REVIEW ARTICLE

The role of microRNAs (miRNA) in circadian rhythmicity

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Abstract

MicroRNA (miRNA) is a recently discovered new class of small RNA molecules that have a significant role in regulating gene and protein expression. These small RNAs (~22 nt) bind to 3′ untranslated regions (3′UTRs) and induce degradation or repression of translation of their mRNA targets. Hundreds of miRNAs have been identified in various organisms and have been shown to play a significant role in development and normal cell functioning. Recently, a few studies have suggested that miRNAs may be an important regulators of circadian rhythmicity, providing a new dimension (posttranscriptional) of our understanding of biological clocks. Here, we describe the mechanisms of miRNA regulation, and recent studies attempting to identify clock miRNAs and their function in the circadian system.

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Introduction

MicroRNA (miRNA) is a recently discovered new class of small RNA molecules that have a significant role in regulating gene and protein expression. A brief survey of the World Wide Web reveals that two of the most common phrases associated with miRNA are 'small RNA revolution' and 'tip of the iceberg'. This reflects well the astonishing fact that the importance of these molecules was obscured for so many years (Lee *et al*. 1993; Wightman *et al*. 1993), and long after the principles of gene regulation were thought to be generally understood. It also conveys the consensus among researchers about the future promise of studying the functional role of miRNAs in diverse fields related to regulation of gene expression. It is, therefore, not surprising that in addition to their role in the development, and normal cell function (Ambros 2004; Bushati and Cohen 2007), evidence began to emerge that miRNAs may play an important role in circadian rhythmicity. Here we describe the mechanism of miRNA regulation, and recent studies attempting to identify clock miRNAs and their function in circadian systems.

How are miRNAs produced?

miRNAs are short, single stranded RNA molecules, 22–24 nucleotides long, transcribed from noncoding genomic regions (Ambros *et al*. 2003; Bushati and Cohen 2007). This process is carried out by RNA polymerase II through long pri-miRNA precursors that encode one or more miRNAs, each of which is organized in a 60–70 nucleotide hairpin structure separated by a single-stranded RNA (figure 1) (Ambros *et al*. 2003; Rana 2007).

In the cell nucleus, the pri-miRNA hairpin is processed by a multi-protein complex that includes the RNAse III Drosha, and the double-stranded RNA binding protein Pasha (Denli *et al*. 2004; Landthaler *et al*. 2004), producing stem loop pre-miRNA sequences (figure 2). Exportin-5 (a Ran-GTP dependent transporter) exports these pre-miRNAs to the cytoplasm, where the RNase III Dicer cuts out the single-stranded loop producing a miRNA duplex (Bernstein *et al*. 2001; Bohnsack *et al*. 2004; Lee *et al*. 2004). Dicer also participates in the assembly of mi-RISC (miRNA RNA-induced silencing complex) where one of the two strands of miRNA duplex is degraded (Matranga *et al*. 2005; Miyoshi *et al*. 2005).

How do miRNAs work?

Accumulating evidence suggests that miRNAs regulate expression in various context-dependent ways by different miRNA processing complexes. The interaction of miRNA

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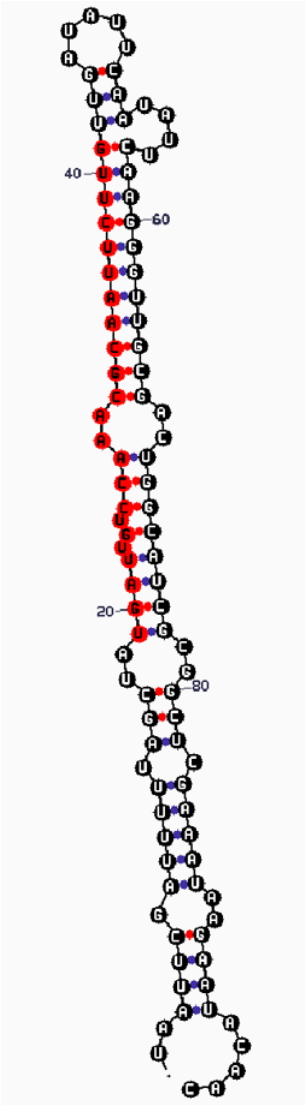

Figure 1. Secondary structure of pre-miRNA. The predicted stem-loop structure of pre-*mir-219-1*, a representative circadian miRNA in the mouse (see text) is depicted. The mature sequence is shown in red. The free energy ΔG predicted for this structure is −30.2 kcal/mole. Pre-miRNA sequence was obtained from miRNAMap (Hsu *et al*. 2006) (http://mirnamap.mbc.nctu.edu.tw) and the structure was derived using the mfold algorithm (Zuker 2003) available at (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi).

with target transcripts can be broadly grouped into two different pathways (figure 2). Perfect base pairing between a miRNA and mRNA-target induces mRNA cleavage through the 'silencing' mechanism. In contrast, imperfect pairing of miRNA with 3′UTR of target mRNA drives binding of miRISC to the target, leading to repression of translation.

The mechanism of action of miRNAs is not completely understood but it involves base pairing between the 5′ end of the miRNA (7–8 nucleotides, named 'seed'), and the 3′UTR of the target mRNA (Lewis *et al*. 2003; Farh *et al*. 2005; Stark *et al*. 2005). This pairing may either lead to mRNA degradation, or can affect the efficiency of translation by either promoting the ribosomal drop-off from the mRNA, or inhibiting the formation of 80S ribosome at the beginning of the translation (Humphreys *et al*. 2005; Pillai *et al*. 2005; Nottrott *et al*. 2006; Petersen *et al*. 2006). It was shown that miRNAs can also affect the stability of mRNA by de-adenylation and de-capping of the target mRNA (Behm-Ansmant *et al*. 2006; Giraldez *et al*. 2006; Mishima *et al*. 2006; Wu *et al*. 2006).

Identifying targets of miRNA is tricky. Different algorithms have been developed to recognize the 'seed' region to identify putative target genes, and their predictions may not always overlap. Currently, four different depositories of miRNA exist, offering different methods for target searching: the EMBL server (Brennecke *et al*. 2005), mirBase at the Wellcome Trust Sanger Institute (Griffiths-Jones *et al*. 2008), TargetScan (Grimson *et al*. 2007), and PicTar (Krek *et al*. 2005). Recently, efforts were made to integrate information of sequences, secondary structure and multiple polyA sites from 3′UTR regions (Vella *et al*. 2004a,b; Brennecke *et al*. 2005; Didiano and Hobert 2006; Rajewsky 2006; Long *et al*. 2007). To add to the complexity, recent studies indicate that base pairing of sequence outside the miRNA's 'seed' region can also contribute to the binding to the target (Brennecke *et al*. 2005; Grun *et al*. 2005; Stark *et al*. 2005; Long *et al*. 2007).

Finally, a recent study showed that in contrast to their traditional role in downregulation, miRNA can also increase protein translation (Vasudevan *et al*. 2007). HeLa cell quiescence induced by serum starvation is associated with translational upregulation mediated by tumour necrosis factor-α (TNFα). Vasudevan *et al*. (2007) found that the human miRNA miR369-3 that targets TNFα 3′UTR is required for the interaction of this protein with two other proteins AGO and FXR1, leading to upregulation of the protein translation. Two other miRNAs, Let-7 and miRcxcr4, that usually repress translation in proliferating cells, were also found to cause upregulation of translation of their targets during cell quiescence (Vasudevan *et al*. 2007).

Circadian clocks: posttranscriptional and translational regulation

The daily cycling of light and temperature, generated by the earth's rotation, is one of the most important driving forces in the evolution of the circadian clock, allowing organisms to anticipate and adapt to their daily (and seasonally) changing environment (Zheng and Sehgal 2008). Although the molecular details of the circadian clock may vary somewhat in different taxa, the principle of a system of selfsustained transcriptional–translational feedback loops is well conserved (Dunlap 1999; Gallego and Virshup 2007; Zheng and Sehgal 2008): Transcription factors, such as the

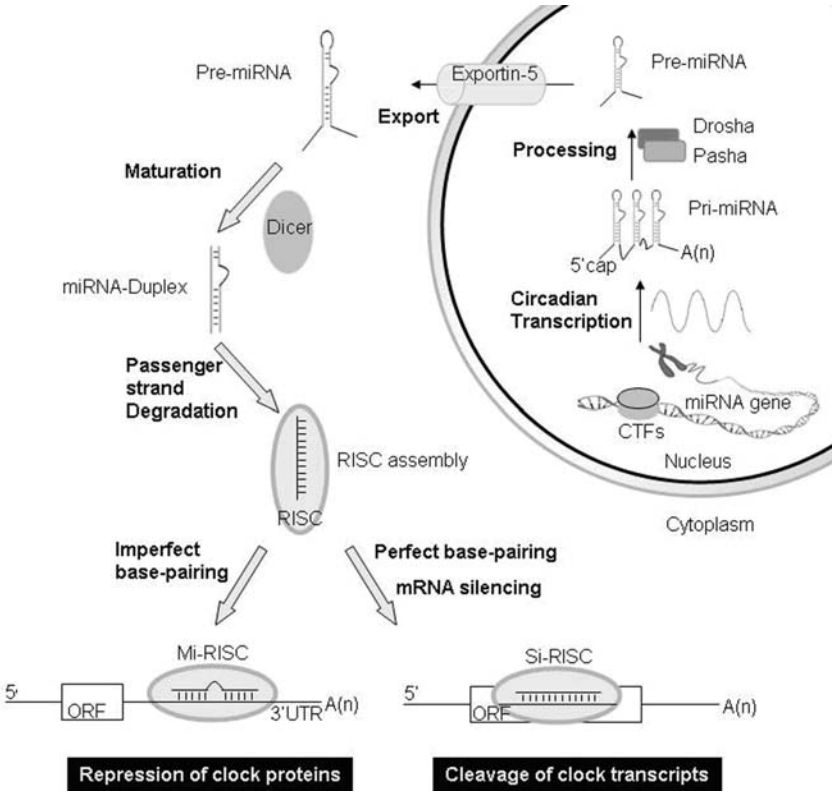

Figure 2. Synthesis and function of miRNA. Circadian transcription factors (CTFs) induce the transcription of miRNA genes (pri-miRNA). Drosha–Pasha multi-protein complex processes the pri-miRNA and produces 60–70 nt pre-miRNA . Exportin-5 translocates the pre-miRNA from the nucleus to the cytoplasm where Dicer cuts the pre-miRNA single stranded loop and generates miRNA duplex. At the RISC, the passenger strands of the miRNA duplex are degraded. The mature single strand miRNA is loaded at the RISC. Perfect base pairing between miRNA and target mRNA induces mRNA cleavage (si-RISC dependent silencing), while imperfect base-pairing between 3′UTR of target mRNA and miRNA induces mi-RISC-dependent inhibition of translation.

mammalian CLOCK and BMAL1, bind to clock-specific motifs (e.g. E-box) and drive the transcription of negative regulators, which are exported to the cytoplasm and then translated. These negative factors, which physically interact with each other, producing heterodimers (e.g. mouse PER-CRY, or fly PER-TIM), are later shuttled back to the nucleus where they repress the positive circadian transcription factors, thereby downregulating their own transcription (Darlington *et al*. 1998; Kume *et al*. 1999; Lee *et al*. 2000; Alabadi *et al*. 2001). The decrease in abundance in the negative factors later in the circadian cycle gradually leads to their own derepression, allowing the positive transcription factor to drive a new circadian cycle.

The daily oscillation of the core clock proteins drives a transcription rhythm of downstream clock-controlled genes (ccgs). Genomewide expression analysis from several microarray studies in different organisms indicates that the expression of as much as 5%–10% of transcripts in a specific tissue oscillates in a circadian manner (Claridge-Chang *et al*. 2001; McDonald and Rosbash 2001; Akhtar *et al*. 2002; Ceriani *et al*. 2002; Lin *et al*. 2002; Panda *et al*. 2002; Storch *et al*. 2002; Ueda *et al*. 2002; Duffield 2003; Keegan *et al*. 2007).

Yet, analysis of the mammalian cycling proteome revealed that the proportion of cycling cytosolic proteins is rather higher than that found in microarray studies, and that many of the cycling proteins show a constant abundance at the level of the mature transcript (Reddy *et al*. 2006). This discrepancy indicates that posttranscriptional and posttranslational mechanisms (largely unknown) are important components of circadian rhythmicity. Additional evidence (reviewed in Zheng and Sehgal 2008) shows that cycling of clock transcripts and proteins is not entirely essential for clock function, indicating that the 'central dogma' of the transcriptional-negative-feedback loop is probably sufficient but not necessary, and posttranscriptional/translational mechanisms are an important part of the circadian clock.

Before we embark on reviewing miRNA as a possible circadian posttranscriptional mechanism, we should point out that regulation of the circadian clock by endogenous antisense RNA (which conceptually includes miRNA) has already been reported in the past (reviewed by Crosthwaite 2004). For example, in *Neurospora*, several (5–5.5 kb) antisense *frequency* (*frq*) RNA are present (Kramer *et al*. 2003) which completely overlap with the sense transcript. The levels of the *frq* antisense RNAs cycle in antiphase with the sense *frq* RNA under free-running conditions, suggesting that the *frq*-antisense transcript has a clock function. This was elegantly demonstrated using mutant strains where the expression of the antisense, which is normally light-induced, is abolished. In these strains, circadian rhythmicity was delayed and phase response to light pulses was dramatically enhanced compared with the response of the wild-type (Kramer *et al*. 2003).

Role of miRNA in the circadian clock

A recent microarray study of *Drosophila* heads tested the expression of 78 miRNAs from flies entrained to light–dark cycles and compared to the corresponding expression in the clock mutant cyc^{01} (Yang *et al*. 2008). Two miRNAs *dme-miR-263a* and *263b*, showed significant cycling that was abolished in the mutant. The expression of these miRNAs was validated by qPCR, which also revealed that both these products cycled in continuous darkness. The fold-change of mir-*263a* and *263b* was rather small (1.7-fold and 2-fold oscillations, respectively). Compared with the fold-change of circadian clock transcripts (e.g. 4–5: McDonald and Rosbash (2001)), the modest change of miRNA may suggest that they are not biologically relevant. However, Yang *et al*. (2008) profiled the expression in whole heads, and it is rather likely that expression of these specific miRNAs change in individual neurons at a higher level (which could be tested by in situ hybridization). Also, it is possible that moderate-fold change is inherent property of miRNAs, reflecting the role of these regulators in fine tuning of expression. For example, miRNA levels after sleep deprivation in the rat's brain also show modest magnitude of fold-change (1.5–2.5: Davis *et al*. (2007)).

Clearly, some clock miRNA may not show any daily oscillation and yet play an important role in circadian regulation. The constant level of miRNA may serve as a threshold that 'gates' circadian oscillations. These miRNA may respond to various cues (e.g. temperature) by changing the gating of oscillation of other clock proteins. Indeed, Yang *et al*. (2008) identified six miRNAs that did not cycle, but had a significantly different profile compared with that of the cyc^{0} mutant.

Using the different prediction algorithms (Yang *et al*. 2008), a number of clock genes were identified that might provide a target for these circadian miRNAs, including *per, Clock, tim, dbt, cwo* and *tws*. However, how the cycling of *dme-miR-263a* and *263b* is generated is not yet clear. Interestingly, expression levels of both miRNAs, which have similar profiles, are high in the mutant, suggesting that their expression is normally repressed by CYC in the wild-type, indirectly by driving an as-yet-unknown transcriptional inhibitor.

A different approach was made in a survey of circadian miRNA in the mouse (Cheng *et al*. 2007). Here, previously published data on genomewide targets of the transcription factor CREB in the SCN in response to light were used (Impey *et al*. 2004). One of the targets of CREB was found to be a region upstream of *miR-132*. This prediction was confirmed using chromatin immunoprecipitation (ChIP) and RT-PCR. Interestingly, in addition to *miR-132*, *miR-219-1* (figure 1) was also enriched in the CREB immunoprecipitate. To identify miRNA whose transcription is driven by *Clock*, another ChIP experiment was carried using CLK antibodies; here, only *miR-219-1* was identified (Cheng *et al*. 2007).

When the promoter region of *miR-219-1* was analysed, E-box and CRE motifs (CRE-1 and CRE-2) were identified. In addition, the coexpression of BMAL1 and CLK in mammalian PC12 cells induced increased expression of *miR-219-1* (Cheng *et al*. 2007). However, *miR-132* was not responding to BMAL1 and CLK expression. In continuous darkness, both miRNAs show daily oscillation in the SCN (but not in other regions), peaking during the subjective day, which was abolished in *mCry1*/*mCry2* double mutant, strongly indicating a clock control of the expression of these miRNAs *in vivo* (Cheng *et al*. 2007).

The role of *miR-219* was further tested using antagomirs, oligoribonucleotides complementary to the miRNAs that block miRNA activity. Antagomir against *miR-219* reduced the level of this miRNA in the SCN and induced a lengthening of the circadian period. The *miR-132* antagomir induced two-fold increase in phase shift in light-pulse experiments, suggesting that *miR-132* is a negative regulator of the light-dependent resetting of the clock.

To identify the targets of *miR-132* and *miR-219-1*, the prediction algorithms TargetScan, miRanda and miRBase were used, revealing an overall enrichment of ion-channel proteins, suggesting that these two miRNAs modulate cellular excitability. Indeed, when cortical neuronal cultures were transfected with each of the miRNAs, a significant change in internal Ca^{2+} level was observed in response to depolarization or activation of the glutamate receptor: introduction of *miR-132* increased Ca^{2+} responsiveness, while *miR-219* slightly reduced it (Cheng *et al*. 2007). Transfecting HEK293T cells with either of the two miRNAs in combination with CLK and BMAL1 increased *Per1::LUC* reporter activity. Increase in *Per1::LUC* reporter was also shown in primary neuronal cultures, indicating that these miRNAs are involved in the core clock timing mechanism.

In another recent study on the mouse, the daily cycling of expression of a number of miRNAs (*miR-96, miR-124a, miR-103, miR-182, miR-106b, miR-422a*, and *miR-422b*) was found in the retina (Xu *et al*. 2007). It was found that the

mRNA encoding adenylyl cyclase VI (*Adcy6*), which was shown earlier to be expressed rhythmically (Han *et al*. 2005), is a predicted target (using PicTar and TargetScan) of two putative circadian miRNAs: *miR-182* and *miR-96*. The expression of these miRNAs is in anti-phase to the *Adcy6* transcript. In a luciferase assay, *miR-182* and *miR-96* are able to repress *Adcy6* expression.

As in the circadian clock in mammals, posttranscriptional regulation also appears to play an important role in the avian circadian system (Karaganis *et al*. 2008). For example, although global cycling of the transcriptome is reduced in the chick pineal gland, a robust cycling of melatonin still persists. This cycling may be driven as a posttranscriptional process by four recently identified miRNAs that show circadian oscillation in their expression levels (Shende *et al*. 2008). Among the predicted targets of these miRNAs are the chicken orthologues of mammalian clock genes *NPAS2* and *Period3*.

Some miRNA may not show diurnal cycling but still have a significant impact in regulation of clock, or clock-controlled proteins. Nocturnin, for example is a cycling deadenylase downstream of the circadian clockwork, serving as the clock output in metabolic regulation (Green *et al*. 2007). Nocturnin has recently been shown to be targeted by miR-122 (Kojima *et al*. 2008), which is consistent with an earlier study suggesting that this miRNA is involved in lipid metabolism (Esau *et al*. 2006). As large proportions of the transcriptome and proteome are under circadian control, one may assume that many more of these circadian-controlled genes are targeted by miRNAs. The miRNA system may represent an additional module that integrates various stimuli and modulates circadian rhythmicity either directly by targeting clock genes, or indirectly by acting on clock-controlled proteins.

Finally, miRNA also seems to be important for seasonal timing. In *Arabidopsis thaliana*, the circadian clock is involved in photoperiodic timing of flowering, with many flowering genes exhibiting circadian rhythmicity (Samach and Coupland 2000; Hayama and Coupland 2003). GIGANTEA (GI), is an *Arabidopsis* clock protein that links the circadian pacemaker and the photoperiodic flowering response through interaction with COSTANT (CO) and FLOWERING LOCUS T (FT) (Yanovsky and Kay 2003; Mizoguchi *et al*. 2005). Interestingly, a plant miRNA (*miR-172*) was identified that responds to day length (Schmid *et al*. 2003). A recent study showed that GI regulates the response of *miR-172* to change in day length (Jung *et al*. 2007). The *miR-172* and its targets, mediate flowering by regulating FT and constitute a separate pathway for seasonal timing.

Conclusion

For almost two decades, the transcription–translation feedback model provided a framework for understanding how the molecular clock works (Hardin *et al*. 1990). Yet, accumulating evidence indicates a gap in our understanding that was often assumed to represent an elusive posttranscriptional mechanism. The recent discovery of miRNAs and their function will probably contribute to narrowing this gap. MicroRNAs play an important role in various biological functions and it was only a matter of time before their role in the circadian system would be revealed. Although research on circadian miRNAs is still in its infancy, we are confident that future research will show that this type of regulation has a major impact on the circadian clock. The 'small revolution' is here.

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