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Towards a synthetic circadian clock in mammals

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The molecular mechanism of circadian clocks is complex: it involves many genes and several interlocked positive and negative feedback loops. However mathematical models predict that a simple delayed negative regulatory feedback involving a single gene would be sufficient to produce self-sustained 24 hours oscillations. The design principles of the genetic network responsible for oscillations are not yet elucidated. Synthetic biology provides a means to tackle this issue. A recent publication in *Nature* addressed this question by designing an artificial clock that relies on a minimal mechanism. Guided by a mathematical model, this system was implemented in cultured mammalian cells and produced *in vivo* self-sustained oscillations. Besides providing insights in the functioning of a genetic oscillator, this first realization of a synthetic clock in a mammalian cell opens promising perspectives for cell therapy.

Circadian oscillations represent one of the more conspicuous examples of biological rhythms. Although present at the physiological, behavioral, and cellular levels, these 24 hours rhythms originate at the molecular level. Identifying the so-called clock genes and their regulations is thus of primary importance in understanding the origin and the dynamical properties of circadian oscillations (*e.g.* their entrainment by light-dark cycles). Since alterations of the circadian clockwork have been linked to physiological disorders (Toh *et al*., 2001; Fu *et al*., 2002), a better understanding of the molecular functioning of the clock could also lead to medical applications (Lévi, 2006; Liu *et al*., 2007).

The genetic bases of the circadian clock started to be elucidated in the early seventies when Konopka & Benzer (1971) identified the first clock gene, called *per* (for *period*), whose mutations affected the period of the circadian locomotor activity rhythm in *Drosophila* flies. Nearly twenty years later, Hardin *et al*. (1990) showed that both the expression of the *per* gene and the level of the PER protein undergo circadian oscillations. The authors explained the origin of these oscillations by a mechanism based on a negative feedback loop. Since this

pioneering work, the study of the molecular regulatory bases of circadian rhythms was undertaken in many model organisms and has attracted much interest from geneticists and molecular biologists (Yu & Hardin, 2006; Dunlap *et al*., 2007; Johnson *et al*., 2008), but also from modelers (Goldbeter, 2002; Roenneberg *et al*., 2008).

The first mathematical model accounting for the molecular mechanism of circadian rhythms was built up for the *Drosophila* clock (Goldbeter, 1995). The model structure was based on the Goodwin's oscillator (Goodwin, 1965) and incorporated the negative auto-regulation of the *per* gene by its protein product, PER, as well as the phosphorylation and nuclear translocation of this protein. Numerical simulations of this minimal five-variable model predict that such a delayed negative feedback loop would be sufficient to generate self-sustained 24h oscillations. However, the actual mechanism happened to be more complex. Shortly after the discovery of the *per* gene, several additional genes have been shown to play a crucial role in the regulatory mechanism of circadian rhythms in *Drosophila* (Glossop *et al*., 1999). In the late nineties, the first clock genes homologous to the *Drosophila per* gene were found in mammals (Albrecht *et al*., 1997) and the molecular regulatory mechanisms in these two organisms were shown to be highly similar (Yu & Hardin, 2006).

In the current picture, the core of the mammalian clock includes some tens genes and involves several interlocked positive and negative feedback loops (Shearman *et al*., 2000; Yu & Hardin, 2006). In the positive limb of the clock, the *Clock* and *Bmal1* genes encode two proteins that interact together to form a CLOCK-BMAL1 complex. This complex acts as a transcription factor for many genes such as *Per*, *Cry*, *Rev-Erbα* or *Rora*. In the negative limb, the products of the *Per* and *Cry* genes form a PER-CRY complex that inhibits the function of the CLOCK-BMAL1 activator. Additional feedback loops involve the products of the *Rev-Erbα* and *Rora* genes that negatively or positively regulate the transcription of *Bmal1*, respectively. Finally, the mammalian circadian clock is also regulated by several post-translational processes such as phosphorylation (Gallego & Virshup, 2007), acetylation (Etchegaray *et al*., 2003), sumoylation (Cardone *et al*., 2005), ubiquitination (Lee *et al*., 2008a) or proteasomal degradation (Ohsaki *et al*., 2008).

Mathematical approaches are of great help to apprehend the dynamical properties of such intricate networks (Forger *et al*., 2007). Currently, many theoretical models tend to incorporate as many elements (genes, proteins, feedback loops) as possible in order to reproduce the complexity of circadian clocks (Leloup & Goldbeter, 2003; Forger & Peskin, 2003). These models have been used to investigate various properties of the circadian clock linked to physiological disorders (Leloup & Goldbeter, 2003; Leloup & Goldbeter, 2008), robustness to molecular noise (Forger & Peskin, 2005), or the role of phosphorylation (Gallego *et al*., 2006).

New high-throughput experimental techniques and bioinformatics approaches allow a systematic screening of the genome to detect clock-controlled genes (Ueda *et al*., 2002; Kumaki *et al*., 2008; Ukai-Tadenuma *et al*., 2008; Yan *et al*., 2008), as well as the regulatory circuits they are involved in (Ueda *et al*., 2005). The goal of combining large-scale

experiments and modeling is to characterize and analyze the full circadian network and to provide an integrated view of this molecular system. Such a system-level approach is sometimes referred to as ‘top-down’ and is part of the so-called ‘Systems biology’. It highlights the complexity of the circadian network, but does not explain the necessity of such complexity. Indeed, theoretical models showed that one gene and one negative regulatory feedback loop would already be sufficient to generate self-sustained circadian oscillations. To unravel the design principles (Rand *et al*., 2004; Novak & Tyson, 2008) of the genetic network responsible for oscillations, it is thus possible to take the problem by the other side and build a minimal experimental regulatory network that provides the basic characteristics of a clock. This ‘bottom-up’ approach is now to be considered thanks to new genetic techniques that allow the construction of an artificial genetic network in a cell. The latter approach is often referred to as ‘synthetic biology’.

Synthetic biology consists of designing, modeling, and engineering artificial functional modules *in vivo* (Hasty *et al*., 2002b; Endy, 2005; Andrianantoandro *et al*., 2006). Important advances in this domain were recently achieved thanks to high-resolution single-cell measurements (using the GFP and fluorescence microscopy) and the possibility to construct new gene/promoter assemblies, and thereby rewiring genetic regulatory networks. Pioneering works in this field include the realization of a synthetic bistable toggle switch in bacteria (Gardner *et al*., 2000) and of a synthetic oscillator, called ‘Repressilator’ (Elowitz & Leibler, 2000).

Engineered transcriptional regulation and genetic networks have then been used to compare the dynamical properties of alternative signaling pathways (Kollman *et al*., 2005), to study the propagation of noise in transcriptional cascades (Hooshangi *et al*., 2005), to build an artificial biosensor module controlling a natural signaling pathway (Kobayashi *et al*., 2004), to create spatio-temporal patterns (Basu *et al*., 2005), to design a genetic circuit that can exhibit either a toggle switch or an oscillatory behavior (Atkinson *et al*., 2003), and to construct a synthetic circuit which generates oscillations in glycolysis (Fung *et al*., 2005). More recently, Stricker *et al*. (2008) engineered an artificial oscillator based on interlocked positive and negative transcriptional feedback loops. Constructed with synthetic gene designs in *E. coli*, this system confirmed the predictions of the mathematical model: the delayed negative feedback loop is required to produce oscillations, while the positive feedback loop is responsible for an increase of the robustness of the oscillations and allows for a greater tuning of the period. These findings are also in agreement with the predictions of previous computational studies (Hasty *et al*., 2002a; Tsai *et al*., 2008). All of the above-mentioned oscillatory systems were built up in bacteria and were not aimed at producing circadian oscillations.

A first attempt to reconstruct a circadian oscillator with clock components in mammalian cells was performed using the known clock genes (*Cry*/*Per* and *Clock*/*Bmal1*) and genetically modified promoters (Chilov & Fussenegger, 2004). The authors designed a generic oscillator assumed to reflect the core mechanism of the mammalian circadian clock. The genetically modified plasmids carrying the new gene/promoter assemblies were transferred in cultured mammalian (Hela) cells. The system was not capable of producing sustained oscillation but

did nevertheless exhibit a single cycle of a clock-like oscillation. This suggests that homologous regulatory components and regulatory design can be used for oscillatory synthetic constructions.

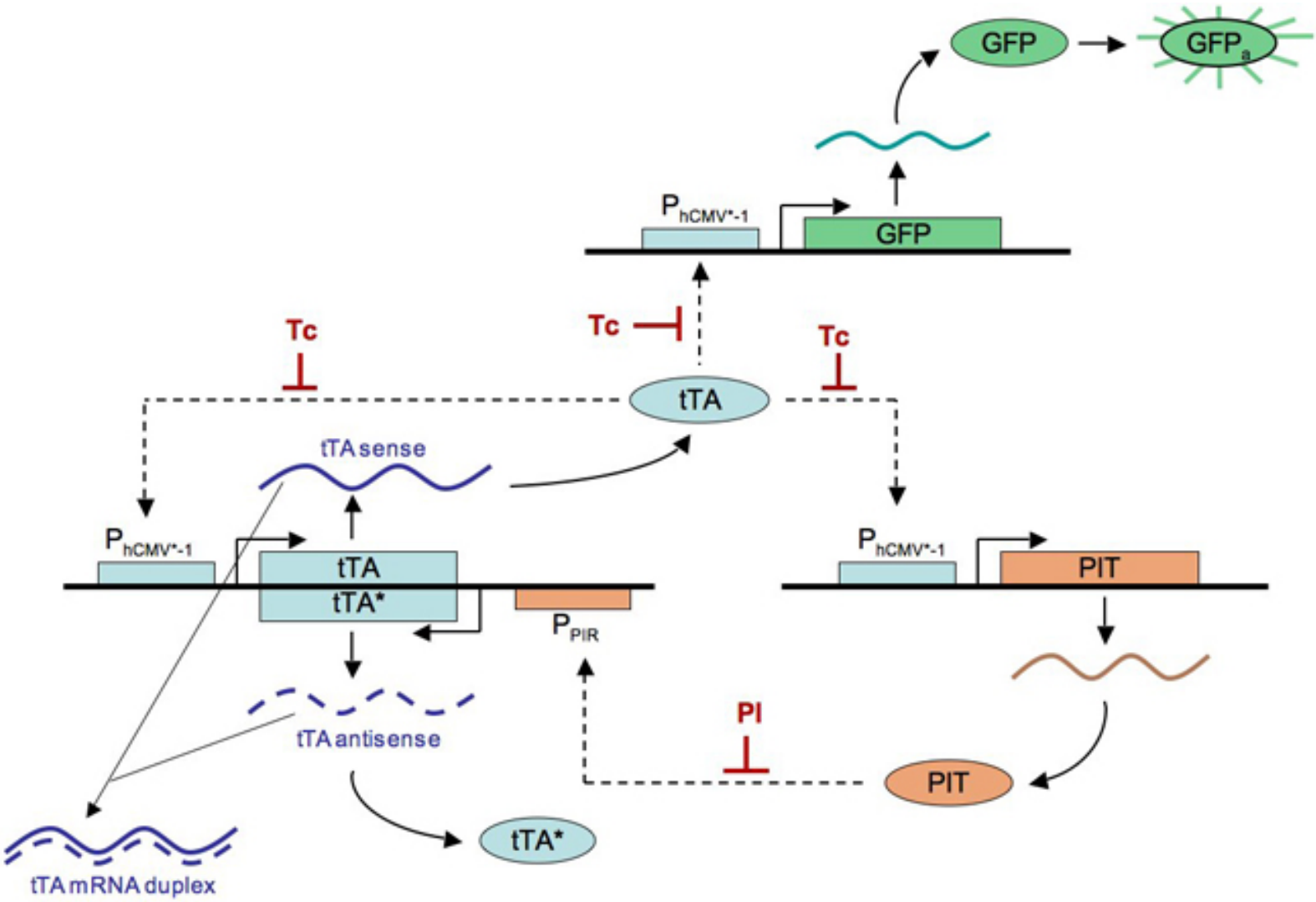

Figure 1. Design of the synthetic mammalian oscillator. The core unit of the system is the tetracycline-dependent transactivator (tTA) which is placed under the control of two promoters: $P_{hCMV*-1}$ (activated by tTA) which directs the sense transcription and P_{PIR} (activated by the pristinamycin-dependent transactivator, PIT) which directs the antisense transcription. Expression of PIT gene is controlled by the $P_{hCMV*-1}$ promoter. The green fluorescent protein (GFP), also under the control of the $P_{hCMV*-1}$ promoter, serves as a reporter. The period of the oscillations can be tuned by gene dosage (*i.e.* the number of plasmids carrying the tTA gene and the number of plasmids carrying the PIT gene) and by the transcriptional inhibitors Tc (tetracycline) and PI (pristinamycin). *Redrawn from Tigges et al, 2009.*

More recently, Professor Fussenegger and his collaborators (Tigges *et al*., 2009) designed a synthetic oscillator based on an auto-regulatory sense-antisense transcriptional control circuit combining a positive and a negative feedback loop (**Figure 1**). They used the pristinamycin- (PIT) and tetracycline (tTA)-dependent transactivator that were previously used to control mammalian cell growth (Fux *et al*., 2001). On the one hand, the tTA protein activates its own

production, forming the positive feedback loop. On the other hand, the tTA protein activates the PIT gene expression. Then the PIT protein activates the synthesis of antisense tTA transcript, which binds to tTA mRNA, thereby preventing the synthesis of tTA protein. This closes the second, negative feedback loop. The tTA and PIT genes are inserted in two different plasmids, allowing a differential gene dosage. The green fluorescent protein (GFP), whose gene is under the same promoter as tTA, is used as reporter.

A mathematical model of this synthetic oscillator was first developed to determine the conditions under which self-sustained oscillations can take place (Tigges *et al*., 2009). Simulations of the model pointed to gene dosage and reporter stability as key parameters for controlling the period of the oscillations. Guided by the predictions of the model, the system was artificially implemented in a mammalian cell: the genetic constructions were inserted in plasmids that were injected into Chinese Hamster ovary cells. In vivo self-sustained oscillations were revealed at the single cell level by the periodic activation of the GFP. The authors found that the dependency of the period to the plasmid ratio were consistent with the model prediction, indicating that the interlocked feedback loop system is tunable thanks to gene dosage. The period however varies from one cell to another and some cells did not oscillate. Stochastic simulations of the theoretical model show that the cell-to-cell variability of the period can be attributed to the molecular noise, inherent in the system. These simulations also predict that not all the cells would oscillate, as observed in the experimental data.

The work of Tigges *et al*. (2009) thus demonstrated that a mechanism based on an interlocked negative and positive feedback loop is an efficient design to generate self-sustained and robust oscillations as required for circadian rhythms. But if the main effect of the positive feedback loop is to increase the tunability of the oscillator, an intriguing question still remains: what would be the advantage of having a tunable circadian clock whose function is precisely to keep the 24h period constant? The possibility arises that the positive feedback loop plays a role in the phase locking of the circadian oscillator when entrained by light-dark cycles.

A major difference between the natural circadian clock and the synthetic oscillator of Tigges *et al*. (2009) pertains to the molecular mechanism responsible for the oscillations. The sense-antisense mechanism of the synthetic clock does not reflect the actual picture of the mammalian circadian clock, which mainly relies on transcription-translation feedback loops (TTFLs). Interestingly however, a mechanism based on sense-antisense regulation of a clock gene was shown to be involved in the regulation of the clock gene *frq* in *Neurospora* fungi (Kramer *et al*., 2003, Crosthwaite, 2004) and could play a role in the generation of circadian oscillations of the expression of the *per* gene in *Antheraea pernyi* silkmoth (Sauman & Reppert, 1996). It is also worth highlighting that - although the TTFL is commonly accepted by experimentalists and modelers - there is actually no proof that it is the core oscillatory mechanism for circadian oscillations. As a matter of fact, there is some evidence pointing to other complementary mechanisms. Recently, in a remarkable experiment, Nakajima *et al*. (2005) succeeded to reconstitute the self-sustainable circadian clock of cyanobacteria without

any TTFLs. By incubating in a test tube only three clock proteins (KaiA, KaiB and KaiC) with ATP, they measured a 24h rhythm in the phosphorylation of KaiC.

It should be noticed that the artificial clock of Tigges *et al*. (2009) is, at least at this stage, not a substitution to the actual circadian clock. First, the period of the oscillations is typically around 150 minutes, and, even upon tuning by plasmid dosage, the period of oscillations does not exceed a few hours. The origin of the large period of circadian oscillations is still not explained. Secondly, the synthetic clock relies on well-characterized genes and promoters, which are not the known circadian clock components. This means that the clock-controlled genes, *i.e.* the genes which are normally under the control of the transcription factor CLOCK-BMAL1 will not be regulated by the artificial clock. To solve this issue, in a future extension of the synthetic oscillator, the promoters of the *Clock* and *Bmal1* genes could also be modified in order to be regulated by the synthetic clock. Finally, since a major role of the circadian clock is to convey the light signal and to synchronize the biological rhythms with the external day-night cycles, the synthetic clock should also be able to respond to light and be entrained by light-dark cycles.

The experiments of Tigges *et al*. (2009) were performed in a cellular culture, which is useful in understanding how circadian rhythms are generated at the level of a single cell. However, the ultimate goal will be to implement the artificial clock in the pacemaker cells of a living organism. It will then be crucial to understand the synchronization between individual cells and the coordination between the pacemaker cells and the peripheral clocks in order to control the clock-related physiological and behavioral rhythms.

Understanding the relationship between the design (*i.e.* the topology of the regulatory network) and the dynamical properties of the network offers promising perspectives. Engineering bacteria or yeast cells could lead to applications in biocleaning (*e.g.* the treatment of biological wastes or the clean-up of toxic spills) and could complement or enhance current methods for the production of drugs, drug precursors, or other organic substances (Weber & Fussenegger, 2002; McDaniel & Weiss, 2005; Lee *et al*., 2008b). Prototypical pharmaceutical applications have already been successful. Ro *et al*. (2006) engineered a yeast cell to produce an antimalarial drug precursor, and Anderson *et al*. (2006) used engineered bacteria to destroy cancer cells.

Current research projects aim at extending this area of research in mammalian cells (Greber & Fussenegger, 2007), as examplified by the work of Tigges *et al*. (2009). Besides providing insight into the dynamics of biological oscillators, the development and analysis of an artificial circadian clock open numerous therapeutic perspectives in clock-related pathologies such as sleep disorders (Liu *et al*., 2007), Huntington (Morton *et al*, 2005) and Alzheimer's (Wu *et al*., 2006) diseases, or may be used in cancer chronotherapy (Lévi, 2006). The road to reach these objectives is still long, but the results of Tigges *et al*. (2009) constitute an important step in this direction.

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