A synthetic low-frequency mammalian oscillator

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ABSTRACT

Circadian clocks have long been known to be essential for the maintenance of physiological and behavioral processes in a variety of organisms ranging from plants to humans. Dysfunctions that subvert gene expression of oscillatory circadian-clock components may result in severe pathologies, including tumors and metabolic disorders. While the underlying molecular mechanisms and dynamics of complex gene behavior are not fully understood, synthetic approaches have provided substantial insight into the operation of complex control circuits, including that of oscillatory networks. Using iterative cycles of mathematical model-guided design and experimental analyses, we have developed a novel low-frequency mammalian oscillator. It incorporates intronically encoded siRNA-based silencing of the tetracycline-dependent transactivator to enable the autonomous and robust expression of a fluorescent transgene with periods of 26 h, a circadian clock-like oscillatory behavior. Using fluorescence-based time-lapse microscopy of engineered CHO-K1 cells, we profiled expression dynamics of a destabilized yellow fluorescent protein variant in single cells and real time. The novel oscillator design may enable further insights into the system dynamics of natural periodic processes as well as into siRNA-mediated transcription silencing. It may foster advances in design, analysis and application of complex synthetic systems in future gene therapy initiatives.

INTRODUCTION

The rapidly developing field of synthetic biology has emerged as a discipline capable of linking theoretical model predictions to experimental implementations consisting of minimal modular building blocks arranged to form synthetic networks (1) with different levels of complexity (2–9). Substantial information on such minimal units and circuitry originated from prokaryotes (10–18) and the relatively recent development of heterologous mammalian gene regulation technologies (19–21) enabled subsequent applications in complex mammalian systems (2,3,22,23).

In synthetic biology, oscillatory behavior has gained substantial attention due to its impact on repair (24), metabolic (25) and signaling pathways (26–27). Such pathways, like the p53-mdm2-triggered apoptosis cascade (28), NF-κB-regulated immune responses (29) or Hes1-mediated developmental control (30), are based on periodic induction of negative feedback loops controlling expression of key pathway factors (31,32).

Utilizing antisense-mediated silencing of a transactivator, we have recently created a synthetic gene network capable of oscillatory expression of the green fluorescent protein (22). Future applications, however, are expected to require fine-tuning of oscillatory properties, such as frequency and amplitude. This could be achieved by alternative selection and reconfiguration of the underlying modular building blocks. Here, we present an alternative oscillatory network that displays robust low-frequency fluctuations in target gene expression. Capitalizing on recent progress showing that RNA interference may be a powerful component of synthetic gene networks (33,34), we have designed a synthetic mammalian clock that combines siRNA-mediated, time-delayed negative feedback loop with autoregulated expression of the tetracycline-dependent transactivator (tTA). The experimental data thus obtained were in good agreement with a mathematical model based on ordinary differential equations. Information gained from such combined dynamic studies should facilitate advances in our understanding of natural circadian clocks and rhythms (35).

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MATERIALS AND METHODS

Expression vector design

pBP283 (P_{ETR3}\rightarrow d2EYFP), harboring a destabilized enhanced yellow fluorescent protein (d2EYFP) under control of the erythromycin-responsive promoter (P_{ETR3}), was constructed by excising d2EYFP from pd2EYFP (Clontech) using EcoRI/NotI and cloning it into the corresponding sites (EcoRI/NotI) of pBP90 (36). pDG156 (P_{hCMV\ast}\rightarrow ET1), containing the erythromycin-dependent transactivator [ET1, E-VP16; (37)] driven by the tetracycline-responsive promoter (P_{hCMV\ast}) was designed by excising P_{hCMV\ast}\rightarrow tTA from pMF111 (38) by SspI/EcoRI and cloning it into the corresponding sites (SspI/EcoRI) of pWW35 (19). pDG157 (P_{hCMV\ast}\rightarrow E-siRNA_{LUC}\cdot VP16) expresses the ET1, E-VP16; (37) containing an intronically encoded siRNA specific for firefly luciferase mRNAs (siRNA_{LUC}; nt 62–80). pDG157 was obtained by replacing P_{SV40} of pDG17 (39) with P_{hCMV}\ast (40) via SspI/EcoRI. pDG178 (P_{hCMV\ast}\rightarrow tTA) harboring an autoregulated expression unit for tetracycline-responsive control of the tTA was obtained by excising tTA from pSAM200 (38) by using EcoRI/BssHII into pDG156, thereby replacing ET1. pND10 (P_{hCMV\ast}\rightarrow TAG_LUC-tTA) contains an expression unit for P_{hCMV\ast}\rightarrow driven expression of the tTA preceded by a noncoding sequence tag derived from the firefly luciferase coding sequence (nt 62–80). pND10 was constructed in two steps, first by replacing P_{hEF1}\ast of pDG54 via SspI/EcoRI and then by replacing P_{hEF1}\ast with P_{hCMV\ast}\rightarrow of pDG178 via SspI/KpnI.

Cell culture, transfection and gene regulation

Chinese hamster ovary cells (CHO-K1, ATCC CCL61) were cultivated in ChoMaster® HTS (Cell Culture Technologies, Graveseano, Switzerland) supplemented with 5% fetal calf serum (Pan Biotech GmbH, Aidenbach, Germany; cat. no. 3302-P231902, lot no. P231902). CHO-K1 were cultivated at 37°C in a humidified atmosphere containing 5% CO₂ and 35 000 cells were transfected using the FuGENE®6 transfection reagent (Roche Molecular Biochemicals, lot. no. 93535720) and a network-encoding plasmid mixture calibrated to a total of 1.2 μg DNA using the extender vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Transfection experiments were standardized to provide efficiencies of up to 35 ± 5%. The number of oscillating cells was indicated as percent relative to the total number of transfected cells (100%). Transfected cells were arrested in the G1 phase by a down-shift of the cultivation temperature to 32°C (41), and monitoring of the fluorescence dynamics by time-lapse microscopy was started 18 h posttransfection (t = 0).

Fluorescence imaging

Time-lapse fluorescence microscopy was performed using an inverted Leica fluorescence microscope (Leica Microsystems DMI 6000B, 11888107) equipped with an incubation chamber, a Leica DFC350FX R2 digital camera (Leica, cat. no: 112730043), an 10× objective (Leica, Obj. HC PL FL 10×/0.30 PH1 /D 11.0, cat. no. 11506507) and the following excitation/emission filter set: d2EYFP (513/527 nm; C/Y/R, cat. no. 11513897). Time-lapse movies were produced with the LAS AF imaging software (Leica FW4000-TZ, cat. no. 12723979) to set exposure times of 610 ms every 20 min. Fluorescence was quantified for single cells using a custom-designed software implemented in MATLAB® (Mathworks, Nantucket/MA, USA) All details on software development are available as Supplementary Data.

Computational modeling

All details on mathematical models and computational methods are provided as Supplementary Data. Simulations were performed with MATLAB®, Version R2007b.

RESULTS

Design of the low-frequency mammalian oscillator

The low-frequency mammalian oscillator (Figure 1a) consists of: (i) an autoregulated expression unit harboring the tTA and an untranslated Photinus pyralis firefly luciferase-derived sequence tag (TAG_{LUC}; 42, 43) targeted by a luciferase-specific siRNA (siRNA_{LUC}; 42) under control of the tTA-specific tetracycline-responsive promoter (P_{hCMV\ast}) driving an autoregulated positive feedback loop sustaining oscillatory behavior, we developed a mathematical model to initiate an autoregulated positive feedback loop sustaining high-level expression of tTA and consequently of P_{hCMV\ast}\rightarrow E-siRNA_{LUC}\cdot VP16 expression. Constitutive splicing of E-siRNA_{LUC}\cdot VP16 produces equimolar amounts of ET1 (E-VP16) and siRNA_{LUC}, which results in concomitantly increasing ET1-triggered expression and siRNA_{LUC}-mediated breakdown of LUC-tagged tTA transcripts. siRNA_{LUC}-based depletion of TAG_{LUC}\cdot tTA transcripts will reduce tTA levels and temporarily shut down the network. This results in low d2EYFP expression and low siRNA_{LUC} production, which eventually allows the circuit to start over again. The gene network can be tuned by the addition of tetracycline and erythromycin to regulate p_{hCMV}\ast and P_{ETR3} promoter activity, respectively.

Model-based analysis of circuit behavior

To evaluate the general capability of the network to generate oscillatory behavior, we developed a mathematical...
model based on ordinary differential equations (see Figure 1b and Supplementary Data). This deterministic model describes the circuitry behavior in a mechanistic fashion. A first model prediction based on experimentally determined, as well as literature-based, parameter sets (see Supplementary Data) demonstrated the general capacity of the circuit to display oscillatory gene expression with periods similar to those observed for natural circadian clocks (Figure 1c and d).

Evaluation of circuit behavior in CHO-K1 cells by monitoring d2EYFP expression

According to model simulations, any inactivation of transactivators tTA and/or ET1 by the addition of antibiotics would disrupt the rhythmic expression pattern (Supplementary Figure 1). The impact of regulating antibiotics on the system was therefore first analyzed to validate the functionality of the single components. For this purpose, pND10 (P_hCMV*-1→TAGLUC-tTA), pDG157 (P_hCMV*-1→E-siRNA_LUC-VP16) and pBP283 (P_ETR3→d2EYFP) were co-transfected at equimolar ratios (1:1:1), followed by subsequent addition of (i) 200 ng/500 μl tetracycline (Figure 2a) or (ii) 200 ng/500 μl erythromycin (Figure 2b). As expected, addition of either antibiotic disrupted the oscillatory behavior, leading to decreasing levels of d2EYFP fluorescence overtime.

In contrast, model predictions suggested that the system would be insensitive to variations in the amount of transfected single components (Figure 3 and Supplementary Data). Therefore, we next evaluated the effect of plasmid dosage variations by co-transfecting pND10 (P_hCMV*-1→TAGLUC-tTA), pDG157 (P_ETR3→E-siRNA_LUC-VP16) and pBP283 (P_ETR3→d2EYFP) at differing amounts, namely 1:1:1 (100 ng each, Figure 4a), 0.5:0.5:1 (Figure 4b), 2:2:1 (Figure 4c), and varying ratios 2:1:1 (Figure 5a) and 1:2:1 (Figure 5b). All variations displayed oscillatory behavior with similar periods of ~26 h and with substantial amplitudes. Hence, the experimental data confirmed the theoretically predicted robustness of
the network to moderate changes in the amounts of single components. This contrasts with the observed and predicted sensitivity of the oscillatory behavior to plasmid dosage in our earlier synthetic oscillator based on antisense-mediated silencing of the tTA (22). There, changes in ratio and/or amount of system components influence the period and amplitude of oscillatory gene expression significantly.

When expressing excessive amounts of either pND10 (PhCMV*1→TAGLUC-tTA) or pDG157 (Petr3→E-siRNALUC-VP16) using ratios of 10:1:1 (Figure 5c) and 1:10:1 (Figure 5d), the oscillatory circuit was substantially repressed. Interestingly, even with a 10-fold excess in tTA gene dosage, silencing induced visible fluctuations in reporter gene expression (Figure 5c). In contrast, a 10-fold excess of siRNA_LUC, which removes TAGLUC-tTA transcripts from the system, completely eliminated such behavior (Figure 5d). These results point to a major role of the amplifying and long-lasting siRNA-mediated silencing mechanism in generating the observed network behavior.

**Figure 2.** Impact of regulating antibiotics on the mammalian oscillator components and systems behavior using single cell time-lapse fluorescence microscopy of transfected CHO-K1. (a) CHO-K1 co-transfected with pND10 (PhCMV*1→TAGLUC-tTA), pDG157 (PhCMV*1→E-siRNALUC-VP16) and pBP283 (Petr3→d2EYFP); 200 ng tetracycline was added to the culture, (b) CHO-K1 transfected with pND10, pDG157 and pBP283; 200 ng erythromycin was added to the culture. Left panels show fluorescence dynamics and micrographs of a representative individual cell, and right panels show the fluorescence profiles of several cells with t = 0 corresponding to the onset of fluorescence.

**Figure 3.** Impact of plasmid dosage on oscillation frequency. Alignment of mathematically simulated and predicted period (gray disc) with the experimental data (black diamonds with error bars) for the conditions (i) pND10 (PhCMV*1→TAGLUC-tTA):pDG157 (PhCMV*1→E-siRNALUC-VP16):pBP283 (Petr3→d2EYFP); 50 ng: 50 ng:100 ng, (ii) 100 ng:100 ng:100 ng, (iii) 200 ng:200 ng:100 ng.
To further characterize the impact of siRNA LUC activity and specificity, we co-transfected CHO-K1 cells with (i) pDG178 (P \text{hCMV}^*\text{C0}_{1!}\text{tTA}) lacking the 5'-TAGLUC, together with pDG157 (P \text{hCMV}^*_{1!}\text{E-siRNA}_{\text{LUC}}\text{-VP16}) and pBP283 (P \text{ETR3}\text{d2EYFP}) and (ii) pND10 (P \text{hCMV}^*_{1!}\text{TAGLUC-tTA}), pDG156 (P \text{ETR3}\text{E-VP16}) lacking the intronically encoded siRNA LUC and pBP283 (P \text{ETR3}\text{d2EYFP}) (Figure 6a and b). For both conditions, we detected no oscillations but a constitutive increase in fluorescent intensity.
Figure 5. Characterization of oscillator dynamics using single-cell time-lapse fluorescence microscopy of CHO-K1 transfected with different ratios of oscillator components. (a–d) Single-cell time-lapse fluorescence microscopy of CHO-K1 co-transfected with pND10 (P_{CMV-1}→TAGLUC-tTA), pDG157 (P_{CMV-1}→E-siRNA_{LUC-VP16}) and pBP283 (P_{ETR3}→d2EYFP): (a) plasmid ratio of 2:1:1, no antibiotics; (b) 1:2:1, no antibiotics; (c) 10:1:1, no antibiotics; and (d) 1:10:1, no antibiotics. Left panels show fluorescence dynamics and micrographs of a representative individual cell, and right panels show the fluorescence profiles of several cells with t = 0 corresponding to the onset of fluorescence.
over time. This was comparable with conditions in which only pDG157 (P_{hCMV*}→E-siRNA_{LUC}-VP16) and pBP283 (P_{ETR3}→d2EYFP) were co-transfected (Figure 6c). The observed oscillatory behavior is therefore specific and directly linked to the siRNA_{LUC}-mediated elimination of TAG_{LUC}-tTA transcripts that leads to silencing of tTA expression. These observations confirm functional siRNA-mediated breakdown of target mRNAs and corroborate modeling results based on a specific set of known RNA-interference parameters (see Supplementary
### Table 1. Overview on oscillation characteristics resulting from different conditions

| Transfected plasmid | Amount (ng) | Antibiotic          | No. of cells analyzed | Oscillating cells (%) | Period (h)       | Relative amplitude (%) |
|---------------------|-------------|---------------------|-----------------------|------------------------|-------------------|------------------------|
| pND10:pDG157:pBP283 | 100:100:100 | Erythromycin        | 109                   | 0 (0.0)                | -                | -                      |
| pND10:pDG157:pBP283 | 100:100:100 | Tetracycline        | 23                    | 0 (0.0)                | -                | -                      |
| pND10:pDG157:pBP283 | 100:100:100 | None                | 336                   | 39 (11.6)              | 25.8 ± 7.6       | 34.8 ± 22.5             |
| pND10:pDG157:pBP283 | 50:50:100   | None                | 34                    | 6 (17.6)               | 23.1 ± 11.5      | 28.3 ± 17.5             |
| pND10:pDG157:pBP283 | 200:200:100 | None                | 588                   | 42 (7.1)               | 26.7 ± 7.0       | 41.7 ± 21.4             |
| pND10:pDG157:pBP283 | 200:100:100 | None                | 318                   | 31 (9.7)               | 26.0 ± 8.5       | 29.7 ± 16.6             |
| pND10:pDG157:pBP283 | 100:200:100 | None                | 425                   | 35 (8.2)               | 25.3 ± 7.5       | 33.1 ± 19.1             |
| pND10:pDG157:pBP283 | 1000:100:100| None                | 192                   | 9 (4.7)                | 26.7 ± 10.5      | 33.3 ± 19.7             |
| pND10:pDG157:pBP283 | 100:100:100 | None                | 282                   | 0 (0.0)                | -                | -                      |
| pND10:pDG157:pBP283 | 200:100:100 | None                | 93                    | 0 (0.0)                | -                | 6a                      |
| pND10:pDG157:pBP283 | 100:100:100 | None                | 50                    | 0 (0.0)                | -                | 6b                      |
| pDG178:pDG157:pBP283 | 100:100:100| None                | 152                   | 0 (0.0)                | -                | 6c                      |
| pDG178:pDG157:pBP283 | (no siRNA<sub>LUC</sub>)| Tetracycline | 125                   | 0 (0.0)                | -                | 6d                      |
| pDG157:pBP283 (no vTA) | 100:100:100| None                | 125                   | 0 (0.0)                | -                | 6d                      |

ET1, macrolide-dependent transactivator (E-VP16); pBP283 (P<sub>ETR3</sub>-d2EYFP), expression vector encoding a d2EYFP under control of the erythromycin-responsive promoter; P<sub>ETR3</sub>, macrolide-responsive promoter (ETR-P<sub>SCMV<sub>min</sub></sub>; P<sub>SCMV<sub>min</sub></sub>, promoter of the human cytomegalovirus immediate early promoter; P<sub>CMV</sub></sub>min</sub>: minimal version of P<sub>CMV</sub>; P<sub>CMV<sub>−1</sub></sub> tetracycline-responsive promoter (tetO<sub>7</sub>-P<sub>CMV</sub><sub>min</sub>); pDG156 (P<sub>CMV<sub>−1</sub></sub>-ET1), expression vector encoding the erythromycin-dependent transactivator (ET1, E-VP16); pDG157 (P<sub>CMV<sub>−1</sub></sub>-E-siRNA<sub>LUC</sub>-VP16), expression vector encoding ET1 containing an intronically encoded siRNA specific for firefly luciferase mRNA (siRNA<sub>LUC</sub> nt 62–80); pDG178 (P<sub>CMV<sub>−1</sub></sub>-tTA), autoregulated tTA expression vector; pND10 (P<sub>CMV</sub><sub>−1</sub>-TAG<sub>LUC</sub>-tTA), tTA expression vector containing an siRNA<sub>LUC</sub>-specific tag (TAG<sub>LUC</sub>) in the 5′ untranslated region derived from the firefly luciferase (nt 62–80); siRNA<sub>LUC</sub>, firefly luciferase-specific siRNA; TAG<sub>LUC</sub>, firefly luciferase-specific sequence tag; tTA, tetracycline-dependent transactivator (TetR-VP16); VP16, transactivation domain of Herpes simplex virus. Data). Owing to the strong amplification of the mRNA destruction capacity by RNA interference components such as RISC and DICER (46), even residual siRNA levels were predicted to completely eliminate leaky tTA transcripts and, therefore, prevent tTA production (Figure 5c and d).

**Quantitative analysis of network behavior using automated image tracking and quantification**

To obtain statistically relevant information on the network dynamics, the experimental data were systematically analyzed using customized tracking and image analysis software. Using high-throughput processing of acquired image series (for detailed information see Supplementary Data), we determined a consistent period for all analyzed plasmid ratio variations. In addition, we obtained significant relative amplitudes of >30% for total cellular fluorescence (Table 1).

With respect to the efficiency of oscillator behavior in the cell population, we found the highest number of cells that showed oscillating d2EYFP fluorescence for transfection with equimolar plasmid ratios (1:1:1). A movie showing the oscillatory behavior of the system after transfection of pND10 (P<sub>CMV</sub><sub>−1</sub>-TAG<sub>LUC</sub>-tTA), pDG157 (P<sub>ETR3</sub>-E-siRNA<sub>LUC</sub>-VP16) and pBP283 (P<sub>ETR3</sub>-d2EYFP) at equimolar ratios 1:1:1 (100 ng each) can be found at http://mf-229-serv.ethz.ch/fussi_download/marcel/supportmov.mov. The share of oscillating cells was 11.7% compared to 9.7% (2:1:1), 8.2% (1:2:1), 7.1% (2:2:1) and 17.6% (0.5:0:5:1) (Table 1). In contrast to the first-generation mammalian oscillator (22), which was tunable by varying plasmid ratio and amount (i.e. it exhibited frequency and amplitude changes in response to variations in these parameters), the alternative low-frequency mammalian oscillator is largely insensitive to component fluctuations.

**DISCUSSION**

The design of functional replicas of natural circadian clocks using native circadian clock components has been unsuccessful, suggesting that engineering of oscillatory behavior remains a non-trivial challenge (47). Synthetic biology at this point seems to be a valuable alternative, as several synthetic networks in prokaryotic backgrounds have efficiently produced functional circuits displaying damped (48), self-sustained (11), tunable (49,50) or metabolically (51) controlled oscillations. By incorporating posttranscriptional antisense-based control mechanisms, together with transcriptional regulation, we have previously created self-sustained and tunable oscillatory behavior in mammalian cells (22). However, the understanding and availability of single building blocks for complex network reconstruction is still relatively low. Any successful future initiative for gene therapeutic applications will require a broad spectrum of single components that can be interconnected to generate tunable and predictable oscillatory behavior in terms of frequency and amplitude of target gene expression levels. Our intronic-siRNA-based low frequency and robust oscillator therefore not only provides another tool for oscillatory gene expression. Due to its characteristics (siRNA-based feedback loops, long period, robustness to variations of DNA amounts and ratios), it serves as a valuable addition as well as counterpart to established systems. Besides synchronization of oscillators across a population of cells which was recently achieved in bacteria (17,18), the design of synthetic clocks with increased robustness of oscillatory behavior to variations in relative expression...
of network components and intrinsic noise remain major challenges on the way to use rhythmic transgene expression dosing in future therapeutic applications. Based on extensive simulations of novel oscillator architectures using a mathematical model, we have chosen to combine a two-level transcription cascade (autoregulated Ta expression triggers expression of the ET1 transactivator which induces d2EYFP expression) with an intronic siRNA-based negative feedback loop eliminating Ta-encoding transcripts by RNA interference. This network design enabled oscillating d2EYFP expression with a fixed frequency and amplitude that was almost insensitive to changes in plasmid ratio and dosage. Such extensions will make custom-tailored designs of regulatory feedback loops for specific applications possible. Furthermore, new insights into intronic siRNA-mediated silencing impact and dynamics may advance the understanding of naturally occurring silencing-mediated feedback and feed-forward circuits.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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