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Development and Characterization of the cellular circadian clock of C2C12 cells

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Development and Characterization of the cellular circadian clock of C2C12 cells

by

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List of Abbreviations

SCN - Suprachiasmatic nucleus

Bmal - Brain and Muscle ARNT Like Protein

Clock - Circadian Locomotor Output Cycles Kaput

Cry- cryptochromes

Per - Period

Nr1d1- Rev-Erb alpha

Nr1d2 - Rev-Erb beta

Fbxl3 - F-box/LRR-repeat protein 3

RORs - box/LRR-repeat protein 3

bHLH - Basic helix–loop–helix

MRFs - myogenic regulatory factors

Myf5 - Myogenic factor 5

MyoD - Myoblast determination protein 1

MyoG - Myogenin

MRF4 - Myogenic regulatory factor4

MEF2 - Myocyte Enhancer Factor 2

RNAi - RNA interference

shRNA - Short Hairpin RNA

Gag-pol - Gag Polyprotein

REV - Rev protein

VSV-g - Vesicular Stomatitis Virus Glycoprotein

EGFP - Enhanced Green Fluorescent Protein

DMEM - Dulbecco's Modified Eagle Medium

FBS - Fetal Bovine Serum

PSG - Penicillin Streptomycin Glutamine

PBS - Phosphate-buffered saline

qPCR - Quantitative Polymerase Chain Reaction

KD - Knockdown

KO - Knock out

WT – Wild Type

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Abstract

Circadian rhythms in mammalian physiology and behavior are generated through coherent rhythmic crosstalk between the central clock in the brain and peripheral clocks throughout the organs in our body. The molecular clocks responsible for these intrinsic rhythmic oscillations work based on interlocked transcription/translation feedback loops, which work together with various environmental and metabolic cues to produce internal 24-hour timing. This project established a stable cellular circadian clock model using the C2C12 cell line. This genetically tractable model contains an integrated luciferase reporter, which enables continuous luminescence recording of each circadian cycle. In order to examine this cellular circadian model, I created a panel of short hairpin RNAs (shRNAs) targeting ten of the primary known clock genes (*Bmal1, Clock, Per1, Per2, Per3, Cry1, Cry2, Nr1d1, Nr1d2, Fbxl3*). I evaluated their functional role in circadian rhythm generation. Knockdown of *Bmal1* resulted in arrhythmicity. Knockdown of the *Clock* caused a shorter period length and a lower amplitude. Knockdown of *Per1* resulted in shorter period lengths and Knockdown of *Per2* resulted in shorter period and high amplitude. Per3 knockdown caused a high amplitude phenotype. Knockdown of *Cry2* caused short period length and higher amplitude. On the other hand, the knockdown of *Cry1* led to lower amplitude. Knock down of *Nr1d1*, and *Fbxl3* both resulted in lower amplitude with no changes in period length. In summary, I established a cellular clock model of the C2C12 cell line, which is amenable to genetic manipulation and would be ideal for studying the crosstalk between clock and muscle physiology and metabolism.

Introduction

Circadian clock

Circadian in Latin word (*'Circa Diem'*) means 'about a day.' [1] Many physiological and behavioral aspects of organisms are controlled by an internal time-keeping circadian system[2], which is conserved in most organisms, from unicellular algae to human beings[3]. Synchronization of the circadian clock anticipates the potential environmental changes that affect metabolism, physiology, or behavior[4]. At a molecular level, circadian rhythms let organisms reach temporal homeostasis by regulating their gene expression[5]. This results in a peak of protein expressions once every 24 hours while a specific physiological process is at the most active level according to the solar day[6]. Generally, mammals have central and peripheral clocks in their body[7]. Every cell in every tissue has its molecular clock, which originates from internal timing of about 24 hours when external cues are absent[5 8]. All the molecular clocks in all peripheral tissues are synchronized by a 'master' clock[9] called the suprachiasmatic nucleus (*SCN*), which creates a hierarchical coherent clock machinery system[10 11]. In mammals, the suprachiasmatic nucleus (*SCN*) is located in the anterior hypothalamus[12]. In a 12:12 hour light-dark cycle, light information passes through the eye to the *SCN*[13]. The *SCN* is the only molecular clock that assimilates the light information and sends the circadian information to the extra-SCN oscillators and then to peripheral tissues and organs to provide circadian control of physiological and behavioral functions.[14] This hierarchical system ensures that all the physiological and behavioral events occur at optimal times about local time[15]. Conversely, any disruption in the circadian system leads to desynchronization of the components of the circadian system and results in sleep, metabolic, psychological, and cardiovascular problems[16 17 18 19]. The circadian clock comprises a dozen clock genes that function as positive and negative transcriptional regulators in the

molecular clock loops[5 20]. The circadian clock is a network of three interlocking transcriptional negative feedback loops at the molecular level[21]. *E-box*-mediated gene expression comprises the core clock loop in which *Bmal1* and *CLOCK* are the positive transcriptional factors that dimerize and turn on the transcription of *Per* and *Cry* through *E-box* elements[22]. The transcribed *Per* and *Cry* genes are translated, form a heterodimer, enter the nucleus, and inhibit their synthesis[23]. This entire process takes about 24 hours[24]. The other two interlocking loops comprise *RRE* and *D-box* regulatory elements, stabilizing the core clock loop. In the *RRE* element loop, the *RORs* (*RORa, RORb,* and *RORc*) function as transcriptional activators, and the Reverbs (Rev-erbα and Rev-erbβ) function as the transcriptional repressors. In the *D-box* element loop, *DBP* acts as the activator, and the *E4BP4* acts as the repressor[25 26]. Whether the animals are active at night or day, the molecular and biochemical (transcriptional and translational negative feedback loop) features of the circadian clock are similar in the *SCN*, extra-*SCN*, and all peripheral clocks. *Clock* genes are expressed in the SCN, various brain regions, and most peripheral tissues and organs[27]. However, the expression level of known clock genes in various peripheral tissues differs. In addition, evidence from different studies has suggested that the functions of clock genes are tissue specific [28 29 30]. Genome-wide transcriptome studies have revealed that thousands of genes of the whole transcriptome in each organ or tissue are expressed rhythmically[31 32 33].

Mouse muscle tissue and C2C12 Cell

Skeletal muscle is the most abundant tissue throughout the human body, making up around 45% of the total body mass, although its significance to health is sometimes underestimated[34]. Skeletal muscle regulates locomotion and postural activity and plays an essential role in nutritional homeostasis and modulation of muscle physiology, including glucose metabolism,

energy cycle, muscle growth, maintenance, and contractile performance [35]. Several studies used animal models with selective clock gene deletions to analyze the functions of the circadian clock network in skeletal muscle. Like almost all other cells throughout the body, the circadian clock exists in skeletal muscle [36]. Disruption of circadian clock machinery in skeletal muscle is associated with many metabolic diseases which strongly affect muscle physiology [37]. More specifically, disruption of the circadian clock in muscle tissue can contribute to metabolic and physiological imbalances such as oxidation of macronutrients and sarcopenia[37 38]. Recent epidemiological studies show that shift workers with changing shifts are more likely at risk of immune defects and metabolic and mental diseases caused by circadian clock disruption [39 40 41]. These data suggest that the clock regulates muscle-specific clock function, but little is known about the crosstalk between the clock and muscle physiology.

C2C12 is an eternalized subclone of the mouse myoblast cell line, which is very useful in biomedical research [42]. C2C12 cells are used for in vitro studies in the initial form of myoblasts which differentiates into myotubes by changing the condition of the cell environment [43]. C2C12 cells develop and mature rapidly, forming functional skeletal or cardiac muscle cells with the contraction ability to generate force. Myoblast differentiation is a multi-step process in which myoblasts become multinucleated myotubes and mature muscle fibers (myofibers) [44 45]. Myoblast differentiation and myofiber formation depend on two groups of *transcriptional* factors, which are basic helix-loop-helix (*bHLH*) myogenic regulatory factors (MRFs) such as *Myf5* (muscle regulatory factor 5), *MyoD* (myogenic determination factor)[38,] *MyoG* (myogenin), and *MRF4* and myocyte enhancer factor 2 (*MEF2*) factors including *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D*. These transcriptional factors are expressed rhythmically and regulated by the circadian clock in muscle tissue[46 47]. In the circadian field, in-vitro cellular clocks are becoming valuable tools for

studying cell-type specific circadian clock function because in-vitro cellular systems are devoid of all the confounding factors that influence local physiology. The advantage is what phenotypes are observed that are specific to the particular effects[48]. In mice, muscle tissues express about 10 to 30% of non-clock genes rhythmically, which plays a pivotal role in various muscle cellular processes [49 50]. Little is known about how the clock regulates rhythmic output genes and how the output genes provide feedback to the core clock mechanisms in muscle tissue. The in-vitro cellular circadian model would be an idea to study underlying mechanisms of input and output clock-regulated functions. This background information tempted us to develop a mouse muscle cellular clock model and confirmed whether the model is ideal for genetic manipulation.

Hypothesis

I hypothesized that a negative feedback mechanism underlies myoblast circadian rhythms like other cellular oscillators. To study this, I generated a myocyte C2C12 circadian cellular clock model, and I genetically characterized this model using RNAi (RNA interference) method.

Material and Methods

Cell Culture

C2C12 myoblast lines were purchased from ATCC (Cat #CRL-1772), and the 293T cells were received from Dr. Liu's lab. Both cell lines were revived by culturing them in complete media (DMEM/HIGH GLUCOSE, Cat#SH30243, Cytiva) supplemented with 10% FBS and 1x penicillin-streptomycin-glutamine (PSG)) on 10 cm culture dishes. The culture dishes were

incubated at 5% CO_2 in 37°C incubators. The cells were used for experiments once the cells achieved 90% confluency.

Lentiviral Reporter Productions:

I used the previously generated lentiviral Bmal1-luciferase and Per2-luciferase reporter constructs to generate the C2C12 reporter cell line [51]. Using the transient transfection method (viral packaging vectors Gag-pal, VSV-G, Rev), Bmal1 and Per2 reporter viral particles were produced in 293T cells. The viral particles were concentrated by using ultracentrifugation. The high- concentrated viral particles were used to infect the C2C12 cell. After the recovery of infection, the transduced cells were selected by 10 µg/mL blasticidin. The selected cells were then recorded in a luminometer to see whether cells expressed the rhythmic oscillation of the luciferase rhythm. Once the rhythm was established, single-cell clones were generated by serially diluting the cells and culturing them in 96-well plates. The single-cell clones were then assayed for bioluminescence rhythm to select a perfect clone for genetic characterization.

Lentiviral shRNA production:

I used a panel (Table 2) of shRNA constructs targeting the following ten clock genes and *NS*: *Bmal1*, Clock, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Fbxl3*, *Nr1d1*, and *Nr1d2* [51]. The reporter was genetically characterized by the knockdown of known clock genes expression in the selected C2C12 reporter cell lines to determine the clock phenotypes for each gene. As previously described, the transient transfection method was used to produce the lentiviral shRNA for each known clock gene. The viral particles were concentrated using ultracentrifugation, and then the

reporter cells were infected with the shRNA individually. EGFP expression was used in every experiment to estimate the transfection and infection efficiency. The infected cells were selected with puromycin to effectively knock down the genes. The selected cells were then used to assay the luminescence rhythm to determine the knockdown phenotype.

Bioluminescence recording

The reporter cells which were infected with individual shRNA were cultured in complete media on 6-well plates. After being confluent, cells were passaged into pre-collagen-coated 35-mm plates. One day after being 100% confluent, cells were synchronized for 2 hours with synchronization media (DMEM without any serum containing 1nM Forskolin [51 52 53]), and then a conditioning media (1mM DMEM, 14.4µM of H2CO3, 20µM of HEPES, 2% B27, 8.4 µM of NaOH) was added. The dishes were entirely covered by microscope glass coverslips using grease and incubated for 24 hours at 37°C in the incubator. After 24 hours, the conditioning media was replaced by newly prepared bioluminescence recording media containing Luciferin (1mM DMEM, 14.4µM of H2CO3, 20µM of HEPES, 2% B27, 8.4 µM of NaOH, 1% Luciferin) was added and then dishes containing cells were loaded into luminometer for bioluminescence rhythm recording [52 54 55 56 57].

Analysis of Bioluminescence rhythm

A LumiCycle luminometer machine containing 32 channels (*version 2.31, Acti-metrics*) was used for circadian rhythm recording (using 35 mm culture dishes). The LumiCycle Analysis tool *version 2.53 (Actimetrics)* was used to determine circadian parameters. To summarize, raw data

were fitted to a linear baseline, and baseline-subtracted data were fitted to a sine wave (damped), from which period length, quality of fit, and damping constant were calculated. A goodness-of-fit of 90% was frequently obtained for samples with persistent rhythms. The first 30 minutes of data points were often removed from rhythm analysis because of significant transient luminescence upon the medium shift. Raw data from days 0.5 to 3 were fitted to a linear baseline for amplitude analysis, and baseline-subtracted (polynomial number = 1) data were fitted to a sine wave for amplitude determination [51].

Quantitative PCR (qPCR)

The expression level of the mRNA of targeted clock genes was evaluated using qPCR to assess the KD efficiency for endogenous gene expression. Cells were cultured to reach 100% confluence, and then the total RNA was isolated using a miRNeasy®Mini Kit (Cat#217004, QIAGEN). Reverse transcription was done to produce cDNA to perform qPCR. *SYBR* green PCR master mix (miRNeasy®Mini Kit (Cat#217004, QIAGEN) was used, and the primers used for qPCR are mentioned in Table 1. The transcription level of both clock genes and NS control cells were normalized to *Gapdh*[51].

Table1. The list of forward and reverse primers used for qPCR[51].

Genes	***Forward primer***	***Reverse primer***
Per1	TGTGTCAAGCAGGTTCAGG	TGTCCTGGTTTCGAAGTGTG
Per2	TGTTCCGACATGCTTGCG	GAAACAGCTTCCTCTGCTCCAG
Per3	CCCTACGGTTGCTATCTTCAG	CTTTCGTTTGTGCTTCTGCC
Gapdh	GCCTTCCGTGTTCCTACC	CCTCAGTGTAGCCCAAGATG

Data Analysis

The SAS *(9.4 Version)* software package was used for statistical analysis. Data are expressed mean ± STD. T-tests were used to compare the means between groups. Significance is established at $\alpha = 0.05$

Result

Generation of C2C12 reporter cell line

To characterize the cellular circadian clock in muscle tissue, I chose the C2C12 cell line, a subclone of mouse myoblasts with rapid proliferation and good efficiency for *in-vitro* systems in biomedical sciences. I generated a lentiviral reporter carrying the *dLuc* gene, controlled by either the mouse *per2* or *Bmal1* gene promoters. C2C12 cells were cultured in 35 mm dishes, recorded rhythmic expression of bioluminescence rhythm, and these cells exhibited sustained bioluminescence rhythms (Figure 1). while the *per2-dLuc* reporter displayed a less sustained bioluminescence rhythm, the *Bmal1-dLuc* reporters showed a robust bioluminescence rhythm with a period length of approximately 24 hours (23.35h) (Figure 1). Due to the less robust rhythm, and period length of the *Per2-dLuc* reporter, I continued our study using the *Bmal1-dLuc* reporter cell line.

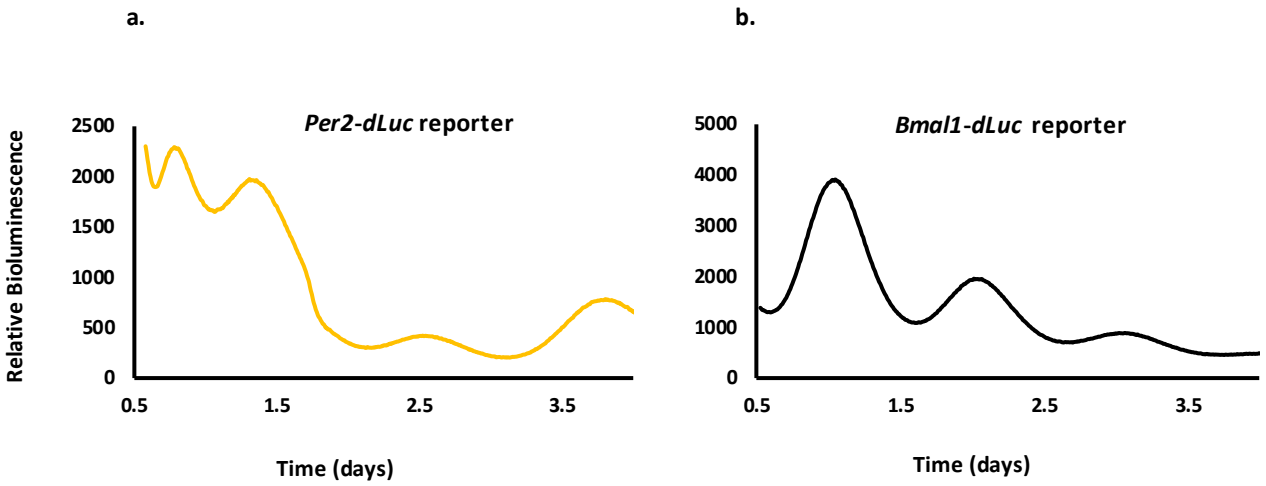
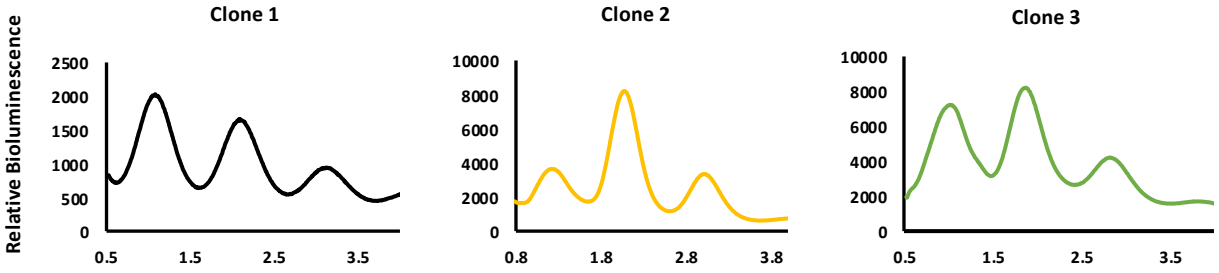


Figure1. C2C12 cell displays a circadian oscillation of bioluminescence rhythm.

Representative reporter cell bioluminescence rhythms were recorded on 35 mm dishes using a LumiCycle luminometer. I used a Lentivirus-mediated shRNA delivery system to generate either Per2-dLuc or Bmal1-dLuc luciferase reporter cells, and then I recorded the circadian rhythm of the infected cell population using a LumiCycle. (a) Per2 dLuc reporter had a less robust rhythm in our cell line (b); conversely, the Bmal1 dLuc reporter had a robust rhythm with a period length close to 24 hours (23.35h).

I established a single cell cloning protocol to obtain a homogeneous C2C12 Bmal1 reporter cell population. Among three clones, I selected clone 1, which displayed the most consistent rhythm with a period length of close to 24 hours, similar to the reporter population cells. Each clone exhibited a robust oscillation of bioluminescence rhythms (Figure 2).

a.



b.

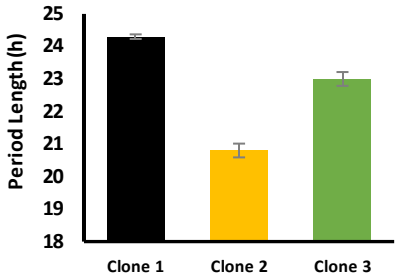


Figure 2. C2C12 single-cell clone exhibits clone-specific bioluminescence rhythms

Representative bioluminescence rhythms of single-cell clones of C2C12 cells recorded on 35mm culture dishes in a *Lumicycle* luminometer (a). Graph (b) depicts the Mean ± SD of the period lengths of each clone.

Genetic manipulation of the reporter cell line using lentiviral shRNA-mediated gene knockdown

In this study, I successfully generated a high-quality lentiviral shRNA system for the targeted knockdown of mouse clock genes. By opting for stable transfection using lentiviral shRNAs instead of transient transfection with siRNAs, I ensured that the genetic manipulation would be passed on to subsequent cell generations *in-vitro*. I designed a panel of *shRNA* constructs targeting ten preselected clock genes, which included core loop activators (*Bmal1*, *Clock*), core loop repressors (*Per1*, *Per2*, *Cry1*, *Cry2*), a core loop post-translational modifier (*Fbxl3*), and *RORE* repressors (*Nr1d1* and *Nr1d2*). NS shRNA was used for control. To assess the lentiviral shRNAs' knockdown (KD) efficiency, I measured the endogenous *mRNA* levels in *Per1*, *Per2*, and *Per3* genes using quantitative polymerase chain reaction (*qPCR*). For each gene, two *shRNAs* were found to be effective in knocking down gene expression. The high efficiency of the lentiviral *shRNAs* allowed us to investigate the phenotypic consequences of knocking down individual clock genes in our cell line.

Phenotypes of RRE promoter element genes (Bmal1, Clock, and Fbxl3)

Our findings demonstrated that knocking down *Bmal1* in the C2C12 reporter cell line resulted in a phenotype consistent with previous studies in mouse or human cell lines. As shown in Figure 3, Compared with the non-specific control group (NS), *Bmal1* knockdown (*KD*) displayed arrhythmic bioluminescence rhythm, confirming the crucial role of *Bmal1* in maintaining rhythmicity. Interestingly, the *Clock KD* phenotype in C2C12 cells exhibited a different pattern from what has been previously reported in other cellular models. In our study, *Clock KD* led to a shorter period length and reduced amplitude in circadian oscillations compared to control cells (*NS*). In contrast to *Clock KD*, *Fbxl3 KD* resulted in a no period length change to wild-type (*WT*) cells but exhibited a lower amplitude. This observation implies that *Fbxl3* may not significantly impact the period length of circadian rhythms, but it does contribute to regulating clock amplitude.

In summary, our results confirm the importance of *Bmal1* in maintaining circadian rhythmicity, which is a similar observation with other cell lines. Clock and Fbxl3 KD phenotypes in the C2C12 cell line differ from those observed in different cell lines. These observations warrant further investigation into the roles of *Clock* and *Fbxl3* in circadian regulation and the potential mechanisms underlying the observed phenotypic differences.

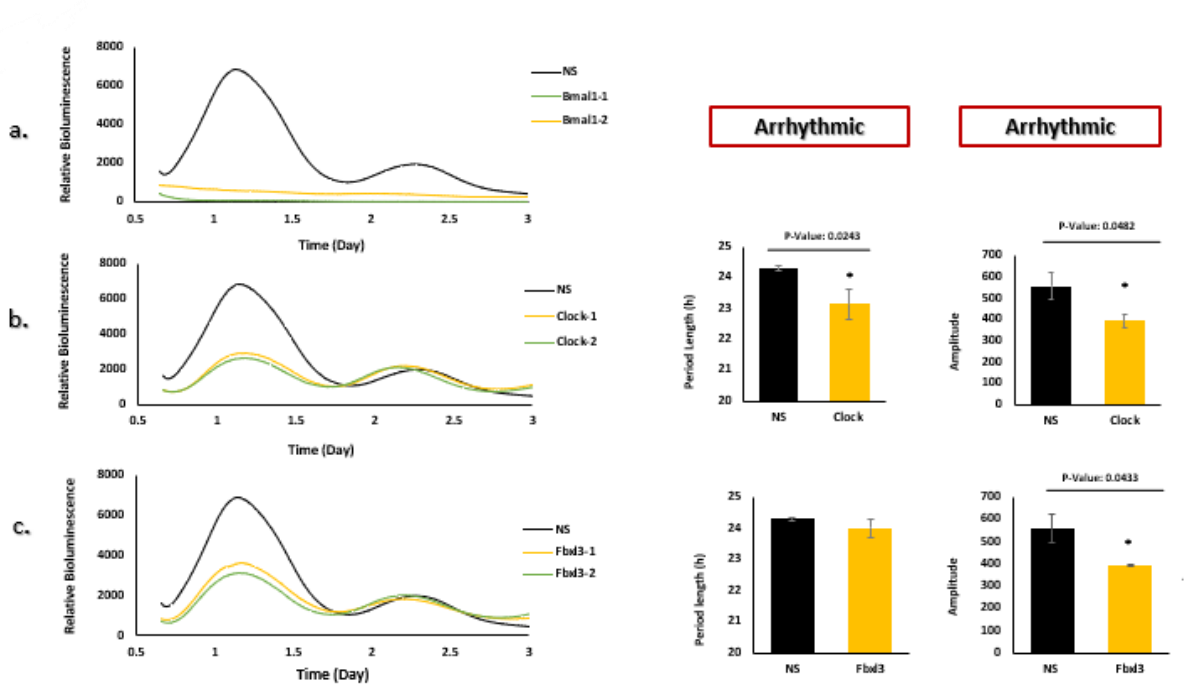


Figure 3. Phenotypes of shRNA-mediated KD of Bmal1, Clock, and Fbxl3

In the C2C12 cell line, KD of Bmal1 exhibits arrhythmicity (a), KD of Clock showed a short period length (P-value=0.0243) and lower amplitude (P-value=0.0482) (b), and KD of Fbxl3 displayed showed no period change and lower amplitude (c) (P-value=0.0433).

Phenotypes of E-box and D-box promoter genes (Per1, Per2, Per3)

Our study aimed to investigate the impact of knocking down *Per1*, *Per2*, and *Per3* genes on the circadian clock in *C2C12* cells. In *Per1* knockdown (*KD*) cells, I found a significant decrease in the period length, indicating that the absence of *Per1* led to a shorter circadian period in *C2C12* cells (Figure 4a). This suggests that *Per1* plays a crucial role in determining the circadian clock's period. Knocking down *Per2* resulted in a shorter period, similar to *Per1 KD* cells. However, *Per2 KD* cells also exhibited a significant increase in amplitude, indicating that the absence of *Per2* affects the period length and leads to a more robust oscillation in the circadian clock

(Figure 4b). Knocking down *Per3* produced different effects on the circadian clock than *Per1* and *Per2*. While *Per3 KD* cells also displayed a significant increase in amplitude, there was no notable difference in period length (Figure 4c). This implies that *Per3* may primarily contribute to the regulation of clock amplitude without significantly influencing the period length.

In summary, our results demonstrate that knocking down *Per1*, *Per2*, and *Per3* genes leads to distinct period length and amplitude alterations in C2C12 cells. These findings highlight the individual roles of these genes in modulating the circadian clock and provide valuable insights into the complex regulatory mechanisms governing circadian rhythms in mammalian cells. In this study, I aimed to assess the knockdown efficiency of *per1*, *per2*, and *per3* genes in our cell line. The relative expression levels of these genes were evaluated using quantitative real-time *PCR* (*qRT-PCR*) in both knockdown (*KD*) and control groups. Our findings demonstrated a significant reduction in the relative expression levels of *per1*, per2, and *per3* genes in the *Per1*, *Per2*, and *Per3 KD* cells as compared to the control group *(P-value < 0.05)* (Figure 4 a, b & c inset). Our results demonstrate the high knockdown efficiency of the *per1*, *per2*, and *per3* genes in the respective *KD* cells, providing a solid foundation for further functional and mechanistic studies on the role of these genes in regulating cellular and physiological processes.

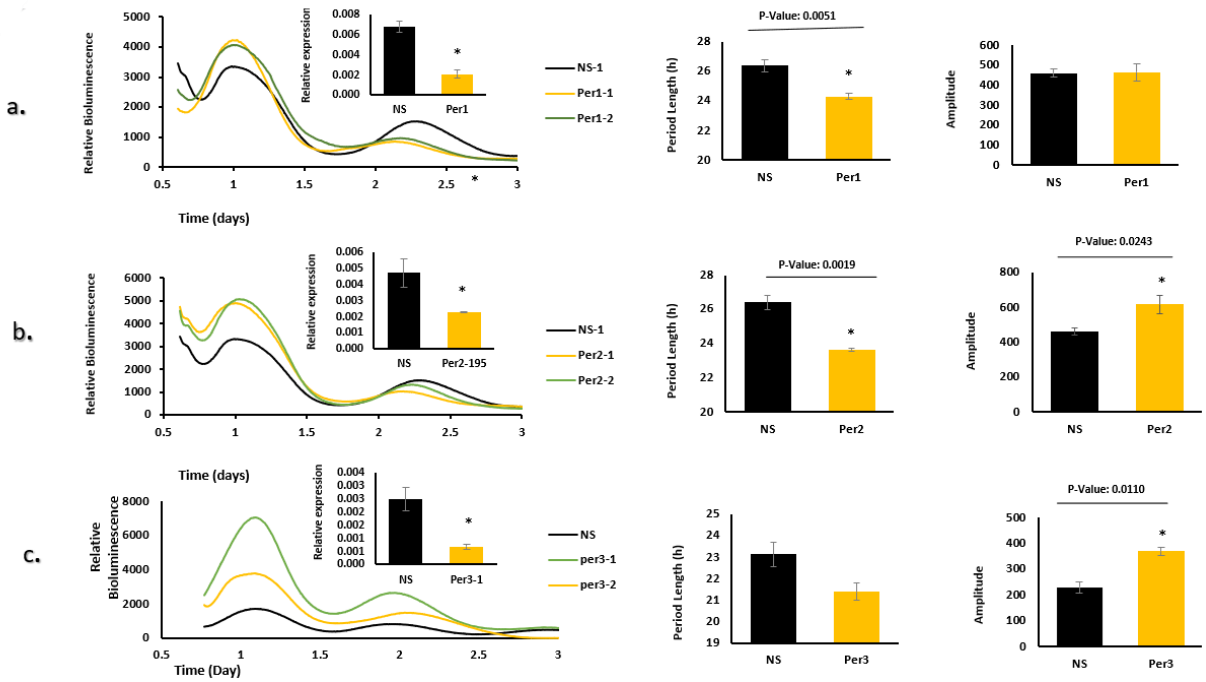


Figure 4. Phenotypes of shRNA-mediated KD of *Per1*, *Per2*, and *Per3*

qPCR results showed a significantly lower level of relative expression of the Per1 gene in Per1 KD cells compared to the control group (a). KD of Per1 caused short period length (P-value=0.005). KD of *per2* showed a shorter period length (*P-value*=0.0019) and higher amplitude (*P-value*=0.0243). qPCR result showed an excellent knockdown efficiency of the *Per2* gene (b). KD of *Per3* caused higher amplitude (*P-value*=0.0110). qPCR results showed a significantly lower level of relative expression of the *Per3* gene in *Per3* KD cells compared to the control group (c).

Phenotypes of E-box promoter genes (Nr1d1 and Nr1d2)

I utilized C2C12 cellular clock models to investigate the impact of *Nr1d1* or *Nr1d2* knockdown (*KD*) on clock function. *Nr1d1* knockdown leads to no period length change and reduced amplitude. This indicates that *Nr1d1* is mainly involved in regulating the amplitude of the cellular clock. *Nr1d2* knockdown results in reduced amplitude and shorter period length in C2C12 cells. When *Nr1d2* was knocked down in our cellular clock model, I observed a lower amplitude and shorter period length. This finding suggests that *Nr1d2* plays a crucial role in

maintaining proper clock function and regulating the amplitude and periodicity of the cellular clock. The differential effects observed upon knockdown of either gene imply that *Nr1d2* may compensate for the function of *Nr1d1* in the clock machinery when *Nr1d1* is knocked down. This compensation mechanism highlights the importance of both *Nr1d1* and *Nr1d2* in maintaining proper clock function.

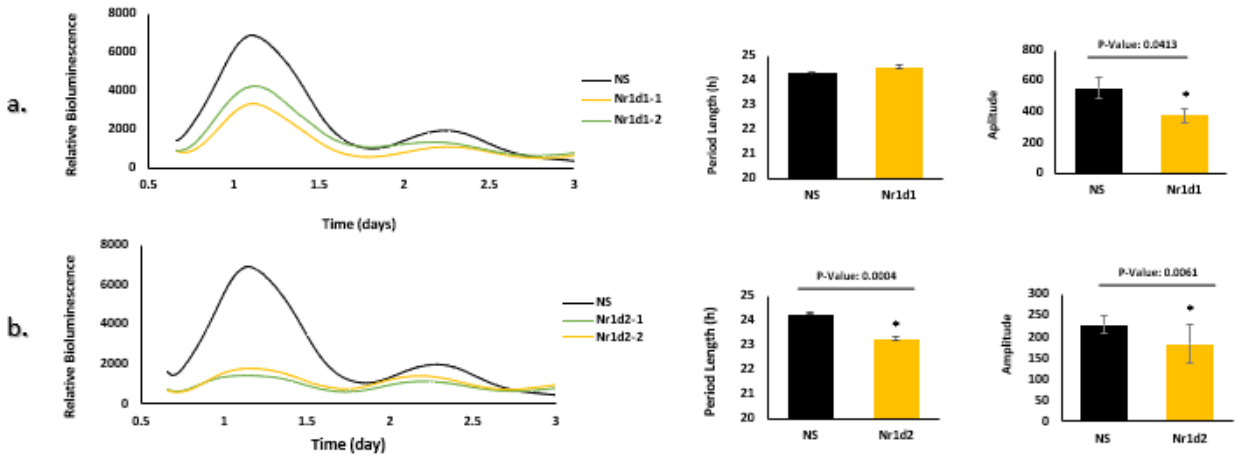


Figure 5. shRNA mediated KD of *Nr1d1*, and *Nr1d2*

KD of *Nr1d1* caused no significant difference n period length, but it caused lower amplitude (*P-value*=0.0413) (a). KD of *Nr1d2* caused a shorter period length (*P-value*=0.0004) and lower amplitude. (*P-value*=0.0061) (b).

E-box and RRE promoter genes (Cry1 and Cry2)

The knockdown of *Cry1* showed no significant impact on the period length of the circadian rhythm, as the experimental group exhibited a similar period length to the control group.

However, a noticeable reduction in the amplitude of the rhythm was observed when *Cry1* knockdown group compared to the control ($p < 0.05$). This finding suggests that *Cry1* might be more involved in modulating the amplitude of the circadian rhythm rather than influencing its period length.

In contrast to *Cry1*, the knockdown of *Cry2* led to a significant increase in the amplitude of the circadian rhythm when compared to the control group. Additionally, a shorter period length was observed in the *Cry2* knockdown group, indicating a critical role for *Cry2* in determining the period length of the circadian rhythm. In summary, our data demonstrate that knocking down *Cry1* predominantly affects the amplitude of the circadian rhythm without altering its period length. On the other hand, *Cry2* knockdown results in increased amplitude and a shortened period length, highlighting the distinct roles of these two cryptochromes genes in regulating circadian rhythms.

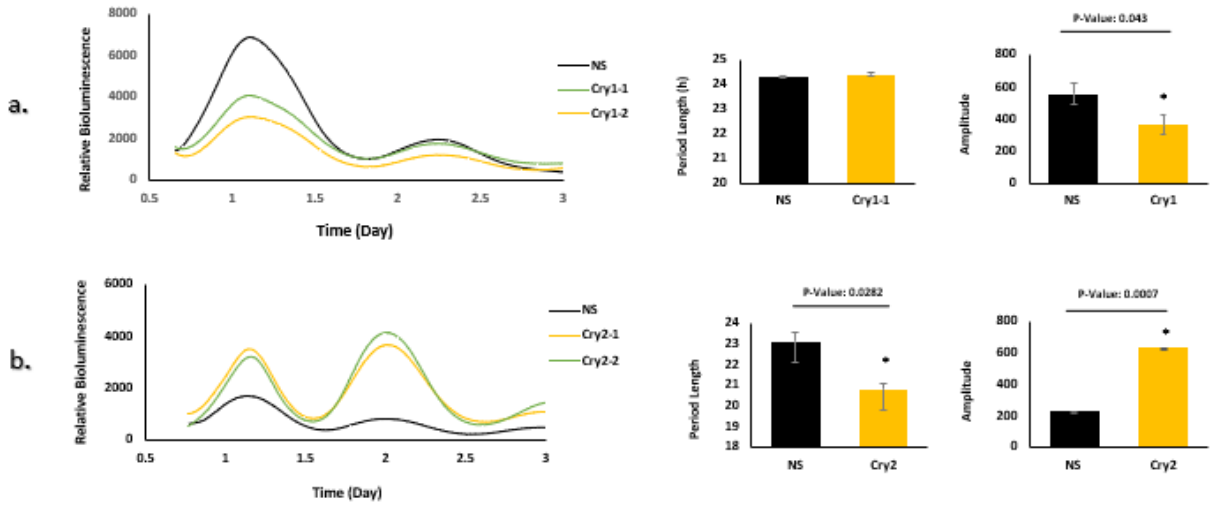


Figure 6. Phenotypes of shRNA-mediated KD of *Cry1* and *Cry2*

KD of *Cry1* caused lower amplitude (*P-value*=0.043) with no significant effect on period length (a). KD of *Cry2* showed a shorter period length (*P-value*=0.0282) and higher amplitude (*P-value*=0.0007) (b).

Discussion

In vitro, cell-autonomous clock studies will help uncover tissue-ubiquitous and tissue-specific properties of circadian clocks. Cell-based models are amenable to genetic and pharmacologic perturbations [58]. Since only few circadian studies have been done in vivo muscle tissue and animal model to study clock and muscle physiology, limited information is available about the clock mechanisms in mouse in vitro cell lines. Many areas of research, specifically metabolic research, use the mouse myocyte C2C12 cell line for mechanistic studies, However, limited information is available on circadian research [59]. In this project, I developed an in vitro cellular clock model using the commercially available stable mouse *C2C12* cell line. This reporter cell line robustly oscillates the bioluminescence rhythm. To check whether the cell line amenable to genetic manipulation, I knock down ten known clock genes and determined the individual gene contribution to the clock mechanism in *C2C12* cells. Our results rejected our hypothesis and confirmed that despite some similarities, some phenotypes significantly differ from previously established in vitro clock models, such as *MMH-D3* hepatocytes and mouse *3T3* fibroblasts.[51]

Bmal1 is the central and primary regulator of clock machinery [60]; knockdown of Bmal1 had the arrhythmic phenotype in our model, similar to other cellular circadian models (*MMH-D3* hepatocytes and mouse *3T3* fibroblasts [51 61 62 63]. The Clock gene is another primary regulator of this system. Our results demonstrate that *CLOCK* knockdown led to significant alterations in the amplitude and period length of the circadian rhythm in the *C2C12* cell line, supporting the critical role of *CLOCK* in regulating cellular rhythmicity. Upon knockdown of *CLOCK*, I observed low amplitude and short period length in the circadian rhythm. This finding is

consistent with other recent studies that have highlighted *CLOCK's* essential role in maintaining circadian oscillations' amplitude and period length [51]. The reduced amplitude and shortened period length suggest that the absence of functional *CLOCK* protein disrupts the normal functioning of the circadian clock machinery, leading to alterations in the molecular feedback loops that generate alter rhythmic patterns. *Fbxl3* KD caused the circadian rhythm compared to the control group. However, in other cellular models, knockdown in *FBxl3* showed different phenotypes in cellular and animal models [51 64.]

Knockdown of the Per1 in our clock model caused a short period length; however, other models showed different tissue-specific phenotypes. Knockdown of Per2 caused short period length and high amplitude, a unique phenotype among all circadian models. Per3 caused higher amplitude, another unique phenotype compared to all other established cell lines [51 20]. Recent studies showed that the Per3 gene has a strong expression level in peripheral tissues in mice, including muscle, liver, lung, brain and heart, kidneys, and testis [65 66 67]. A study has shown that the Per3 expression level is associated with anxiety and other neurological phenotypes. These suggest that Per3 plays a pivotal role in peripheral organ regulation of clock function and local physiological processes. [65 66].

KD of *Cry1* and *Cry2* phenotypes are very different in the *C2C12* cell line compared with *MMH-D3* hepatocytes and mouse *3T3* fibroblasts. In *MMH-D3* hepatocytes and mouse *3T3* fibroblasts, *KD* of *Cry1* resulted in a short period and *KD* of *Cry2* long period and higher amplitude. In *C2C12* cells, *KD* of *Cry1* had no period change, and *KD* of *Cry2* had a short period and higher amplitude. The contribution of *Cry1* and *Cry2* on clock mechanisms is interesting in c2C12 cells

and different from other cell types. A much more detailed study needs to tease out the actual mechanisms behind the contribution of each clock gene to clock mechanisms in the C2C12 cell line.

Knockdown of *Nr1d1* resulted in lower amplitude in our model, which is different from other studies; for example, Nr*1d1* KD and *KO* in cellular and animal models showed normal circadian rhythm and behavior [51] [20] [68]. However, *Nr1d2 KD* in our model made a rhythm with a lower amplitude similar to the phenotype in *3T3* Fibroblast. Recent studies showed that *Nr1d2* is a tissue and sex-specific gene which plays an essential role in clock machinery and tissue aging [51] [20] [67]. Many studies support these two genes overlapping clock functions in most cell lines. [69].

Table 2 Summary of KD phenotypes and comparison to other established models This table summarize our circadian clock model in C2C12 cell line compared to other tissue specific cellular models established by *Ch. Ramanathan, et al, 2014* and *J E. Baggs. et al, 2009.* [51] [20]

Gene KD	C2C12*	3T3 Fibroblasts	3T3-L1 Adipocytes	MMH-D3 Hepatocytes	U2OS Osteosarcoma cells
Bmal1	AR	AR	RD	AR	AR
CLOCK	Short, LA	AR	RD	AR	AR, LA
Per1	Short	WT	WT	Short, LA	AR, LA
Per2	Short	Short	WT	Short, LA	Long, LA
Per3	HA, Short	Short	Short	Short	Short
Cry1	WT	RD	RD	LA	Short
Cry2	HA, Short	Long	Long	Long, HA	Long
Nr1d1	WT	WT	WT	WT	Long
Nr1d2	LA	LA	Short	Short	WT
Fbxl3	WT	Long, LA	Long	RD, LA	Long

Limitations

Despite the novelty of this project, it has some limitations as well. I observed the phenotypes

from only one shRNA, and I need to confirm whether similar phenotypes were observed a second shRNA. Knockdown efficiency is not verified for most of the shRNA, and I need to determine the KD efficiency for both shRNs before concluding the actual phenotype for each clock gene.

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