks performing basic computational functions

(A) Boolean algebra in mammalian cells. NAND gate based on a transcriptional cascade. In the absence of erythromycin (EM = 0), ET1 activates its promoter P_{ETR}, driving expression of PIT, which activates its cognate promoter P_{PIR} in the absence of pristinamycin I (PI = 0). Only in the absence of both antibiotics (EM = 0 AND PI = 0) SEAP expression is induced (SEAP = 1). In all other configurations, SEAP expression is repressed (SEAP = 0).

(B) AND gate based on modulation of posttranscriptional expression. Addition of siRNA triggers destruction of its target mRNA harboring a specific complementary TAG sequence. mRNA decay prevents LacI translation, thereby leading to de-repression of P_{CAG} and expression of the reporter dsRed. Only in the presence of both siRNAs ($siRNA_1$ = 1 AND $siRNA_2$ = 1) is dsRed expression active (dsRed = 1).

(C) Bi-stable memory. The bi-stable memory circuit consists of two promoters, each driving expression of a repressor to silence the other one. PIP-KRAB binding to PIR silences the adjacent promoter P_{SV40} in the absence of PI, whereas E-KRAB binding to ETR silences the upstream promoter in the absence of EM. The reporter SEAP is cocistronically expressed with E-KRAB via the internal ribosome entry site (IRES). Once one of the promoters has been activated by the addition of the respective antibiotic, the expression state is maintained, even after removal of the regulating drug (without antibiotics).

(D) Time-delay and band-detection circuit. Addition of biotin triggers BirA-catalyzed covalent biotinylation of the herpes simplex virus protein 16 (VP16) transactivation domain fused to the avitag biotinylation signal (AT). Biotinylated VP16 binds to streptavidin (SA) fused to the TET repressor TetR, which binds to its cognate promoter P_{TET} and results in VP16-activated transcription of the reporter SEAP. Time-delay circuit: A short pulse of biotin triggers formation of biotinylated VP16, which accumulates in the cell. Therefore, even after removal of exogenous biotin, SEAP expression is sustained (time-delay function) until biotinylated VP16 has been degraded by the proteasome. Addition of TET (+ TET) overrides the time-delay switch by dissociating TetR from P_{TET}. Band-detection function: Addition of increasing concentrations of biotin to the circuit first correlates with increasing SEAP expression due to the increase in biotinylated VP16. A further increase in the biotin concentration in the medium results in a dose-dependent decrease in SEAP production since binding of biotinylated VP16 to TetR-Streptavidin is competitively inhibited by free biotin.

the absence of EM, binds to the ETR operator 3′ of $P_{SV40}(1)$ and represses this promoter. Administration of EM releases E-KRAB from ETR, which results in derepression of $P_{SV40}(1)$ and coordinated expression of PIP-KRAB and SEAP (Figure 2C). As validated in transgenic mammalian cells and in mice for up to 3 weeks, this epigenetic toggle switch maintained and inherited the SEAP expression status unless SEAP expression switches were induced by addition of either PI (SEAP repression) or EM (SEAP induction) (Kramer et al., 2004b). The dynamic range of this first-generation mammalian epigenetic memory device was significantly expanded by engineering intronically encoded shRNAs into the transsilencers, which eliminated leaky transcripts, bypassing from transsilencer-mediated transcription shut down, via siRNA interference (Greber et al., 2008).

Synthetic Time-Delay and Band-Detection Circuits

Genetic circuitries, exhibiting time-delayed expression kinetics, were shown to modulate nuclear factor kappa B activation, to manage quorum-sensing crosstalk and to control the circadian clock (Weber et al., 2007c). In order to rationally design such time-delayed expression circuits, vitamin H (biotin) was applied to act as the signaling molecule (Weber et al., 2007c, Figure 2D). In the presence of regulation-effective concentrations of biotin constitutive expression of BirA mediates biotinylation of a synthetic peptide tag (avitag, AT). Biotinylation of an avitag-VP16 fusion protein enables heterodimerization with a streptavidin (SA)-tagged TetR that leads to reconstitution of a synthetic TET-dependent transcription factor (TetR-SA-biotin-AT-VP16), which induces expression from TET-responsive promoters (P_{TET}, Weber et al., 2007b). Time-delayed transgene expression is observed when applying a short biotin pulse to the system, thus triggering the accumulation of biotinylated VP16, which persists in the cell and triggers SEAP expression even after removal of biotin from the culture medium. The duration of time-delayed gene expression is, therefore, determined by the amount of biotinylated VP16 at the time of biotin removal and by the degradation rate of AT-VP16, which can be modified by fusion to proteasome-targeting PEST sequences. A complementary study (Weber et al., 2007b) showed that such time-delayed gene expression can act as a filter to eliminate noise originating from inducer fluctuations and provide rather uniform expression kinetics.

Besides time-delayed expression kinetics, it was shown that the same biotin-triggered circuit acts as a band-detection network, responding only to a specific range of biotin concentrations. Gradual increase of biotin concentrations triggers dose-dependent SEAP production until a plateau is reached, indicating that at least one component of the signaling network had become limiting; the P_{TET} promoter was probably saturated with bound synthetic transcriptions factors. As the biotin concentration continues to increase, free biotin competes with biotinylated VP16 for the SA binding sites (TetR-SA-biotin, biotin-AT-VP16), which produces a transactivation-incompetent TetR-SA-biotin fusion protein and leads to graded deactivation of the target promoter (Figure 2D). Such band-detection devices play crucial roles in the differentiation of the developing embryo, where they translate the local concentration of a gradient-forming signaling molecule into a threshold-based steady activation or repression of differentiation-determining genes.

From Individual Cells to Interconnected Multicellular Assemblies and Synthetic Ecosystems

All the above-mentioned genetic networks operated within single mammalian cells and required the stable transfection of up to six expression cassettes, which is technically challenging and time-consuming (Weber et al., 2007c). The next generation of mammalian cell-based synthetic gene networks will rely on the design principle of electronic devices, whereby specialized integrated circuits (microchips), which fulfill basic computational functions (memory, logic functions, i/o interface), are interconnected to form higher-order computational units that process signals not only inside individual cells but also within and across cell populations of the same or different species as well as in tissues, organs, and entire organisms. In order to establish intercellular crosstalk among specialized mammalian cells, cell-to-cell communication channels must be designed to coordinate and orchestrate the overall response of the multicellular assemblies.

Cell-to-Cell Communication: Molecular Cell Phones

Basic principles of **s**ynthetic cell-cell communication networks were established and developed first in bacteria (Bulter et al., 2004) and then in yeast (Chen and Weiss, 2005). The first synthetic mammalian communication device entirely assembled from genetic parts was recently shown to manage information transfer from a sender to a receiver cell in a contact-free manner (we coined the term "molecular cell phone" for a cell-to-cell communication device, Figure 3A, Weber et al., 2007a). Such molecular "wireless" broadcasting included a sender cell, engineered for constitutive or inducible expression of a liver-derived alcohol dehydrogenase to convert traces (‰ range) of spiked ethanol into acetaldehyde, which, having a boiling point of 21°C, diffuses via the gas or liquid phase to the receiver cells (Weber et al., 2007a). The receiver cells were engineered for an acetaldehyde-inducible expression unit consisting of the *Aspergillus nidulans*-derived transcription factor AlcR that activates the chimeric target promoter P_{AIR} in the presence of acetaldehyde (Weber et al., 2004). Considering the acetaldehyde production kinetics and its transfer from the sender to the receiver cells according to Fick's law of diffusion, this molecular cell phone can be used to design distance and cell-density-controlled expression scenarios. In order to evaluate dependence on distance, culture wells containing the receiver cells were placed at gradually increasing distances to the culture wells harboring the sender cells, resulting in a gradual decrease in SEAP production and demonstrating that this system translates the spatial configuration of two cell populations into a graded protein production profile (Figure 3A, inset i; Weber et al., 2007a). It could also be demonstrated that the cell-to-cell communication system enables cell-density-controlled induction of SEAP expression; high numbers of sender cells triggered an immediate onset of SEAP production, whereas decreasing populations of sender cells correlated with a longer time delay until SEAP expression was initiated (Figure 3A, inset ii; Weber et al., 2007a). Such cell-density-dependent induction of transgene expression can be used to design autonomously operating bioprocesses, where expression of product genes is induced automatically once an optimum cell density has been reached. An increasing size of the sender cell population correlated with increasing levels of SEAP production, demonstrating the potential use of this

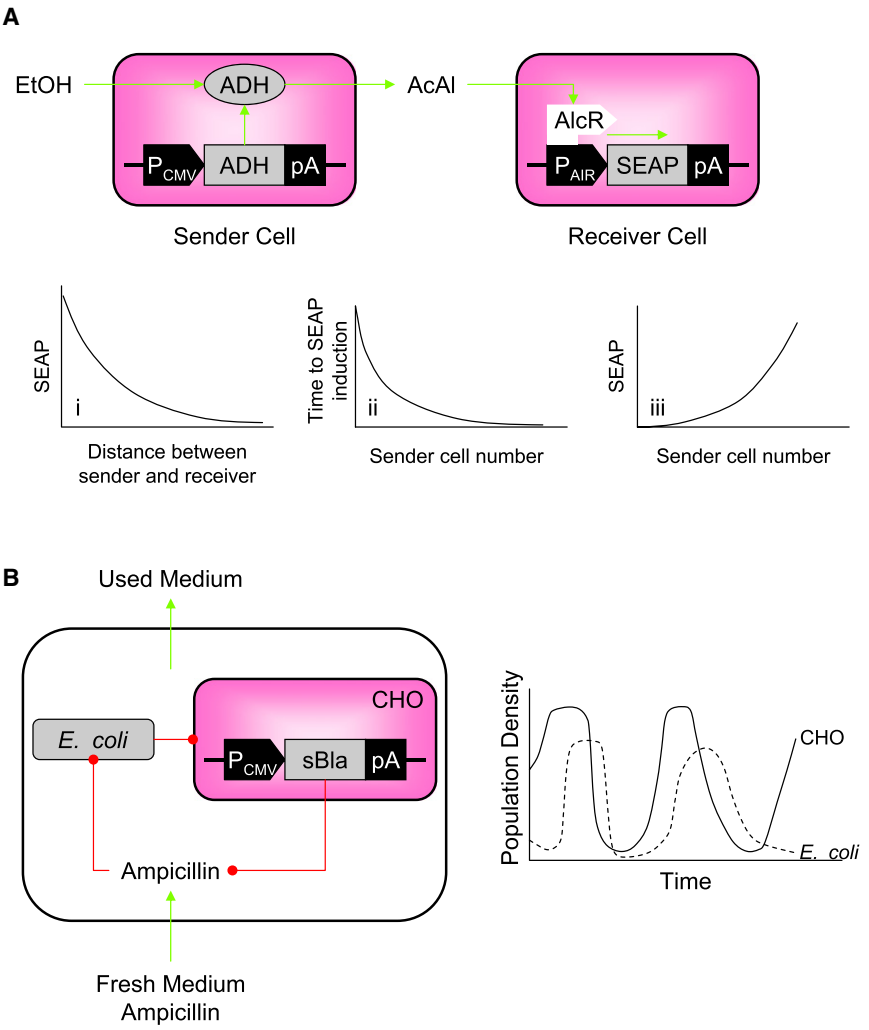

Figure 3. Cell-to-cell communication and synthetic ecosystems

(A) Cell-to-cell communication. The sender cell is genetically engineered to express alcohol dehydrogenase (ADH), resulting in the conversion of ethanol to acetaldehyde, which, with a boiling point of 21°C, diffuses through the gas or liquid phase to the receiver cells. The receiver cell harbors the *Aspergillus nidulans*-derived transcription factor AlcR that activates its cognate promoter P_{AIR} in the presence of acetaldehyde, resulting in the expression of the reporter SEAP. (i) Dependence on distance: SEAP expression decreases with increasing distance between the sender and receiver cultures. (ii) Dependence on time: Early onset of SEAP production in the receiver cells correlates with a high number of sender cells, whereas a lower number of sender cells correlates with a longer delay before to the induction of gene expression. (iii) Dependence on concentration: After a fixed period, the level of SEAP expression reflects the population density of the sender cells. (B) Synthetic predator-prey interactions. Chinese hamster ovary (CHO) cells were genetically engineered to express β-lactamase fused to an IgG-derived secretion signal (sBla). Secreted β-lactamase triggers degradation of ampicillin in the medium, thereby enabling the survival of *E. coli* cells, which grow, consume nutrients, and produce toxic by-products, which in turn reduces the viability of the CHO cells. When the whole system is semi continuously supplied with fresh medium containing ampicillin, the population densities of the parasite (*E. coli*) and the host (CHO) oscillated with shifted phases.

communication system in mammalian cell-based quorum-sensing (biopharmaceutical manufacturing) configurations (Figure 3A, inset iii; Weber et al., 2007a). This acetaldehyde-based communication system was also shown to function in multistep signal processing cascades and was successfully assembled into the first synthetic hormone system in mice in which sender cell inserts were communicating across the living animal to instruct remote receiver cell implants to produce a human glycoprotein in the bloodstream of treated mice (Weber et al., 2007a). More recently, mammalian system was described in which sender cells were engineered to produce nitric oxide as diffusible messenger that triggers *c-fos* promoter and reporter gene expression in engineered receiver cells (Wang et al., 2008). Here, quorum-sensing like behavior was produced by integrating sender module into receiver cells under negative feedback control.

Synthetic Ecosystems

The capacity to connect several cells or organisms with each other by molecular communication mechanisms enables the design of synthetic ecosystems to emulate and analyze the coexistence and interactions of different species (Weber et al., 2007a). For example, a synthetic predator-prey ecosystem with oscillating population dynamics was recently described. It consists of *E. coli* as the predator and of Chinese hamster ovary (CHO-K1) cells, engineered for constitutive production of a secreted β-lactamase, as the prey. Both species were cocultivated in standard culture dish equipped for semicontinuous supply of fresh medium and ampicillin. Upon start of the predator-prey ecosystem, *E. coli* growth is restricted by ampicillin and CHO cells proliferate, thereby secreting β-lactamase, which lowers ampicillin levels. As ampicillin concentrations decrease, *E. coli* recovers and resumes growth, which compromises viability of the mammalian cells due to bacteria-mediated nutrient depletion. Because a declining CHO cell population produces lower amounts of secreted β-lactamase, ampicillin levels continue to increase, which decimates *E. coli* and allows recovery of the mammalian cell population to initiate another cycle of oscillating population dynamics (Figure 3B).

This synthetic prokaryote-eukaryote ecosystem could serve as a model for the design of synthetic host-pathogen ecosystems that characterize disease dynamics with unmatched precision and help devise or refine therapeutic strategies accordingly.

Future Directions of Mammalian Synthetic Biology

Mammalian cell-based synthetic biology is expected to develop into two major directions: (i) the knowledge-producing "basic synthetic biology" enabling fundamental scientific discoveries and providing insight into operation dynamics of complex

natural systems using rationally designed synthetic replicas, and (ii) ''translational synthetic biology,'' which leads from evidence-based ''basic synthetic biology'' to sustainable solutions for pertinent public health problems. Both synthetic biology disciplines will gather tremendous momentum by integrating recent advances in ''enabling technologies'' such as large-scale DNA synthesis (Gibson et al., 2008), (single-cell) analytics (Cordey et al., 2008), nanotechnology (Maerkl and Quake, 2007), and materials science (Lutolf and Hubbell, 2005), as well as ''enabling sciences'' including systems biology and bioinformatics, which quantitatively capture the dynamics of biological phenomena at a systems scale (Aebersold and Mann, 2003).

Future developments in ''basic synthetic biology'' will largely be dependent on standardized orthogonal mammalian cell-compatible biologic devices developed de novo or being adapted for use in mammalian cells following blueprints established in prokaryotes. Such devices might include novel input interfaces triggered by light (Levskaya et al., 2005) or by electricity to enable electrogenetic interfaces and harness advances in electronics for control of living systems. Mammalian versions of complex synthetic gene networks showing oscillating expression dynamics such as described for *E. coli* (Elowitz and Leibler, 2000; Stricker et al., 2008) have just been developed (Tigges et al., 2009). Rewiring of intracellular signaling pathways enabling synthetic connection of any receptor-mediated input with any desired target gene such as recently established in yeast still lacks similar implementation in mammalian cells (Yeh et al., 2007). Also, mammalian homologs of complex two-way communication at multicellular scale such as recently established in *E. coli* by engineering of orthogonal acylhomoserine-lactone-based circuits (Balagadde et al., 2008) could enable multichannel intercell signaling to coordinate cell growth and differentiation of organ-like cellular assemblies or might be used to design prosthetic hormone signaling for future therapies.

Mammalian cell-based ''translational synthetic biology'' is expected to foster advances in drug discovery, gene therapy, tissue engineering, and biopharmaceutical manufacturing. For drug discovery, the synthetic reconstruction of healthy or pathologic signaling cascades in an orthogonal host could be an ideal approach to elucidate signal processing dynamics as exemplified at the reconstruction of the B cell antigen receptor signal transduction pathway in insect cells (Wossning and Reth, 2004). For gene therapy, semisynthetic gene networks are expected to emerge that enable engineered cells to autonomously sense a pathologic state (e.g., abnormal concentrations of glucose) and coordinate a corresponding therapeutic action (e.g., secretion of insulin) in a self-sufficient manner. For tissue engineering, reprogramming of differentiation pathways to coordinate (stem) cell fate in artificial tissues using synthetic signaling and transcription networks could become an essential technology for the treatment of tissue-specific diseases. For biopharmaceutical manufacturing, mammalian-cell-based translational synthetic biology might become fundamental for targeted manipulation of product quality (Tomiya et al., 2003) or expanding protein function by introduction of non-canonical amino acids (Summerer et al., 2006).

Although mammalian cell-based translational synthetic biology is unique in having a great potential to improve human therapies in the not-so-distant future, applications of synthetic biology are already in use today, for example for pest control. The Mediterranean fruit fly (*Ceratitis capitata*) is an insect pest that is inflicting heavy economic damage on agriculture. Fu et al. (2007) designed a synthetic circuit for autoregulated expression of tTA, which was engineered for female-specific splicing and could only accumulate to toxic levels in female flies living in a TET-free environment. Production and release of transgenic male flies will compete with wild-type flies for mating with female flies, thereby infiltrating the fly population with the synthetic circuitry that will rapidly disseminate within the wild-type male population, whereas the female population is constantly decreasing until the fly will be extinct (Fu et al., 2007).

In conclusion, **s**ynthetic biology is rapidly developing from a loose network of scientists with different education and scientific background into an interdisciplinary global community convinced that the engineering of biology—in essence the rational design of biological systems in a systematic manner—might offer solutions to major societal and health-associated challenges of the 21st century.

ACKNOWLEDGMENTS

Synthetic-biology-related work in the laboratory of W.W. and M.F was in part supported by Swiss National Science Foundation and Cistronics Cell Technology, P.O.B. 145, 8093 Zurich, Switzerland.

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