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**Original Article**

# MiR-29a/b/c regulate human circadian gene *hPER1* expression by targeting its 3′UTR

Xiyan Zhao[1,2†], Xueqiang Zhu[3†], Shuting Cheng[1], Yizhou Xie[1], Zhengrong Wang[1], Yanyou Liu[1], Zhou Jiang[1], Jing Xiao[1], Huiling Guo[1], and Yuhui Wang[1*]

[1]Health Ministry Key Laboratory of Chronobiology, Pre-clinic and Forensic Medical School, Sichuan University, Chengdu 610041, China
[2]Laboratory of Human Function, Hainan Medical College, Haikou 571199, China
[3]Department of oncology, Sichuan Provincial People's Hospital, Chengdu 610072, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel/Fax: +86-28-85501282; E-mail: wangyuhui1972@163.com

**Several essential biological progresses in mammals are regulated by circadian rhythms. Though the molecular mechanisms of oscillating these circadian rhythms have been uncovered, the specific functions of the circadian genes are not very clear. It has been reported that knocking down circadian genes by microRNA is a useful strategy to explore the function of the circadian rhythms. In this study, through a forward bioinformatics screening approach, we identified miR-29a/b/c as potent inhibitors for the human circadian gene *hPER1*. We further found that miR-29a/b/c could directly target *hPER1* 3′untranslated region (UTR) and down-regulate *hPER1* at both mRNA and protein expression levels in human A549 cells. Thus, our findings suggested that the expression of *hPER1* is regulated by miR-29a/b/c, which may also provide a new clue for the function of *hPER1*.**

*Keywords* circadian rhythms; miR-29a/b/c; *hPER1*; 3′UTR

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## Introduction

Circadian clock orchestrates the intrinsic period of ~24 h in mammals [1]. The suprachiasmatic nuclei of the hypothalamus functions as the key circadian clock drives endogenous rhythms, which is regulated by transcriptional and post-transcriptional factors leading to molecular oscillation [2]. The circadian circuit is composed of the negative and positive loops which form the basic construction of molecular circadian oscillation [3,4]. In the positive feedback process, circadian locomotor output cycles kaput/aryl hydrocarbon receptor nuclear translocator-like heterodimer (also known as CLOCK/BMAL1) plays a significant role in circadian oscillation by binding to E-box in circadian genes to activate the transcription of cryptochromes (*CRYs*) and period (*PERs*) [4,5]. In the negative feedback, the phosphorylated CRYs and PERs enter the nucleolus to suppress the transcriptional activity of CLOCK/BMAL1 heterodimer.

*PERs* genes, which were first found in *Drosophila,* play important roles in circadian clock. The mutation of *PERs* genes can alter the circadian rhythmic period of *Drosophila*, so they were named as *period*. Human period genes (*hPERs*) were cloned by a polymerase chain reaction (PCR) method. The analysis of a human genomic draft has identified three *hPERs*, named *hPER1*, *hPER2*, and *hPER3. hPER1* gene is not only involved in the regulation of the physiological rhythm, but also related to the regulation of cell cycle, DNA damage repairing, and tumor progress [6].

MicroRNAs (MiRNAs) are small, non-coding RNAs endogenously expressed with 19–25 nucleotides in length. By binding to the complementary sites in their target genes, miRNAs post-transcriptionally modulate the target gene expression [7]. MiRNAs have been shown to be involved in many cellular processes, including the control of the circadian clock genes by binding to their 3′untranslated region (UTR) [8]. The role of miRNAs in the regulation of circadian clock has been well investigated. The miR-206 was demonstrated to regulate the circadian clock in the skeletal muscle. The miR-192/194 cluster was identified as a powerful negative regulator of the *PERs* family. The over-expression of *PERs* results in an alteration of circadian rhythm. Recently, a report revealed that miR-494 and miR-142-3p can target the 3′UTR of *BMAL1* in mouse. Therefore, the accumulating data suggested that miRNAs might act as the potent regulators of the circadian clock.

In this study, we explored the function of miRNAs in the regulation of *hPER1*. By using a forward bioinformatic algorithm, miR-29a, miR-29b, and miR-29c were identified as potent regulators toward *hPER1*. It was also found that their expressions significantly down-regulated the expression of hPER1 at both mRNA and protein levels in human A549 cells.

## Materials and Methods

### Cell culture and transfection

Human non-small-cell lung carcinoma-derived cell line (A549), provided by Sichuan University (Chengdu, China), were cultured in Dulbecco's modified Eagle's medium high-glucose (Sigma-Aldrich, St Louis, USA) supplemented with 10% (v/v) fetal bovine serum under standard conditions at 37°C in the humidified atmosphere containing 5% $CO_2$. Mimics of miR-29a/b/c or negative control (NC, Ruibo Bio, Changzhou, China) were transfected into A549 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, USA), which were marked as 29a, 29b, 29c, and NC, respectively. The blank control group was marked as blank.

### Luciferase assay

The human 3′UTR of the *hPER1* gene was amplified by PCR and cloned into the pmiR-RB-REPORT™ (PMIR) vector (Ruibo Bio) between the restriction enzyme cutting sites of *Xho*I and *Not*I to construct pmiR-RB-REPORT™-3′UTR (PMIR-3′UTR) dual-luciferase reporter gene system. The A549 cells were cultured in 96-well plates and were transfected using Lipofectamine 2000 with 200 ng of either PMIR-3′UTR or PMIR control vector and 50 pmol of miR-29a/b/c or NCs. At 24 h after transfection, the firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega, Madison, USA). The experiment was repeated at least three times.

### MiRNA and *hPER1* mRNA reverse transcription

RNA was isolated from the cells of the five different groups using TRIZOL reagent (Invitrogen). One-step reverse-transcription (RT) PCR was carried out. Each sample including 10 ng of total RNA was reverse transcribed to complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA). The cDNA was applied for both miRNA and *hPER1* gene quantification. Five different groups of synthesized cDNA were diluted to 1 : 10 and stored at −20°C prior to quantitative real-time PCR (qPCR).

### qPCR analysis

qPCR was performed in a CFX48 Real-Time PCR Detection System (Bio-Rad, Hercules, USA), to measure the miRNA expression and *hPER1* mRNA expression. *U6* was served as the endogenous reference for miRNA expression. All reactions were performed in triplicate with a calibrator control derived from a pool of all cDNA samples and a non-template control. PCR amplification efficiencies (85%–100%) for each primer pair were calculated using a 10-log serial dilution of the calibrator sample and efficiency correction was applied to the data during analysis. For quantification of *hPER1* gene expression, all five different groups of synthesized cDNA were used. To identify miR-29a/b/c is truly linked to the inhibition of *hPER1*, we also quantified the human *CRY1* gene expression in all five different groups as unrelated transcripts control. All primer sets for mRNAs crossed an exon–exon junction to avoid the amplification of genomic DNA. Gene expression changes were quantified using the delta–delta CT method. Data were normalized to determine the relative fold changes by Equation $2^{-\Delta\Delta Ct}$. All primers used are listed in **Table 1**. *GAPDH* were used as controls for mRNA expression.

**Table 1. The primers used in qPCR for measuring the mRNA expression**

| Primer name | Primer sequence |
|---|---|
| *hPER1* | 5′-AAGTTCGTCTTCTGCCGTATC-3′ |
| | 5′-AGGCGGAATGGCTGGTA-3′ |
| *GAPDH* | 5′-AACGACCCCTTCATTGAC-3′ |
| | 5′-TCCACGACATACTCAGCAC-3′ |
| *CRY1* | 5′-GTTCGCCGGCTCTTCCA-3′ |
| | 5′-CAAGATCCTCAAGACACTGAAGCA-3′ |

### Western blot analysis

Protein expression levels were assessed by western blot analysis. In brief, cells were lysed in an ice-cold RIPA lysis buffer, containing 100 μg/ml phenylmethylsulfonyl fluoride (beyotime, China). Anti-hPER1 antibody (H-120) (1 : 1000; Santa Cruz, Santa Cruz, USA) and HRP-labeled goat anti-rabbit IgG antibody (1 : 20000; ZSGB-BIO, Beijing, China) were used to detect hPER1. Anti-β-Actin antibody (1 : 1000; ZSGB-BIO) and HRP-labeled goat anti-rabbit IgG antibody were used to detect β-actin which served as the internal control.

### Statistical analysis

All data were presented as mean ± standard deviation. Statistical analysis was performed with SPSS software (version 11.0, SPSS, Inc., Chicago, USA). The differences between groups were analyzed by one-way analysis of variance. $P < 0.05$ were considered statistically significant (two-tailed).

## Results

### Bioinformatic algorithms prediction

The screening of miRNAs which are targeted *hPER1* was performed using bioinformatics algorithms TargetScan (http://targetscan.org/). *hPER1* was predicted as the putative target of miR-29a/b/c using the bioinformatics algorithms TargetScan (http://targetscan.org/) (**Fig. 1**).

### MiR-29a/b/c targeted *hPER1* 3′UTR

To confirm *hPER1* is the target of miR-29a/b/c, we constructed the *hPER1* 3′UTR-luciferase reporter gene. The results showed that over-expression of miR-29a/b/c significantly inhibited the activity of the *hPER1* 3′UTR-luciferase compared with the NC (**Fig. 2**), which indicated that miR-29a/b/c could directly target *hPER1* 3′UTR.

### MiR-29a/b/c down-regulated *hPER1* expression at mRNA level

To explore the regulatory effect of miR-29a/b/c on *hPER1* gene expression, we examined the expression of *hPER1* and *CRY1* mRNA in miR-29a/b/c over-expression A549 cells by qPCR. The data showed that the expression of *hPER1* mRNA was significantly decreased in the groups of miR-29a, miR-29b, and miR-29c, compared with that in the group of NC. No significant difference was observed in the expression of *CRY1* (**Fig. 3**).

### MiR-29a/b/c inhibited hPER1 protein expression

To investigate the regulatory effect of miR-29a/b/c on hPER1 protein expression, we examined hPER1 protein expression by western blotting in the five groups. The results showed the down-regulation of hPER1 protein expression in

| | predicted consequential pairing of target region (top) and miRNA (bottom) |
|---|---|
| Position 479-485 of PER1 3' UTR | 5' ...GGACCAUGGAGUCCCUGGUGCUG... |
| hsa-miR-29a | 3' AUUGGCUAAAGUCUACCACGAU |
| Position 479-485 of PER1 3' UTR | 5' ...GGACCAUGGAGUCCCUGGUGCUG... |
| hsa-miR-29c | 3' AUUGGCUAAAGUUUACCACGAU |
| Position 479-485 of PER1 3' UTR | 5' ...GGACCAUGGAGUCCCUGGUGCUG... |
| hsa-miR-29b | 3' UUGUGACUAAAGUUUACCACGAU |

**Figure 1.** Putative-binding site of miR-29a/b/c in 3′UTR of *hPER1* as detected by TargetScan6.2.

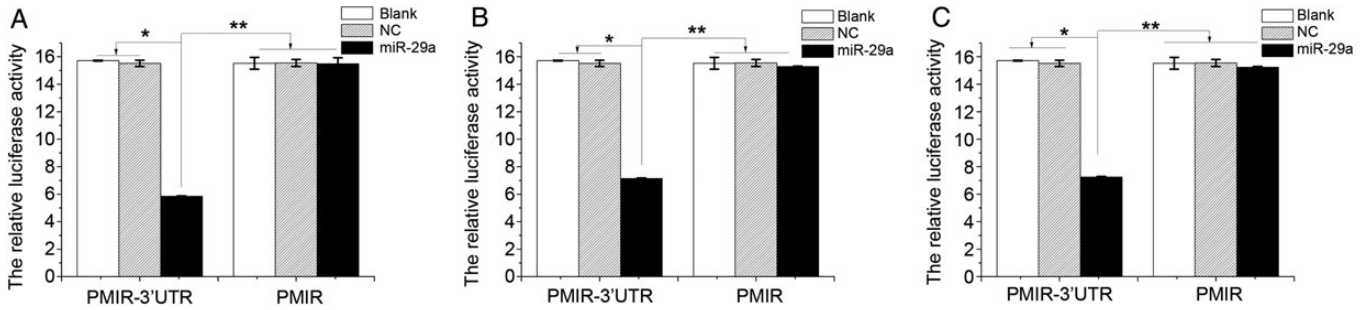


**Figure 2. MiR-29a/b/c targeted *hPER1* 3′UTR** (A)–(C) Relative luciferase activity was measured in A549 cells transfected with PMIR-3′UTR or PMIR vector (blank) and co-transfected with PMIR-3′UTR or PMIR vector and miR-NC mimics (NC) or miR-29a/b/c mimics. Firefly luciferase activity was normalized to Renilla luciferase activity. *$P < 0.05$ vs. cotransfected PMIR-3′UTR and blank or NC group. **$P < 0.05$ vs. cotransfected PMIR vector and blank or NC or miR-29a/b/c group.

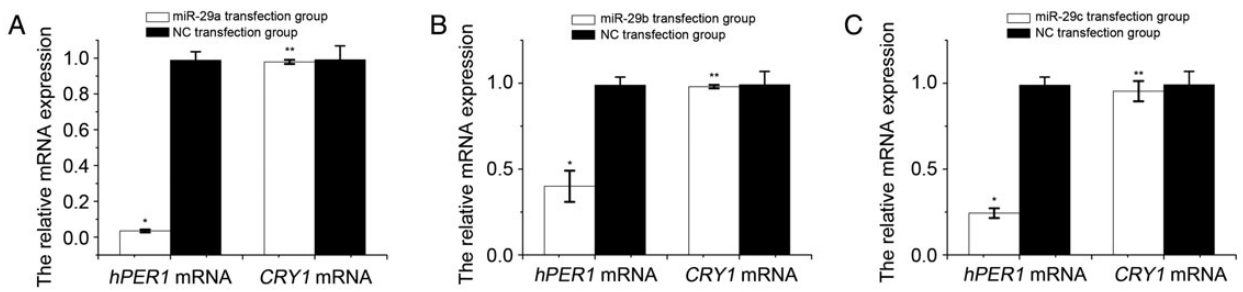


**Figure 3. MiR-29a/b/c down-regulated *hPER1* expression at mRNA level** (A)–(C) The relative expression of *hPER1* and *CRY1* mRNA in miR-29a/b/c mimics and NC-transfected cells quantified by qPCR. *$P < 0.05$, **$P > 0.1$.

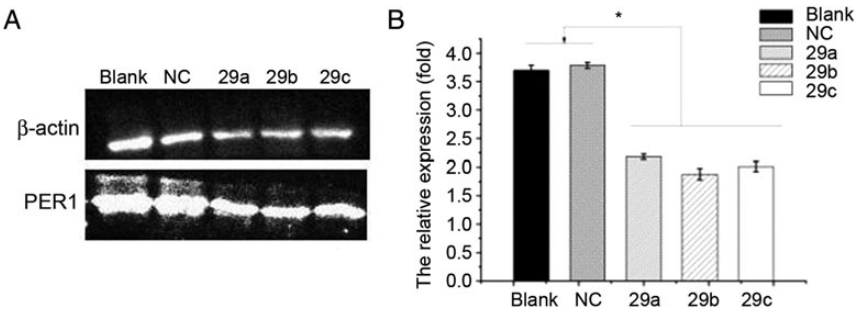


**Figure 4. MiR-29a/b/c down-regulated *hPER1* expression at protein level** Western blot analysis shows the reduction of *hPER1* protein levels in miR-29a (29a), miR-29b (29b), or miR-29c (29c) transfected cells compared with those in the NC and blank control (blank) cells. $*P < 0.05$.

Groups 29a, 29b and 29c compared with that in Group NC and blank, which indicated that *hPER1* may be a target gene of miR-29a/b/c (**Fig. 4**).

## Discussion

Using bioinformatic algorithms, we found that *hPER1* was predicted to be targeted by miR-29a/b/c through binding to its 3′UTR; nevertheless, no studies have been performed to confirm that. Here, in this study, we first showed that miR-29a/b/c can directly target 3′UTR of *hPER1* through construction of a PMIR-*hPER1*-3′UTR dual-luciferase reporter gene system. Furthermore, in order to confirm whether miR-29a/b/c can regulate *hPER1*, the expressions of *hPER1* mRNA as well as the protein were determined, respectively. The results suggested that miR-29a/b/c down-regulated the *hPER1* at both mRNA and protein levels, which uncovered that the core clock proteins were also under post-transcriptional regulation by miRNA.

MiRNAs are small-non-coding RNA molecules that alter gene expression at post-transcriptional level. Recently, it has been reported that miRNAs could regulate the circadian rhythm. MiR-219 and miR-132 have been shown to shorten the circadian period and negatively regulate light-dependent rhythm. Furthermore, miR-219 is a target of the CLOCK/BMAL1 complex. It exhibits robust circadian rhythms of expression, and the *in vivo* knockdown of miR-219 lengthens the circadian period. MiR-132 is induced by photic entrainment cues, modulates *CLOCK* gene expression, and attenuates the entraining effects of light. Collectively, these results suggested that miRNAs provide a mechanistic examination of their roles as effectors of pacemaker activity and entrainment [9].

Indeed, involving miRNAs and circadian gene, a similar regulatory network exists. MiR-182 was reported to be a modulator of *CLOCK* and a feedback loop consisting of miR-182 and *CLOCK* has been proposed. But the specific interaction between the miR-182 and *CLOCK* has not been clarified; therefore, the regulation of *CLOCK* by miRNAs needs to be further studied [10]. It has been reported that miR-142 regulates *BMAL1* both in human and mouse cells. It has also been suggested that miR-142 is transcriptionally regulated by CLOCK/BMAL1 heterodimers only in a mouse cell line (NIH3T3) [11]. Another research suggested that miR-192/194 expression alters the circadian cycle, potentially resulting from the regulation of the *PER* gene family. So far, it cannot be confirmed whether miR-192/194 has other targets besides the *PER* genes that assist in the control of circadian clock [12].

In this study, both mRNA and protein expressions of *hPER1* gene were decreased by over-expression of miR-29a/b/c. *hPER1* is an important component of circadian clock in human beings. Circadian rhythm and molecular oscillation are both affected by the decrease of *hPER1*. Multiple signaling pathways are involved in the modification of circadian molecular oscillation [13]. Further exploration is needed to determine whether *hPER1* has an effect on circadian rhythm, circadian molecular, and interactivity with other circadian genes.

*hPER1* is also an important gene related to human health. Reduction of *hPER1* is correlated with many diseases including cortisol awakening response [14], psychosocial stress-induced alcohol drinking [15], chronic myeloid leukemia [16], and especially in cancer [17]. Furthermore, disorder of circadian rhythm may lead to disruption in sleep [18], metabolism [19], cardiovascular [20], endocrine [21], reproduction [22], cancer [23], and some biological progress [24]. Here, we indicated that miR-29a/b/c could be a potent inhibitor of human circadian gene *hPER1*. Further studies will focus on whether miR-29a/b/c can act on some human diseases through regulating *hPER1*. The detailed mechanism by which this miRNA is capable of affecting the circadian period still remains to be explored.

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