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Model-based investigation of the circadian clock and cell cycle coupling in mouse embryonic fibroblasts: Prediction of *RevErb-α* up-regulation during mitosis

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ABSTRACT

Experimental observations have put in evidence autonomous self-sustained circadian oscillators in most mammalian cells, and proved the existence of molecular links between the circadian clock and the cell cycle. Some mathematical models have also been built to assess conditions of control of the cell cycle by the circadian clock. However, recent studies in individual NIH3T3 fibroblasts have shown an unexpected acceleration of the circadian clock together with the cell cycle when the culture medium is enriched with growth factors, and the absence of such acceleration in confluent cells. In order to explain these observations, we study a possible entrainment of the circadian clock by the cell cycle through a regulation of clock genes around the mitosis phase. We develop a computational model and a formal specification of the observed behavior to investigate the conditions of entrainment in period and phase. We show that either the selective activation of *RevErb-α* or the selective inhibition of *Bmal1* transcription during the mitosis phase, allow us to fit the experimental data on both period and phase, while a uniform inhibition of transcription during mitosis seems incompatible with the phase data. We conclude on the arguments favoring the *RevErb-α* up-regulation hypothesis and on some further predictions of the model.

1. Introduction

In most organisms, from bacteria to plants and animals, spontaneous gene expression oscillations with a period close to 24 h have been observed. A biochemical circadian clock present in each cell is responsible for maintaining these oscillations at this period, generally in the form of a self-sustained genetic oscillator entrained by the day/night cycle through various input pathways.

This circadian clock has many effects on cell signaling and metabolism (Partch et al., 2014). Experimental results have also shown a regulation of the cell division cycle by the circadian clock (Matsuo et al., 2003; Barnes et al., 2003; Ünsal-Kaçmaz et al., 2005), in particular in mammalian cells with possible applications to cancer chronotherapies (Ballesta et al., 2005; De Maria et al., 2011). Molecular links between these two cycles have been exhibited to explain this regulation. In particular the regulation of Wee1, an inhibitor of the G2/M transition, by the clock genes has been proposed to explain the circadian gating of mitosis during the liver regeneration process (Matsuo et al., 2003) with 48 h period doubling phenomena for the cell cycle (Nagoshi et al., 2004). Other similar molecular links going in the same direction, through p21 (Gréchez-Cassiau et al., 2008) and Chk1 and Chk2 (Ünsal-Kaçmaz et al., 2005; Gery et al., 2006), have been shown in different cells in the literature. A few models have also been developed to further investigate those hypotheses, by coupling a model of the cell cycle with a model of the circadian clock through those direct molecular links, and analyzing the conditions of entrainment in period (Gérard and Goldbeter, 2012; Calzone and Soliman, 2006; Glass, 2001).

However, in mouse embryonic fibroblasts NIH3T3, several studies using large-scale time-lapse microscopy to monitor circadian gene expression and cell division events in real time and in individual cells during several days have unveiled unexpected behaviors, hinting that the relationship might be more complex. Nagoshi et al. (2004), have first shown that circadian gene expression in fibroblasts continues during mitosis, but with a consistent pattern in circadian period variation relatively to the circadian phase at division, leading them to hypothesize that mitosis elicits phase shifts

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in circadian cycles. A more recent study of Bieler et al. (2014) relating the same experiments on dividing fibroblasts found the two oscillators synchronized in 1:1 mode-locking leading the authors to hypothesize a predominant influence of the cell cycle on the circadian clock in NIH3T3 cells. This is in agreement with another detailed experimental study of Feillet et al. (2014) which found several synchronization states in NIH3T3 fibroblasts in different conditions of culture. In particular, it was observed in Feillet et al. (2014) that enriching the culture medium with growth factors by increasing the concentration of Fetal Bovine Serum (FBS) not only accelerates the cell division cycle but also the circadian clock. For cells cultured in 10% FBS, both distributions of the cell cycle length and the circadian clock are centered around 22h. For cells cultured in 15 % FBS, both the cell cycle and the circadian clock accelerate, with period distributions centered around 19 h. However, when cells reach confluence and stop dividing, the circadian clock slows down and the period distribution is then centered around 24 h. None of the currently available models coupling the cell cycle and the circadian clock can explain these observations since they are based on an unidirectional influence of the circadian clock on the cell cycle (Gérard and Goldbeter, 2012; Calzone and Soliman, 2006) and not on the other direction.

In this paper, in order to explain these observations, we investigate the reverse influence of the cell cycle on the circadian clock, using computational modeling tools. We develop a mathematical model of the influence of the cell cycle on the circadian clock through the differential regulation of clock genes around the mitosis phase, and study the conditions in which the cycles are entrained in period and phase as observed in Feillet et al. (2014). For this, we use the circadian clock model of Relógio et al. (2011) which has been carefully fitted to phase data on suprachiasmatic cells, and a simple model of the cell cycle by Qu et al. (2003) which focuses on the mitosis phase. In Traynard et al. (2015), we have already shown that the uniform inhibition of transcription during mitosis, as observed in eukaryotes (Weisenberger and Scheer, 1995), could explain the acceleration of the circadian clock in non-confluent cells when the concentration of FBS increases (Feillet et al., 2014). In particular, our model could reproduce the same periods for the cell cycle and the circadian clock for different levels of FBS, modeled by different values for the synthesis parameters of the cell cycle model, but with an incorrect time delay between the cell division and the peak of Reverb-α, which seemed impossible to fix under the hypothesis of a uniform inhibition of transcription during mitosis.

Here, we show that these difficulties can be resolved, using a different hypothesis of selective regulation of one clock gene during the M phase, either the activation of *Reverb-α* or the inhibition of *Bmal1*. Our coupled model under one of these hypotheses is able to reproduce the experimental measures on periods and phases made by Feillet et al. (2014) in individual unperturbed fibroblasts. Furthermore we argue that the complex behaviors observed with high variability after a treatment by dexamethasone to synchronize cellular clocks, modeled by the induction of a high level of *Per* and the inhibition of the other clock core genes, can be explained by the perturbation of the clock after this treatment. Indeed, our model shows that the stabilization time after that pulse appears to be greater than the time horizon of 72 h used in those experiments.

This computational model has been built using the Biocham modeling software (Calzone et al., 2006) for

1. importing and exporting models in SBML, and modeling the molecular interactions of the coupling of the models,
2. specifying the observed behavior in quantitative temporal logic using pattern formulae for periods and phases (Fages and Traynard, 2014; Traynard et al., 2014),
3. searching parameter values (Rizk et al., 2011) and measuring robustness and parameter sensitivity indices (Rizk et al., 2009) with respect to the temporal logic specification of the dynamical behavior[1].

Table 1
Estimated periods of the circadian molecular clock and the cell division cycle measured in Feillet et al. (2014) in fibroblast cells without treatment by dexamethasone, for two concentrations of FBS. The time delay is between the cell division time and the next peak of RevErb-α protein.

Medium	Clock period	Division period	Mean delay
FBS 10%	21.9 h ± 1.1 h	21.3 h ± 1.3 h	8.6 h
FBS 15%	19.4 h ± 0.5 h	18.6 h ± 0.6 h	7.1 h

2. Experimental data and their formal specification in temporal logic

2.1. Experimental observations and measurements

In this section we explain the single cell experiments and analyses performed in Feillet et al. (2014) and the conclusions drawn by the authors. The reported experiments have been done using time lapse videomicroscopy and cell tracking using different fluorescent reporters for the cell cycle and the circadian clock observed during 72 h in proliferating NIH3T3 embryonic mouse fibroblasts. This cell line was modified to include three fluorescent markers of the circadian clock and the cell cycle: the RevErb-α::Venus clock gene reporter (Nagoshi et al., 2004) for measuring the expression of the circadian protein[2] RevErb-α, and the Fluorescence Ubiquitination Cell Cycle Indicators (FUCCI), Cdt1 and Geminin, two cell cycle proteins which accumulate during the G1 and S/G2/M phases respectively, for measuring the cell cycle phases (Sakaue-Sawano et al., 2008).

The cells were left to proliferate in regular medium supplemented with different concentrations of FBS (10% and 15%). Long-term recording was performed in constant conditions with one image taken every 15 min during 72 h. The lengths of the cell cycles were measured as the time interval between two consecutive cell divisions.

The expression traces of RevErb-α proteins were detrended and smoothed. Spectrum resampling was used to estimate the clock period. Cells with less than two RevErb-α peaks within their lifetime, a period length outside the interval between 5 h and 50 h or a relative absolute error (RAE) bigger than 0.25 (showing a confidence interval wider than twice the estimated period) were classified as non-rhythmic and discarded, assuming that they do not have a functioning clock. Finally, the delay between cell division and the next clock marker peak was measured. It revealed that RevErb-α-Venus peaked about 7 h after cell division in all conditions, quite consistently with the delay of 5 h for Reverb-α without Venus observed in Feillet et al. (2015), Bieler et al. (2014).

The quantitative data on the periods of the cell cycle and the circadian clock and the phase between them are summarized in Table 1 (Feillet et al., 2014). Surprisingly, increasing FBS from 10% to 15%, not only decreases the mean period of the cell cycle from 21.3 h to 18.6 h, but also the clock period from 21.9 h to 19.4 h, i.e. to essentially the same period. This shows that both oscillators remain unexpectedly in 1:1 mode locking. While the speedup of the cell cycle can be directly attributed to the growth factors in

[1] The models and the formal specifications used in this paper are available on http://lifeware.inria.fr/wiki/software/biosystems16.
[2] In this paper, the genes are distinguished from the proteins by writing the names of the genes in italics and the proteins in normal text.