

MicroRNA-185 oscillation controls circadian amplitude of mouse *Cryptochrome 1* via translational regulation

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ABSTRACT Mammalian circadian rhythm is observed not only at the suprachiasmatic nucleus, a master pacemaker, but also throughout the peripheral tissues. Investigation of the regulation of clock gene expression has mainly focused on transcriptional and posttranslational modifications, and little is known about the posttranscriptional regulation of these genes. In the present study, we investigate the role of microRNAs (miRNAs) in the posttranscriptional regulation of the 3'-untranslated region (UTR) of the mouse *Cryptochrome 1* (*mCry1*) gene. Knockdown of *Drosha*, *Dicer*, or *Argonaute2* increased *mCry1*-3'UTR reporter activity. The presence of the miRNA recognition element of *mCry1* that is important for miR-185 binding decreased *mCRY1* protein, but not mRNA, level. Cytoplasmic miR-185 levels were nearly antiphase to *mCRY1* protein levels. Furthermore, miR-185 knockdown elevated the amplitude of *mCRY1* protein oscillation. Our results suggest that miR-185 plays a role in the fine-tuned regulation of *mCRY1* protein expression by controlling the rhythmicity of *mCry1* mRNA translation.

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INTRODUCTION

The mammalian molecular circadian clock system is composed of feedback loops and transcriptional, translational, and posttranslational regulatory steps (Lowrey and Takahashi, 2004). The core molecular clock is established by a positive limb, composed of heterodimers of the transcription factors CLOCK and BMAL, which drives the rhythmic expression of the negative limb proteins Period (PER1–3) and Cryptochrome (CRY1/2; Dibner *et al.*, 2010). Accumulation of PER and CRY proteins results in their translocation to the nucleus, where they inhibit BMAL/CLOCK

transcriptional activity, thereby repressing their own expression and initiating a new cycle. The *Cryptochrome1* (*Cry1*) gene, a true circadian oscillator, maintains its rhythmicity in the absence of environmental cues. Mouse *Cry1* (*mCry1*) transcripts persist when mice are kept in a “free-running” state, such as constant darkness (Liu *et al.*, 1999). This has also been observed for other clock genes. Recently posttranscriptional regulation has been recognized as being important for the fine-tuning of the circadian system (Kim *et al.*, 2007; Kojima *et al.*, 2007; Woo *et al.*, 2009; Lee *et al.*, 2012b).

MicroRNAs (miRNAs) act as regulators in fine-tuning gene expression. They comprise an abundant group of endogenous, small, noncoding RNAs and predominantly act at the mRNA level to regulate the expression of protein-coding genes through translational repression and/or degradation of their target mRNAs (Bartel, 2004; Guo *et al.*, 2010). The short (20–22 nucleotides [nt]) miRNAs bind to Argonaute (AGO) proteins and guide the AGO-associated RNA-induced silencing complex (RISC) to mRNA targets (Hutvagner and Simard, 2008). AGO2 then mediates the miRNA-dependent interaction of the RISC complex with the

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Abbreviations used: AGO, Argonaute; Cry, Cryptochrome; miRNA, microRNA; Per, Period; RISC, RNA-induced silencing complex; UTR, untranslated region.

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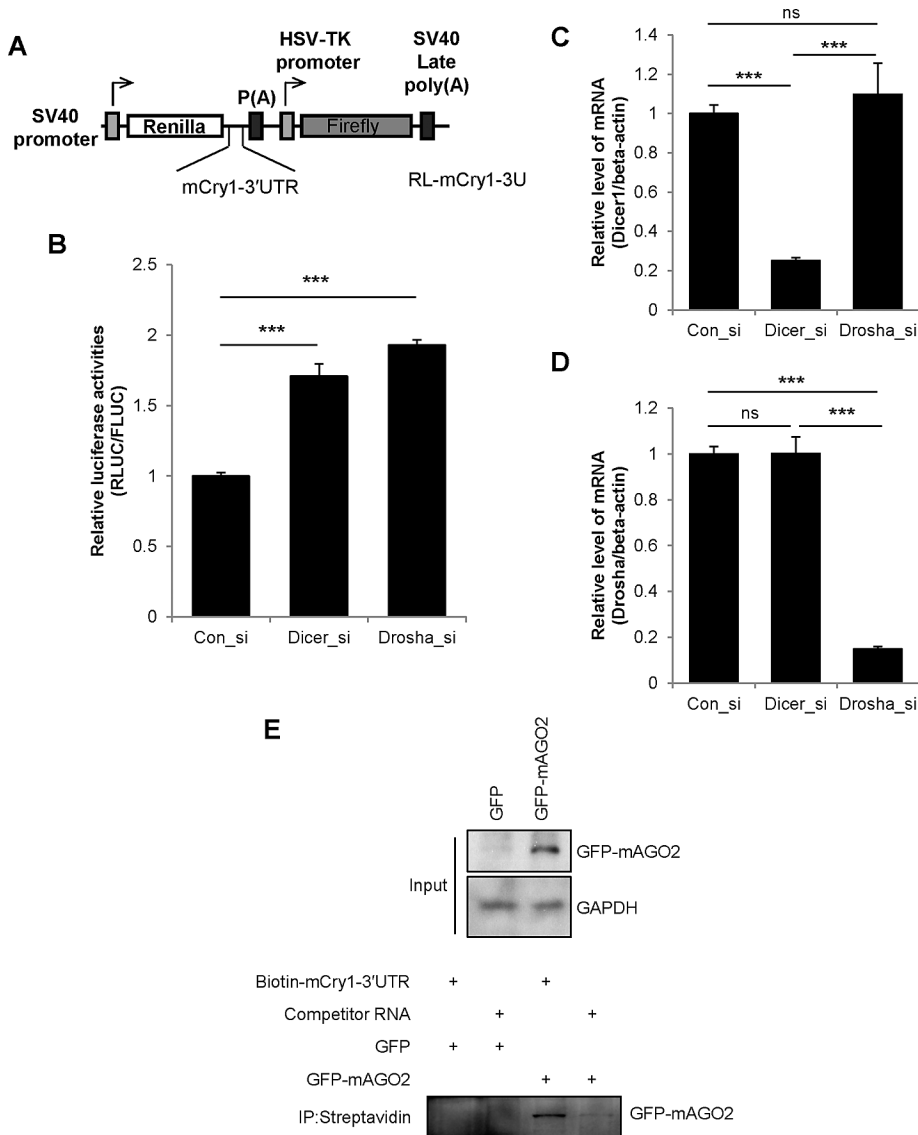


FIGURE 1: *Cry1* expression is regulated by the miRNA machinery. (A) Schematic diagram of the reporter plasmid containing the full-length 3'UTR of *mCry1*. Full-length *mCry1*-3'UTR was fused to the *Renilla* luciferase reporter gene. Firefly luciferase was used as a transfection control. (B) Microportation was used to cotransfect NIH 3T3 cells with the *Renilla* luciferase *mCry1*-3'UTR reporter and siRNAs specific for *Dicer1* (*Dicer_si*) or *Drosha* (*Drosha_si*). Nonspecific siRNA (*Con_si*) was used as a control. After 24 h, cells were harvested and dual-luciferase assays were performed using firefly luciferase as a transfection control. The activity of *Con_si* was set to 1 ($n = 4$; $***p < 0.0001$). The relative mRNA levels of (C) *Dicer1* and (D) *Drosha* were quantified by real-time PCR and normalized to *mActb* ($n = 4$; $***p < 0.0001$). (E) The in vitro-transcribed *mCry1*-3'UTR construct was labeled with biotin-UTP and incubated with cytoplasmic extracts of GFP-mAGO2-overexpressing NIH 3T3 cells. Streptavidin affinity-purified samples were separated by SDS-PAGE and subjected to immunoblotting with anti-GFP or anti-GAPDH antibodies.

target mRNA (Meister et al., 2004). AGO2, the catalytic center of RISC, induces the endonucleolytic cleavage of perfect or nearly perfect base pairings of miRNA and target mRNA or 11 or 12 contiguous Watson-Crick pairs to the center of the miRNA (Meister et al., 2004; Shin et al., 2010). In contrast, imperfect miRNA-mRNA interactions generally cause translational repression or mRNA destabilization (Bushati and Cohen, 2007; Filipowicz et al., 2008). Bioinformatic analyses suggest that each miRNA is capable of controlling hundreds of target genes in humans,

and it was reported that >60% of protein-coding genes are under selective pressure to maintain pairing to miRNAs (Friedman et al., 2009). This indicates that miRNAs have a great potential to regulate the majority of protein-coding genes. Thus the identification of miRNA targets is necessary to understand their mechanisms of action in various physiological responses. However, relatively few miRNA targets have been experimentally validated, even though many miRNAs have been identified. Here we report that miR-185 regulates *CRY1* protein expression by controlling the rhythmicity of *Cry1* translation.

RESULTS

CRY1 expression is regulated by the miRNA machinery

Although miRNA binding is not restricted to the 3' untranslated region (UTR) of mRNA (Chi et al., 2009; Hafner et al., 2010), 3'UTR is the most preferential target of miRNAs. Therefore a *Renilla* luciferase reporter that was fused to *mCry1*-3'UTR was used to investigate the role of miRNAs in the regulation of *Cry1* expression (Figure 1A). A small interfering RNA (siRNA) knockdown approach was used to down-regulate the expression of AGO2, *Dicer*, and *Drosha*, key proteins in miRNA genesis and function. Downregulation of *Dicer* or *Drosha* mRNA stabilized *mCry1*-3'UTR reporter activity (Figure 1B). siRNA-mediated knockdown of *Dicer* and *Drosha* mRNA was confirmed by real-time PCR (Figure 1, C and D). Furthermore, siRNA-mediated reduction of AGO2 expression increased the luciferase activity of the *mCry1*-3'UTR reporter (Supplemental Figure S1, A and B). These results suggested that the miRNA machinery regulates *mCry1*. However, it is possible that miRNAs may mediate this effect indirectly. miRNAs regulate diverse biological functions; therefore alterations in other miRNA functions may have indirectly contributed to the stabilization of *mCry1*-3'UTR reporter activity. To obtain more direct evidence of the regulatory role of miRNAs on *mCry1* expression, we determined AGO2 protein binding with *mCry1*-3'UTR by RNA affinity precipitation. Overexpressed green fluorescent protein (GFP)-AGO2 was coprecipitated with biotin-labeled *mCry1*-3'UTR mRNA (Figure 1E). In contrast, 3'UTR-bound AGO2 was dramatically decreased by nonlabeled *mCry1*-3'UTR. Overexpressed GFP did not coprecipitate with the biotin-labeled *mCry1*-3'UTR. This finding suggested that AGO2 might specifically interact with the 3'UTR of *mCry1*. Collectively these results indicated that miRNAs might play a role in the regulation of *mCry1* expression.

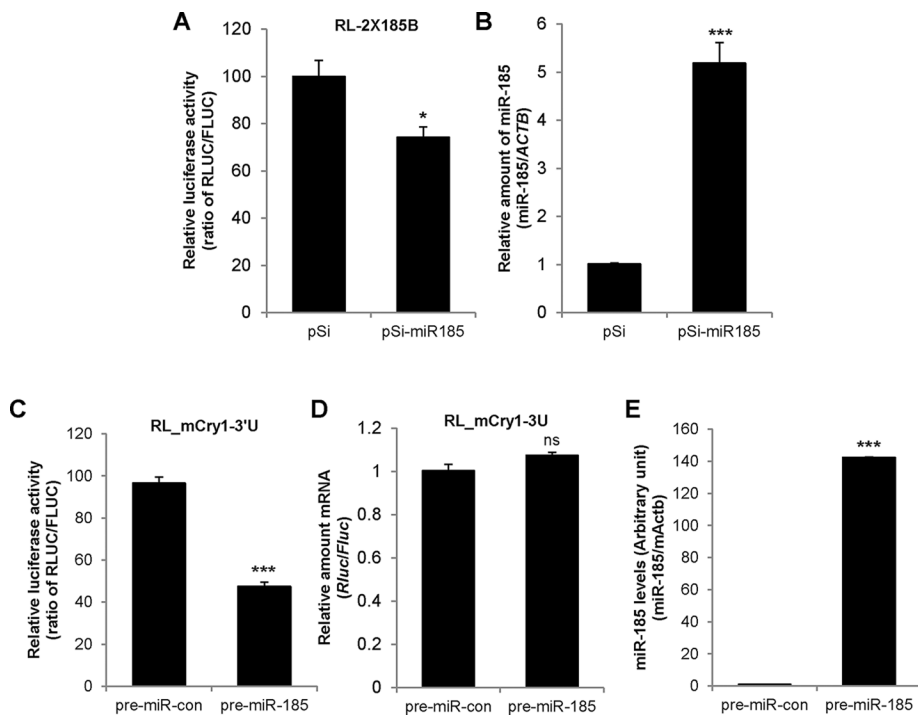


FIGURE 3: miR-185 overexpression represses translation. (A) NIH 3T3 cells were cotransfected with RL-2×185B plasmids and control pSi or miR-185-expressing plasmids (pSi-miR185). After 24 h, dual-luciferase assays were performed. The RLUC/FLUC ratio of pSi was set to 100 ($n = 4$; $*p < 0.05$, $p = 0.0189$). (B) Real-time PCR was used to quantify miR-185 mRNA levels in NIH 3T3 cells cotransfected with RL-2×185B plasmids and pSi or pSi-miR185 plasmids. miR-185 mRNA levels were normalized to *mActb* mRNA levels. Relative miR-185 levels of pSi were set to 1 ($***p < 0.0001$; unpaired *t* test). (C) NIH 3T3 cells were cotransfected with RL-mCry1-3'U (full-length *mCry1*-3'UTR) and pre-miR-con or pre-miR-185 plasmids. After 24 h, luciferase assays were performed ($n = 4$; $***p < 0.0001$). (D) Real-time PCR was used to determine the *Rluc* mRNA levels in NIH 3T3 cells cotransfected with RL-mCry1-3U (full-length *mCry1*-3'UTR) and pre-miR-con or pre-miR-185. *Rluc* mRNA levels were normalized to *Fluc* mRNA levels. The relative luciferase activity (RLUC/FLUC) of pre-miR-con was set to 1 ($n = 6$; $p = 0.0587$). (E) The overexpression of miR-185 was confirmed by real-time PCR ($n = 4$; $***p < 0.0001$). Data shown in C–E represent the mean \pm SEM.

mCry1-3'UTR, we made four point mutations in the miR-185-binding region of the full-length 3'UTR and inserted it into the RL vector (RL-185mut; Figure 4A). Although the mRNA levels of RL-185mut and wild-type full-length *mCry1*-3'UTR (RL-mCry1-3'U) were not dramatically different, the mutant reporter showed an approximately fourfold increase in luciferase activity (Figure 4, B and C). Furthermore, the overexpression of pre-miR-185 decreased the reporter activity of RL-mCry1-3'U but not that of RL-185mut (Figure 4, D and E). These findings suggest that miR-185 specifically binds to the 3'UTR of *mCry1* to regulate *mCry1* expression.

Cytoplasmic miR-185 oscillation regulates CRY1 expression

We demonstrated that miR-185 regulates *mCry1* expression by using a reporter linked to the 3'UTR of *mCry1*. Therefore, to determine whether miR-185 also regulated endogenous *Cry1* expression, we transfected NIH 3T3 cells with anti-miR-185, a chemically modified, single-stranded nucleic sequence designed to specifically bind to and inhibit endogenous miR-185 or pre-miR-185. Inhibition of miR-185 with anti-miR-185 increased CRY1 protein levels, whereas overexpression of pre-miR-185 decreased CRY1 protein levels (Figure 5A). Furthermore, anti-miR-con, but not anti-miR-185, did not significantly change the expression of mCRY1 compared with water treatment (Supplemental Figure S3A). To assess the physiological

role of miR-185-mediated *mCry1* translational regulation, we determined the effect of anti-miR-185 on the circadian expression of CRY1 in NIH 3T3 cells. To do this, we used dexamethasone to synchronize the rhythmicity of the cells (Lee *et al.*, 2012b). Again, inhibition of miR-185 by anti-miR-185 resulted in higher mCRY1 protein levels (Figure 5, B and C). In contrast, anti-miR-185 did not affect the phase of CRY1 expression, suggesting that miR-185 is critical for *mCry1* RNA translation (Supplemental Figure S3B). Because miR-185 was capable of regulating endogenous CRY1 protein levels, we hypothesized that miR-185 levels may oscillate with circadian time. Therefore we determined the expression profile of miR-185 in synchronized NIH 3T3 cells. Synchronized cells showed rhythmic *mCry1* mRNA expression (Supplemental Figure S4A); however, miR-185 did not exhibit an oscillation pattern (Supplemental Figure S4B). Because miRNA-mediated posttranscriptional regulation, especially translational repression, mainly occurs in the cytoplasm, we examined the cytoplasmic levels of miR-185. Synchronized NIH 3T3 cells were found to have a rhythmic *mCry1* mRNA expression profile with phase-delayed mCRY1 protein oscillation (Figure 5D, first and second from top). Of interest, in synchronized cells, the amount of cytoplasmic miR-185 fluctuated according to the specific circadian time period (Figure 5D, third from top). Moreover, the pattern of cytoplasmic miR-185 levels was nearly antiphase to that of mCRY1 protein levels and nuclear miR-185 levels (Figure 5D, top and bottom). These data provide evidence that cytosolic miR-185 has circadian rhythmicity antiphase to the mCRY1 protein profile and that its expression suppresses endogenous mCRY1 expression.

DISCUSSION

CRY1 is a core gene involved in the molecular clock system. Although the periodicity of transcription is crucial for maintaining rhythmic expression of clock genes, posttranscriptional and posttranslational regulation are also important for the fine-tuned regulation of the rhythmic cycle. The posttranscriptional regulation of mRNA stability and translational efficiency are often mediated by *cis* elements in mRNA that interact with *trans*-acting factors, such as RNA-binding proteins or microRNAs. The importance of UTR in the regulation of clock genes has been acknowledged (Garbarino-Pico and Green, 2007; Kojima *et al.*, 2011).

Here we showed that miR-185 interacts with the *mCry1* 3'UTR to regulate *mCry1* expression in a posttranscriptional manner. Of interest, cytoplasmic miR-185 levels were nearly antiphase to mCRY1 protein levels, and miR-185 knockdown elevated the amplitude of mCRY1 protein oscillation. Our results suggest that miR-185 plays a role in fine-tuned regulation of mCRY1 protein expression by controlling the rhythmicity of *mCry1* mRNA translation rate.

It has been estimated that miRNAs target >5300 human genes (Lewis *et al.*, 2003). Multiple miRNAs may regulate one gene,

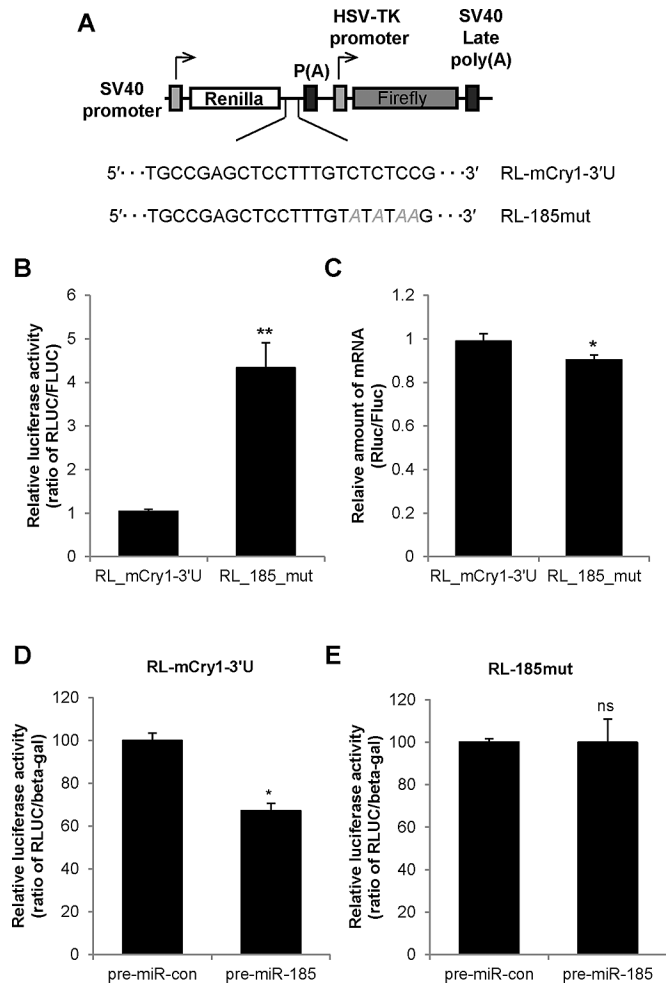


FIGURE 4: Modulation of the miR-185-binding site increases translation. (A) The naive (wild-type) full-length *mCry1*-3'UTR sequence containing the miR-185 target site was amplified and ligated into a pRL reporter vector (pRL-*mCry1*-3U). A plasmid containing the full-length *mCry1*-3'UTR with a selected point mutation in the miR-185 target sequence (pRL-185-mut) was also constructed. (B) Luciferase reporter assays were performed using NIH 3T3 cells transfected with pRL-*mCry1*-3U or pRL-185-mut. The relative luciferase activity of RL-*mCry1*-3U was set to 1 ($n = 4$; $p = 0.0047$). (C) Total RNA extracted from NIH 3T3 cells transfected with pRL-*mCry1*-3U or pRL-185-mut were subjected to real-time PCR to determine the relative *Rluc* mRNA levels (ratio of *Rluc/Fluc*; $n = 4$; $p = 0.0465$). (D) Luciferase assays were performed in NIH 3T3 cells cotransfected with pRL-*mCry1*-3U and pre-miR-con or pre-miR-185 ($n = 3$; $p = 0.0165$). (E) Luciferase assays were performed in NIH 3T3 cells cotransfected with pRL-185-mut and pre-miR-con or pre-miR-185 ($n = 3$; $p = 0.9928$). Data shown represent the mean \pm SEM.

indicative of cooperative translational control, and similarly, a single miRNA may regulate multiple target genes, reflecting target multiplicity (Enright *et al.*, 2003; Yoon and De Micheli, 2006). Therefore, although we identified one miRNA involved in the regulation of *Cry1* expression, other miRNAs may also be involved. Indeed, combinatorial control of a single target by multiple miRNAs is an important feature because the effects of a single miRNA can be marginal. Therefore the identification of other miRNAs that act on *mCry1* may help to elucidate the mechanism of rhythmic *mCry1* expression.

Gene silencing mediated by miRNA has two modes: mRNA degradation and translational repression (Lim *et al.*, 2005). In our study, miR-185 was found to regulate *mCry1* expression by translational repression. However, miRNA-mediated mRNA degradation may additionally modulate the rhythmic *mCry1* mRNA profile. It was reported that 3'UTR-mediated mRNA decay plays a role in the circadian *mCry1* mRNA regulatory mechanism (Woo *et al.*, 2010), and the oscillation of *mCry1* mRNA is well established (Lee *et al.*, 2001). Therefore it is possible that other miRNAs may also regulate *mCry1* mRNA stability, which may consolidate fine-tuned posttranscriptional regulation of rhythmic circadian clock genes.

In the present study, there were some differences in the miR-185-binding site of *mCry1*-3'UTR predicted by TargetScan compared with rats and humans. Based on the output of the prediction program and sequence analysis, it was difficult to determine whether the miR-185-binding site was conserved. The sequences of full-length *Cry1*-3'UTR in mice, rats, and humans are markedly different. Furthermore, significant differences in the circadian expression of clock genes among mice, rats, and humans have been observed (Yan *et al.*, 2008). The expression of most core clock genes showed a 4- to 5-h phase delay in rats compared with mice and an 8- to 12-h phase delay in humans compared with mice, which probably reflects the gap between nocturnal and diurnal animals (Yan *et al.*, 2008). The different expression patterns in mice and rats suggest that diverse and rewirable clock gene-regulatory networks may exist. We also suggest that the miR-185-binding site of *mCry1*-3'UTR may increase the complexity and elaboration of clock gene-regulatory networks and therefore contribute to the sophisticated and fine-tuned molecular basis of physiological and behavioral differences among species. The function of miR-185 in rats or humans was not investigated in the present study, however, and thus further studies are needed to determine the function of miR-185 in different species.

Although we initially believed that the miR-185 level may have circadian rhythm because of the rhythmic expression of its target, total miR-185 levels were found to be constant over the time points investigated. In contrast, the amount of cytoplasmic miR-185 showed an antiphase profile to the pattern of *mCRY1* protein expression. The posttranscriptional regulation of miRNAs, which can occur at the level of miRNA maturation, stability, or localization, can also influence their expression (Cullen, 2004; Kim, 2005; Hwang *et al.*, 2007). Therefore the mechanism of rhythmic cytosolic miR-185 accumulation needs to be studied further. Of importance, this study revealed a novel mechanism of miRNA-mediated rhythmic *mCry1* control that may be important in the regulation of the circadian clock. This regulatory system may induce fine-tuning of the amplitude of *mCRY1* protein expression regardless of mRNA oscillation. A similar circadian time-dependent, miRNA-mediated regulation may also be functional in other clock genes. On the basis of our results, we suggest that physiological circadian rhythm is generated through a complex regulation of gene expression that involves a variety of factors acting in rhythmic transcriptional, time-dependent posttranscriptional, and controlled translational and posttranslational regulation. Therefore our study provides insight into the tightly and finely regulated molecular circadian clock system.

MATERIALS AND METHODS

Cell culture and circadian synchronization

NIH 3T3 cells were cultured in DMEM (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and 1% antibiotics (Welgene, Daegu, South Korea) and maintained in a humidified

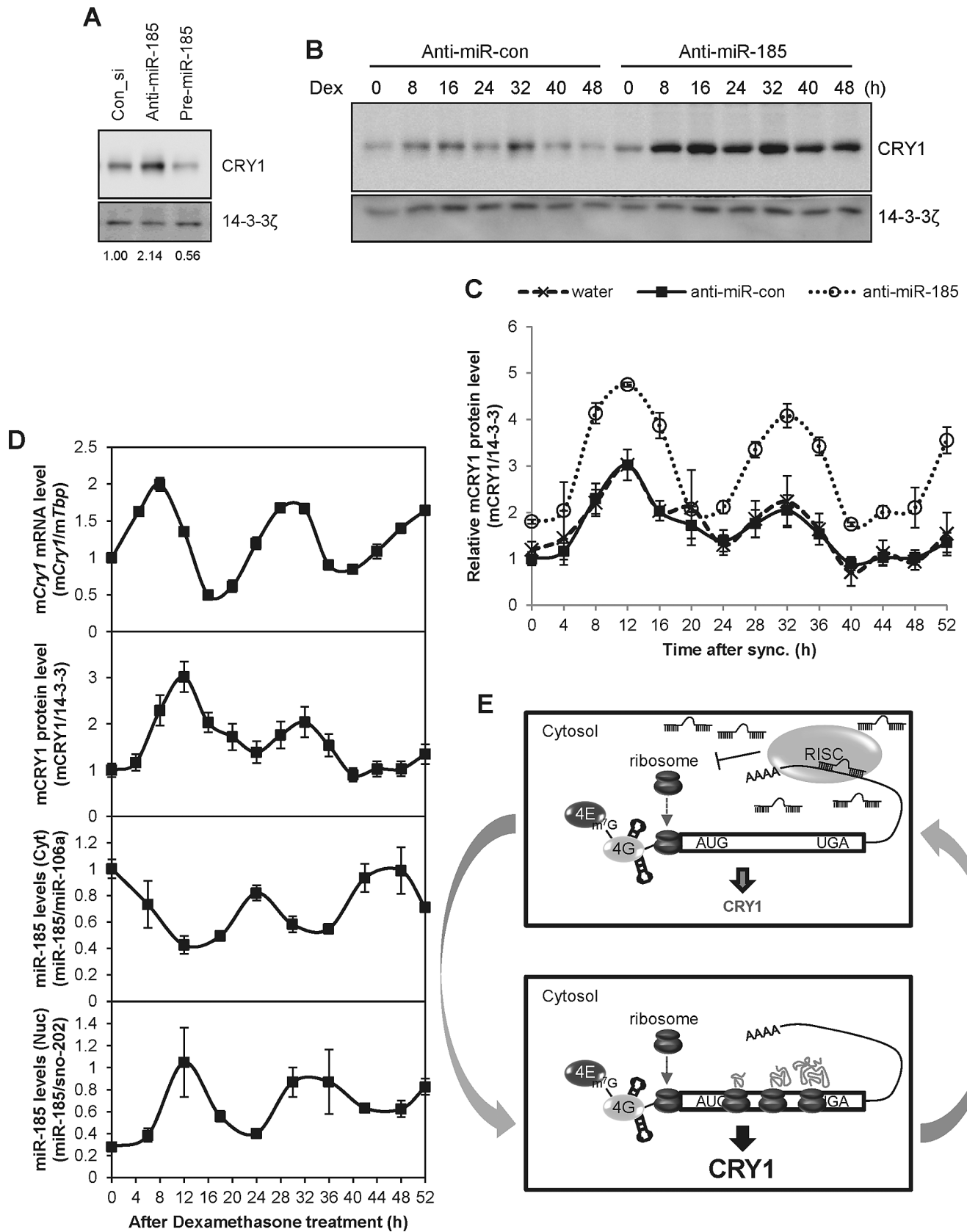


FIGURE 5: Cytoplasmic miR-185 oscillation regulates CRY1 expression. (A) CRY1 and 14-3-3 ζ expression in NIH 3T3 cells transfected with control siRNA (Con_si), anti-miR-185, or pre-miR-185 were determined by immunoblotting. (B) Anti-miR-con and anti-miR-185-transfected NIH 3T3 cells were synchronized with dexamethasone treatment for 2 h. Then cells were harvested at the indicated time points and subjected to immunoblotting for CRY1 and 14-3-3 ζ expression. (C) The relative mCRY1 levels in water-treated (x marks/dashed line), anti-miR-con-transfected (closed

atmosphere containing 5% CO₂. The circadian oscillation of NIH 3T3 cells was synchronized by dexamethasone treatment (100 nM) as described previously (Lee *et al.*, 2012b). After 2 h, the dexamethasone-containing medium was replaced with complete medium (Lee *et al.*, 2012b).

Plasmid construction

The mCry1 3'UTR was amplified from the pcNAT-wt610 plasmid as previously reported (Woo *et al.*, 2010). The resultant products were cloned into the *XhoI/NotI* site of the control pRL vector, which expresses *Renilla* luciferase (psiCHECK-2 vector; Promega, Madison, WI). The pRL-185mut plasmid was constructed by deletion mutagenesis using *DpnI* digestion. The plasmid containing a single or dimer 3'UTR fragment (RL-1×185B or RL-2×185B) was constructed by ligation of an annealed oligonucleotide containing one or two 3'UTR fragments into a pRL vector. For *in vitro* binding assays, the mCry1 3'UTR was amplified, and the resultant PCR products were digested and subcloned into the *EcoRI/XbaI* site of the pSK' vector (Kim *et al.*, 2005) to generate pSK'-mCry1-3U. To overexpress miR-185, we generated pSi-miR185. Pre-miR-185 was amplified from mouse genomic DNA, and the resultant products were cloned into the *BamHI/HindIII* site of the control pSi vector (pSilencer 4.1-CMV neo; Ambion, Austin, TX). Mouse Ago2 coding region was amplified from the cDNA of NIH 3T3 cells, and the resultant products were cloned into the pEGFP-C1 vector (Clontech, Mountain View, CA).

Transient transfection

For transient transfection of NIH 3T3 cells with reporter plasmids, miRNA mimics (Ambion), and antagomirs (Ambion), the Neon Transfection System (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions.

Cellular fractionation, RNA quantification, and reporter assay

Cytoplasmic and nuclear fractionation of NIH 3T3 cells was performed as described previously (Hahm *et al.*, 1998; Kim *et al.*, 2007; Lee *et al.*, 2012b). Fractionated or total small miRNAs were prepared using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions and subjected to reverse transcription (RT) using specific RT primers (Applied Biosystems, Foster City, CA) with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems).

Total RNA was extracted from NIH 3T3 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed using ImProm-II (Promega) according to the manufacturer's instruc-

tions. The mRNA levels of endogenous genes and reporter plasmids were detected by quantitative real-time PCR using a StepOne-Plus Real-Time PCR System (Applied Biosystems) with the FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN) as described previously (Lee *et al.*, 2012b). The specific primer pairs for mCry1, mTbp, Rluc, Fluc, mAgo2, mDicer, mDrosha, and mβ-actin (mActb) used for real-time PCR are shown in Supplemental Table S1. For miRNA quantification, quantitative real-time PCR was performed using the TaqMan MicroRNA Assay (Applied Biosystems) according to the manufacturer's instructions. A comparative C_t method was used for quantification.

The luciferase assay was performed as previously described (Lee *et al.*, 2012a). The ratios between Firefly and *Renilla* luciferase activities (RLUC/FLUC) were calculated. The ratio for the empty pRL vector was set to 1.

In vitro RNA synthesis and in vitro binding

For *in vitro* binding assays, biotin-UTP-labeled RNA was transcribed from the *XbaI*-linearized pSK-mCry1-3U plasmid using T7 RNA polymerase (Promega). Streptavidin-biotin RNA affinity purification was performed as described previously (Kim *et al.*, 2005). In brief, cytoplasmic extracts prepared from NIH 3T3 cells were incubated with biotinylated-mCry1-3'UTR RNA and subjected to streptavidin resin adsorption. Resin-bound proteins were analyzed by SDS-PAGE.

Immunoblot analysis

Immunoblot analyses were performed using polyclonal anti-CRY1 and monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Millipore, Billerica, CA) primary antibodies and horseradish peroxidase-conjugated, species-specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using the SUPLEX ECL solution kit (Neuronex, Daegu, South Korea) and the LAS-4000 chemiluminescence detection system (Fujifilm, Tokyo, Japan). Acquired images were analyzed using Image Gauge (Fujifilm) according to the manufacturer's instructions.

Statistical analysis

All quantitative data are presented as the mean ± SEM. Comparisons between two groups were analyzed by two-tailed unpaired Student's *t* tests. For comparisons between more than two groups, a one-way analysis of variance (ANOVA) was used with a post hoc Tukey's test. A *p* < 0.05 was considered statistically significant. A two-way ANOVA with a post hoc Bonferroni test was used to analyze the effects of anti-miR-185, anti-miR-con, and water treatment on mCRY1 protein levels.

squares/solid line), and anti-miR-185-transfected (open circles/dotted line) NIH 3T3 cells. CRY1 levels were normalized to 14-3-3ζ and plotted. mCRY1 protein levels were not significantly different at any time point between water-treated and anti-miR-con-transfected cells (*p* > 0.05). mCRY1 protein levels were significantly different between the anti-miR-con-transfected and anti-miR-185-transfected groups except at the 0-, 4-, 20-, 24-, and 40-h dexamethasone treatment time points. (D) The rhythmic mCry1 mRNA profiles of anti-miR-con- and anti-miR-185-transfected NIH 3T3 cells (top). Real-time PCR results were calculated using the ratio of mCry1/mTbp. The protein profiles of water-treated, anti-miR-con-transfected, and anti-miR-185-transfected NIH 3T3 cells were quantified and plotted by time (second from top). Cytoplasmic and nuclear miR-185 levels in synchronized NIH 3T3 cells transfected with anti-miR-con and anti-miR-185 (bottom). Cells were treated with dexamethasone for the indicated time points, and cytoplasmic and nuclear extracts were obtained under RNase-free conditions. miR-185, miR-106a, and sno-202 levels were measured by real-time PCR. The cytoplasmic miR-185 levels were normalized to miR-106a, whereas nuclear miR-185 levels were normalized to sno-202. Data shown represent the mean ± SEM. (E) The proposed model for cytosolic miR-185-mediated rhythmic mCry1 translational regulation. Increased cytosolic miR-185 binds to the 3'UTR of mCry1 and inhibits mCry1 translation. When cytosolic miR-185 levels are reduced, miR-185-mediated mCry1 translational inhibition does not occur, and mCRY1 protein is increased.

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