A synthetic time-delay circuit in mammalian cells and mice

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Time-delay circuitries in which a transcription factor processes independent input parameters can modulate NF-κB activation, manage quorum-sensing cross-talk, and control the circadian clock. We have constructed a synthetic mammalian gene network that processes four different input signals to control either immediate or time-delayed transcription of specific target genes. BirA-mediated ligation of biotin to a biotinylation signal-containing VP16 transactivation domain triggers heterodimerization of chimeric VP16 to a streptavidin-linked tetracycline repressor (TetR). At increasing biotin concentrations up to 20 nM, TetR-specific promoters are gradually activated (off to on, input signal 1), are maximally induced at concentrations between 20 nM and 10 μM, and are adjustably shut off at biotin levels exceeding 10 μM (on to off, input signal 2). These specific expression characteristics with a discrete biotin concentration window emulate a biotin-triggered bandpass filter. Removal of biotin from the culture environment (input signal 3) results in time-delayed transgene expression until the intracellular biotinylated VP16 pool is degraded. Because the TetR component of the chimeric transactivator retains its tetracycline responsiveness, addition of this antibiotic (input signal 4) overrides biotin control and immediately shuts off target gene expression. Biotin-responsive immediate, bandpass filter, and time-delay transcription characteristics were predicted by a computational model and have been validated in standard cultivation settings or biopharmaceutical manufacturing scenarios using transgenic CHO-K1 cell derivatives and have been confirmed in mice. Synthetic gene circuitries provide insight into structure–function correlations of native signaling networks and foster advances in gene therapy and biopharmaceutical manufacturing.

Results

Information processing in the synthetic mammalian time-delay circuit consists of (i) administration of the bioavailable and nontoxic input molecule biotin (vitamin H) (switch; Fig. 1A), (ii) irreversible ligation of biotin to the synthetic AVITAG peptide (30) fused to the Herpes simplex-derived VP16 transactivation domain (31) by ectopic expression of heterologous E. coli BirA biotin ligase (32) (diode; Fig. 1A), (iii) an AVITAG-VP16-based buffer, which retains biotin and sustains signal processing even after biotin withdrawal from the culture medium (capacitor; Fig. 1A), (iv) proteasome-dependent degradation of biotin-AVITAG-VP16 domains, which controls capacitor discharge and delays signal referral (resistor; Fig. 1A), and (v) binding of biotin-AVITAG-VP16 to streptavidin fused to the E. coli Tn10-derived tetracycline repressor (TetR) (33), resulting in a chimeric transactivator (TetR-streptavidin-biotin-AVITAG-VP16), which binds and activates TetR-specific target promoters (PcCMV*−1) that contain heptameric operator sites (tetO7; PcCMV*−1, tetO7-PcCMVmin) (transistor; Fig. 1A). This promoter drives transcription of the human glycoprotein SEAP (human placental secreted alkaline phosphatase) in a tetracycline-adjustable manner until the biotin-AVITAG-VP16 buffer is degraded (output; Fig. 1A). Functionality of individual circuitry components was validated by cotransfection of Chinese hamster ovary (CHO-K1) cells with expression vectors encoding

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ynthetic biologists have begun designing artificial gene networks from modular well characterized and compatible genetic components (1–5). Such networks have not only enabled a better understanding of cellular signal processing circuits (6–12), but have provided solutions for economic biopharmaceutical manufacturing of protein therapeutics and small-molecule drugs (13, 14), precise and timely molecular interventions in prototype gene therapy scenarios (15), and rational reprogramming of therapeutic cell phenotypes in tissue engineering (16). Pioneering synthetic networks using repressors to create transcription feedback loops in Escherichia coli have provided insight into noise suppression (7), bistability (10, 17), and oscillations (8). More recently, newly developed transcription regulators (18, 19), interoperating as genetic transistor replicas with adjustable signal processing, have been assembled to create semisynthetic transcription cascades, which interface with host regulatory networks (20), epigenetic expression imprinting (21), and hysteretic expression memories (22) in mammalian cells and mice. However, compared with electronic circuitries that combine diodes, capacitors, resistors, and transistors to achieve complex in silico information processing, the assembly of synthetic mammalian gene networks remains limited to the functional interconnection of transistor-like switches emulated by trigger-inducible promoters (23, 24). By rational assembly of protein modification and transcription-control components we designed a synthetic mammalian time-delay circuit that emulates an electronic switchboard with sequential wiring of diodes, capacitors, resistors, and transistors. Time-delay circuitries are a recurrent control motif in nature, which evolved, for example, to (i) create stationary patterns from a diffusive gradient that grows with time during interpopulation quorum-sensing cross-talk (25, 26), (ii) modulate parallel Toll-like receptor 4 signaling pathways activating NF-κB from isolated damped oscillatory to stable behavior (27), and (iii) adjust the circadian clock by delayed phosphorylation-dependent degradation of FRO (28) or differential ubiquitinylation of CRY2 and CRY1 (29).

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AVITAG-VP16 (pWW800), TetR-streptavidin (pWW801), BirA (pWW804), and a TetR-responsive SEAP expression vector (pMF111) (Fig. 1B). This vector set enabled biotin-triggered SEAP production (heterodimerization and P_cMV−1 binding of TetR-streptavidin-biotin-AVITAG-VP16), which could be repressed by vitamin H deficiency (no heterodimerization) or addition of tetracycline (heterodimerization, no P_cMV−1 binding) (Fig. 1C).

Because biotin is covalently linked to AVITAG-VP16 and thus remains sequestered in the cell, it continues to promote heterodimerization of the chimeric transactivator even after biotin withdrawal from the culture medium. SEAP production continues in a time-delayed manner until it plateaus as the biotin-AVITAG (Fig. 1D). Biotin, circles), biotin-containing (pWW801, pWW804, and pMF111 (Fig. 1B) and cultivated in the presence of 20 nM biotin for 9 h before switching to biotin-free, biotin-containing, or tetracycline-supplemented medium and subsequent SEAP profiling (Fig. 1D). Whereas biotin-supplemented populations exhibited steady increases in SEAP production, the SEAP levels of biotin-deprived cultures reached 50 milliunits/liter during the first 50 h (time delay) and stagnated thereafter (Fig. 1D). Addition of tetracycline overrode biotin-triggered and time-delayed SEAP production (Fig. 1D). The corresponding time delay became obvious when we calculated the specific SEAP production rates: the delay of SEAP production rates in tetracycline-containing medium, which represents shut-off of a simple transcription-control system, occurred ~30 h before a corresponding decrease in the time-delayed circuit (Fig. 1E). Time-delayed SEAP production was also observed in the serum of biotin-deficient mice implanted with CHO111, whereas control animal populations, kept on a normal diet, showed steady increases in their SEAP serum levels (Fig. 1F). In both situations, tetracycline administration repressed transgenic SEAP production (Fig. 1F). This successful in vivo validation...
suggests that the biotin-triggered, time-delay circuitry may provide a temporally defined epigenetic expression window, independent of inducer fluctuations, and so enable stable therapeutic interventions in future gene therapy scenarios.

To characterize the signal processing capacity of the time-delay circuitry in greater detail, we used a differential algebraic equation model, coordinated to molecular and biochemical parameters, to simulate SEAP production profiles (Eqs. 1–6):

\[
\frac{d[V]}{dt} = v_1 \left( \frac{[B]}{K_{SB} + [B]} \right)^2 \left( \frac{[V]}{K_{SV} + [V]} \right) - (d_1 + \mu)[V]
\]

\[
\frac{d[M]}{dt} = v_2 \frac{\varphi_3}{K_{SE} + \varphi_3} - (d_2 + \mu)[M]
\]

\[
\frac{d[S]}{dt} = v_3[M][X] - d_3[S]
\]

\[
\frac{d[X]}{dt} = \mu[X]
\]

\[
\frac{d[G]}{dt} = -d_4[G]
\]

with

\[
[VBT] = \left( \frac{[VB]T_0^6[G]}{[VB] + \varphi_4/k_{BS}}, \frac{\varphi_4}{k_{BS}} \right),
\]

\[
\varphi_4 = 1 + f_wk_{BS}[B] + k_{TT}[TC],
\]

\[
[V] = \frac{v_4(G) - [VB] - [VBT]}{[VB]T_0^6[TG] - \varphi_3(f_w[G], [G]T_0, [TC], k_{TO}, k_{TT})}.
\]

Changes in the concentration of the biotinylated AVITAG-VP16 [VB] depend on the extracellular biotin concentration [B] and the concentration of free AVITAG-VP16 [V]. Transport and conversion by BirA are represented by the first term of Eq. 1 (SI Text for all details of model derivation), while linear degradation (parameter \(d_2\)) and dilution caused by cell growth (growth rate \(\mu\)) diminish the pool of biotinylated AVITAG-VP16. Similarly, the dynamics of SEAP mRNA [M] is described by a Michaelis-Menten-like term for transcription-competent complexes with biotin-AVITAG-VP16. The impact of the buffer size on time-delay adaptation only of experiment-specific parameters such as the specific growth rate. The impact of the buffer size on time-delay dynamics was analyzed by simulating transfection of CHO-K1 cells with expression vectors encoding BirA (pWW804), TetR-streptavidin (pWW801), a TetR-responsive SEAP expression vector (pMF111), and AVITAG-VP16 variant (pWW800, pWW830 or pWW840) in biotin-free medium after 72 h of profiling SEAP production. Maximum SEAP production showed a monotonic relation between the half-life of the VP16 fusion protein and the maximum SEAP production levels, as predicted by theoretical considerations (Fig. 2E).

Furthermore, dose–response profiling of biotin-controlled SEAP expression revealed three independent biotin-response characteristics. Note that these quantitatively accurate theoretical predictions were truly independent (data not used for parameter adjustment). Apart from experiments-specific parameters such as the specific growth rate. The impact of the buffer size on time-delay dynamics was analyzed by simulating transfection of CHO-K1 cells with expression vectors encoding BirA (pWW804), TetR-streptavidin (pWW801), a TetR-responsive SEAP expression vector (pMF111), and AVITAG-VP16 variant (pWW800, pWW830 or pWW840) and subsequent cultivation for 2 h in the presence of biotin to replenish the inducer buffer and then for 72 h in biotin-free medium before profiling SEAP production. Maximum SEAP production showed a monotonic relation between the half-life of the VP16 fusion protein and the maximum SEAP production levels, as predicted by theoretical considerations (Fig. 2E).

Besides providing insight into natural time-delay signal processing (27, 28), biotin-triggered transcription control could impact biopharmaceutical manufacturing and gene therapy applications because of the bioavailability of the nontoxic physiologic inducer biotin (with no known side effects), its excellent regulation performance, and its inducible dimerization-based control characteristics. Furthermore, dose–response profiling of biotin-controlled SEAP expression revealed three independent biotin-response characteristics: (i) adjustable dose-dependent induction of SEAP expression up to 10 nM biotin, (ii) maximum SEAP production plateau within a specific biotin concentration window (20 nM to 10 pM), and (iii) adaptable transgene repression beyond 100 pM (Fig. 3A).

As indicated by the mathematical model, transcription shut-off at high biotin concentrations is probably caused by free biotin-based saturation of the TetR-streptavidin and a corresponding reduction of transcription-competent complexes with biotin-AVITAG-VP16.
to-produce (e.g., cytotoxic) protein therapeutics. In this system, the modalities are particularly valuable for the manufacture of difficult-classical transistor-type control modalities (35). Transgene control molecule for reversal of the transgene expression status when using organisms, which depend on the pharmacokinetics of the inducer be eliminated by cost-intensive medium exchange or filtration, and systems including bioreactors, from which inducer molecules must be attractive for precise transgene expression control in complex tion of a single biocompatible signal molecule, are particularly capacities into a single synthetic transcription factor.

tetracycline (Fig. 1)

Adjustable induction and repression signaling by addition of a single inducer molecule is unique and exhibits band-pass filter-like characteristics, which have thus far been achieved only by combining different transistor-type transcription control modalities (39) or by assembly of complex networks mimicking developmental pattern formation (25). With its off-to-on and on-to-off responsiveness to different biotin concentrations (Fig. 3), the time-delayed expression characteristics after biotin removal, and the sensitivity to tetracycline (Fig. 1C), this system combines four signal processing capacities into a single synthetic transcription factor.

Reversible off-to-on and on-to-off switches, through administration of a single biocompatible signal molecule, are particularly attractive for precise transgene expression control in complex systems including bioreactors, from which inducer molecules must be eliminated by cost-intensive medium exchange or filtration, and organisms, which depend on the pharmacokinetics of the inducer molecule for reversal of the transgene expression status when using classical transistor-type control modalities (35). Transgene control modalities are particularly valuable for the manufacture of difficult-to-produce (e.g., cytotoxic) protein therapeutics. In this system, the administration of biotin, to induce the desired levels of protein production, will result in an identical media composition to that currently found in standard licensed processes, thereby alleviating the need for expensive downstream processing procedures to remove the inducer molecule. To validate the potential of biotin-triggered transgene control for biopharmaceutical manufacturing, we profiled heterologous protein production of CHOTIME, transgenic for biotin-inducible SEAP expression in 2-liter bioreactors.

After a 48-h cultivation period in biotin-free media, which repressed SEAP to the detection limit, the production culture was exposed to 100 nM biotin, which triggered sustained SEAP production until reaching 50% SEAP production rate after transcriptional shut-off caused by switching to tetracycline-containing and biotin-free medium, respectively. (C) Corresponding in silico-determined switching characteristics of the biologic time-delay circuit as a function of the buffer size of total VP16 molecules and the buffer fill time (time of cultivation in 20 nM biotin medium).

(D) Quantification of half-lives of different chimeric transactivators. CHO-K1 were transfected with plasmids pWW830 (P_{PSV40}-AVITAG-EosFP-VP16-pA) or pWW840 (P_{PSV40}-AVITAG-EosFP-VP16-PEST-pA) and cultivated for 48 h before switching the EosFP-fusion proteins from green to red by a 20-s UV (390 nm) light pulse. FACs analysis was used at the indicated time points to score the decrease in red fluorescence. (E) In vitro investigation of the impact of VP16 degradation kinetics on switching characteristics as represented by maximum SEAP production. CHO-K1 was transfected with plasmids pWW801, pWW804, pMF111, and either pWW880, pWW830, or pWW840 and cultivated for 2 h in the presence of biotin before switching to biotin-free conditions for 72 h and profiling of maximum SEAP production. Maximum SEAP production was correlated with the half-lives of AVITAG-VP16 variants (pWW800, 3.5 h; pWW840, 12.4 h; pWW830, 37.3 h) as predicted by the mathematical model (line). (F) In vitro investigation of the impact of the buffer size on switching characteristics represented by maximum SEAP production. CHO-K1 were transfected with plasmids pWW801, pWW804, pWW830, and pMF111 and cultivated in biotin-containing (20 nM) medium for the indicated periods before switching to biotin free conditions for another 48 h. Maximum SEAP production was determined and correlated with the time required to replenish the biotinylated VP16 buffer (line indicates model predictions).
we expressed the apoptosis-inducing death domain of the human receptor-interacting protein (RipDD) (40–42). Because the buffer size defined the maximum RipDD production it could be used to program the optimal balance of RipDD expression and apoptosis to maximize product yield (Fig. 3D). In contrast, constitutive expression resulted in decreased RipDD titer (90.3 ± 0.8 integrated optical density) caused by extensive cell death (68% viability). To validate biotin-induced transgene expression in a prototype gene therapy scenario including a standard biotin-containing diet we implanted microencapsulated CHOsxME i.p. into mice sustained on a standard biotin-containing diet. Administration of increasing vitamin H doses triggered decreasing serum SEAP levels, which we expressed the apoptosis-inducing death domain of the human receptor-interacting protein (RipDD) (40–42). 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(5’-gatgatcgagacctagagccctGCTGTAACGGCCTGACGCGC-3’), annealing sequence upercasse, BamHI site underlined) and ligated (BssHII/BamHI) into pSAM200. The EcoI·boli proteinase K digest (32) was excised (EcoRI/SpeI) from pGEM-SD2 (J. Trono, personal communication) and ligated (EcoRI/XbaI) into pMF150, which resulted in pWW804 (P_CMV•-1-BirA-pA). pWW830 (PCAF•1AVITAG-eosFP-VP16-pA) was constructed by inserting the BssHII-generated eosFP (36) encoding fragment of pd2EosFP (Genscript, Piscataway, NJ) in frame and sense orientation into the BssHII site of pWW800. The PEST domain was PCR-amplified from pd2EFP (Genscript, Piscataway, NJ) and fused in-frame 3’ to the VP16 domain of pWW830 in a multistep cloning procedure (available on request), resulting in pWW840 (P_CMV•-1-SEAP-pA; P_CMV•-1, tetO2-pCMVmini) has been described (18). For construction of pWW811 (P_CMV•-1-RipDD-HIS6-pA) pCMV•-1 was excised from pCF201 (unpublished work) by SspI/AscI and cloned into the SspI/BssHII sites of pWW326 (41).

Cell Culture and Cell Line Design. Chinese hamster ovary cells (CHO-K1, CCL-61; ATCC, Manassas, VA), and the CHO-K1-derived transgenic cell line CHO_TMEM were cultivated in biotin-free DMEM (Invitrogen, Carlsbad, CA) or biotin-free ChoMaster HTS medium (Cell Culture Technology, Gravenzano, Switzerland) supplemented with either 10% FCS (Pan Biotech, Aidenbach, Germany) or 10% biotin-free knockout serum replacement (KOSR; Invitrogen) and selective antibiotics G418 (405 μg/ml) and puromycin (1 μg/ml) (CHO_TMEM only). Transfections were performed according to an optimized calcium phosphate protocol (19). CHO_TMEM was constructed by sequential cotransfection and single-cell cloning of CHO-K1 with (i) pWW800, pWW801, and pSV2neo (Clontech) (selection in G418-containing medium) and (ii) pWW804, pMF111, and pPUR (Clontech) (selection in puromycin-containing medium).

Bioreactor Operation. Cells were cultivated in a Biowave 20SPS-F bioreactor (Wave Biotech, Tagelswangen, Switzerland) equipped with 2-liter Wave Bags and inlet gas humidification (Humicare 200; Gruender Medical, Freedomstadt, Germany) and the following parameter setting: temperature, 37°C; aeration, 100 ml/min humidified air (>95% relative humidity) containing 5% CO2; rocking rate, 16 min−1; rocking angle, 5°.

Analytics. SEAP production was quantified as detailed by Schlatter et al. (34). EosFP-based red fluorescence dynamics was analyzed by using either a DM RB fluorescence microscope (Leica, Vienna) equipped with 520-nm (excitation) and 570-nm (emission) filters or a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA) with CFP analysis software (Beckman Coulter) set for 488-nm excitation and recording at 620 nm. Cell viability and cell death was assessed by FACS using an annexin V/propidium iodide-based apoptosis assay (Invitrogen). Affinity-purified RipDD (Qiagen, Valencia, CA) was quantified by Western blot using anti-heatshock antibodies (Novagen, San Diego, CA) and ECL-Plus detection reagents (Amersham, Piscataway, NJ) and Chemilux hardware (Intas, Göttingen, Germany).

Inductor Molecules. Biotin (Acros Organics, Geel, Belgium) was dissolved in water and used at a final concentration of 20 nM unless stated otherwise. Tetracycline (Sigma, St. Louis, MO) was prepared as a stock solution of 1 mg/ml in water and used at a final concentration of 2 μg/ml.

Animal Studies. CHO_TMEM was encapsulated in 400 μm alginate-PDLL-alginate beads by using the Encapsulator Research IE-50R Encapsulator (Biotronics, Basel, Switzerland) according to the manufacturer’s protocol and the following specific settings: 0.2-mm nozzle, 20-ml syringe at a flow rate of 325 units, nozzle vibration frequency 1,088 s−1, voltage for bead dispersion 1,300 V. Female OF1 mice were obtained from Ifa-Credo (Les Oncins, France) and kept on a biotin-free diet (Societé SAFE, Augy, France) for 2 weeks, whereas the control mice were fed the same diet supplemented with 4 mg/kg biotin. Biotin deficiency was monitored by using the forced swim test (44), resulting in swim times of 58 ± 28 and 132 ± 30 s for biotin-deficient and control mice, respectively. Seven hundred microliters of F8X medium containing 50% capsules (2 × 105 cells, 200 cells per capsule) was injected i.p. into the mice. When appropriate, additional biotin was dissolved in 0.9% (wt/vol) NaCl and administered by i.p. injection 1 h after capsule implantation. Blood samples were collected retroorbitally, and serum was produced by using microtainer SST tubes (Becton Dickinson, Franklin Lakes, NJ). All experiments involving animals were approved by the French Ministry of Agriculture and Fishery and performed by M.D.-EB.

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A + P $\xrightarrow{k_A} AP$

A + I $\xrightarrow{k_I} AI$