MICRORNAS FUNCTION AS CIS- AND TRANS- ACTING MODULATORS OF CLOCK GENE EXPRESSION IN SCN AND PERIPHERAL CIRCADIAN OSCILLATORS

A Dissertation

by

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ABSTRACT

The circadian system in mammals is arranged as a hierarchical network of oscillators, with the master pacemaker of circadian rhythms located in the suprachiasmatic nuclei (SCN) of the hypothalamus and peripheral oscillators in most other organ and tissue systems of the body. The molecular machinery responsible for generating circadian rhythms is composed of interlocked transcriptional-translational feedback loops with the gene Brain Muscle Arnt-like 1 (*Bmal1*) functioning as a core positive regulator. Using the mouse, *Mus musculus* as a model system, we studied the post-transcriptional mechanisms regulating *Bmal1* expression in the SCN pacemaker and in peripheral oscillators.

Target prediction algorithms were used to identify microRNAs (miRNAs) predicted to target *Bmal1*. We profiled the temporal expression of miR-142-3p in the mouse SCN in vivo and in an immortalized SCN cell line and observed robust circadian rhythms in its expression in the SCN. Following luciferase-reporter and site-directed mutagenesis analyses, we identified miR-142-3p as a bona-fide post-transcriptional repressor of *Bmal1*. The temporal expression of potential *Bmal1*-targeting miRNAs was also examined in the circulation in mouse serum. In mice housed in a light-dark cycle, diurnal oscillations were observed in serum levels of miR-152 and miR-494, but not miR-142-3p expression. Luciferase reporter studies indicated that miR-494, both independently and synergistically with miR-142-3p, repressed the *Bmal1* 3′ UTR. Overexpression of these miRNAs disrupted ensemble circadian rhythms of

PER2::LUCIFERASE activity in cultured fibroblasts. Overexpression of the miRNAs also increased their extracellular levels and their intracellular accumulation in recipient cultures exposed to conditioned medium. Furthermore, inhibition of exocytosis and endocytosis affected ensemble circadian rhythms in cultured fibroblasts.

The results thus implicate miR-142-3p and miR-494 in the regulation of *Bmal1* expression in the SCN and peripheral oscillators and suggest that miRNAs may function as both, intracellular and extracellular (*cis*- and *trans*- acting) signals, modulating the core clock mechanism in the SCN and in fine-tuning the synchronization of circadian rhythmicity between cell-autonomous oscillators in the periphery.

DEDICATION

This dissertation is dedicated to my family for their unwavering and unconditional belief, love and support.

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Having grown up in big, crowded cities like Mumbai and Pune, it is almost surreal to realize that I have spent the majority of my adult life in a town called College Station, population 95,000. My situation is certainly not unique; each year hundreds of international students come to A&M, many from my home country, many of those from socio-economic, cultural and educational backgrounds very similar to my own. We all however walk our own journeys, and people along the way, intentionally or not, shape our experiences. Since a PhD was the only reason I was supposed to be here, everything that mattered professional or personally, directly or indirectly, influenced the outcome of this dissertation. I would like to take this opportunity to thank everybody who helped me during my time at A&M, some as drill sergeants, others, as unpaid therapists and counselors, all very much appreciated nonetheless. Being trained as a chronobiologist, it is perhaps fitting that I start in chronological order.

Having never heard the words proctoring, mentoring, tutoring, curving, etc. until 2-weeks before I was supposed to begin teaching a biology laboratory, meant TA'ing could have been a very uneasy experience. I would like to thank the introductory biology administrative and support staff for helping me through that first semester. The 319 lab coordinators and colleagues have since also been very helpful and I have enjoyed each of the 12 semesters that I taught for the Biology department. I also want to thank Kay Goldman, Vickie Shrhak and other Biology advising and administrative staff for their help and patience throughout my interactions with them over the years.

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CHAPTER I

INTRODUCTION

Life on earth has evolved on a planet that rotates on its axis and revolves around a star. This 'eternal' motion, influenced by interactions with the moon orbiting around it, generates distinct, repeating environmental changes that most organisms have adapted to. One of the ways these adaptations manifest are as endogenous rhythms that cycle in synchrony with the cyclic changes occurring in the environment. One cycle can last for ~12h (circatidal), one day (circadian), twenty eight days (circalunar) or one year (circannual).

Circadian Rhythms- Evolutionary Context

Circadian rhythms, derived from latin roots 'circa' meaning 'about' and 'diem' meaning 'day' are cyclic changes in an organisms behavior, physiology, metabolism and gene expression that occur with a period of about one day. Circadian rhythms are pervasive in nature and are observed in photosynthetic cyanobacteria and in most eukaryotes including fungi, plants and mammals. The widespread occurrence of circadian clocks in diverse species is suggestive of evolutionary pressure to maintain them. The presence of an endogenous clock is thought to provide an evolutionary advantage in allowing an organism to anticipate the predictable changes in environmental conditions including temperature, food-availability, and predation risk. However, systematic rigorous tests of the evolutionary importance of clocks are

cumbersome. Additionally, a laboratory, where animals cannot interact with the myriad of other species and factors they are normally exposed to in their natural environment is not an ideal setting for testing evolutionary importance of clocks. There has, hence, been limited experimentation of evolutionary questions.

Increased fitness: The suprachaiasmatic nuclei (SCN), as detailed in the succeeding sections, are the seat of the master pacemaker of circadian rhythms in mammals. Because SCN-lesioned animals are known to loose most circadian rhythms, DeCoursey and colleagues (1997) monitored mortality rates of intact or SCN-lesioned antelope ground squirrel in a semi-natural field setting. The experimenters observed a significantly higher mortality rate in SCN-lesioned animals compared to the intact control counterparts. The second, even more convincing study compared mortality rates of free living, wild caught chipmunks after SCN lesions or Sham operations (DeCoursey et al., 2000). Between 14-60 days after surgeries, the mortality rate of the SCN-lesioned animals was significantly higher than the sham operated or the non-operated animals.

The resonance hypothesis: Using genetic manipulations, it has been possible to carry out more robust studies in plant and cyanobacterial systems by testing the 'circadian resonance' hypothesis. These experiments test the prediction that since life on earth has evolved to take advantage of 24h cycles, genetic mutations generating organisms with endogenous free-running periods (FRP's) different from that of the wild-type's will be at a competitive disadvantage in a 24h cycle. As a corollary, wild-type individuals would be predicted to be outcompeted if they are housed with mutants in an environment where T-cycles ('T' = zeitgeber; 'cycle' = period) are more compatible to

the FRP's of the mutants rather than the wild-types. In one such study on *Arabidopsis,* Dodd and colleagues (2005) exposed wild-type and clock mutant plants to 20h, 24h or 28h light-dark cycles. They measured chlorophyll, carbon-fixing ability and vegetative growth under competition and monoculture conditions. In all cases, accurate matching of the plants endogenous cycle with the external light-dark cycle was most advantageous for growth and survival whereas circadian dissonance (i.e., endogenous rhythms not aligned with environmental cycles) was disruptive for growth and survival.

Some of the best evidence for the adaptive value of endogenous clocks obtained so far has come from similar competition experiments using the cyanobacteria, *Synechococcus elongatus*. Ouyang and colleagues (1998) utilized three strains of the cyanobacteria, which had different endogenous free-running periods. The wild type strain had a FRP of ~25h, the short period mutant had a period of ~23h and the long period mutant had a period of ~30h. Competition experiments were performed by exposing pairwise mixtures containing equal proportions of two strains to light-dark T-cycles of 22h, 24h or 30h. In all instances, the strains with endogenous FRP closest to the environmental cycle out-competed the other strain. These two lines of research provide compelling evidence in support of the importance of preservation of functional circadian rhythms in natural/pseudo-natural environments.

Formal Properties of Circadian Rhythms

The study of circadian clocks involves analysis and understanding of cyclic, mostly sinusoidal, phenomenon and many terms otherwise associated with physics are

used while describing circadian rhythms. Zeitgeber ('zeit' meaning 'time' and 'geber' meaning 'giver') is an exogenous rhythmic environmental stimulus that can act as an indicator of external time. Phase of a rhythm is any defined instantaneous point on a sinusoidal wave. Peak is the phase of highest expression and a trough is the phase of lowest expression. Period of a rhythm is the time taken for a defined reference phase to recur during the subsequent cycle (e.g. time between two subsequent peaks, or two subsequent troughs). Amplitude of a rhythm is a measure of the mean difference between the peak and trough phase of one cycle.

Because multiple examples of biological processes repeating with rhythmic sinusoidal patterns can be otherwise found in nature, three basic characteristics need to be fulfilled before a cyclic biological phenomenon can be considered as a circadian rhythm (Pittendrigh, 1960; Aschoff et al., 1971; Bell-Pedersen et al., 2005). These are as follows: **1**) the process cycles with a period close to 24h and the cycles are maintained under constant conditions (Fig. 1A). The persistence of circadian rhythms under constant environmental conditions such that processes 'free-run' with a period determined by an internal clock demonstrates the 'endogenous' biological origin of the rhythm. The free-running periods of circadian rhythms differ slightly from the 24h period of environmental light-dark cycles. **2**) The phase and period of the rhythm can be entrained by environmental cycles (Fig. 1B). Considering that clocks are an adaptation to ensure that different biological processes occur at the optimal time during an environmental cycle, it is necessary for these clocks to adjust to potential changes in the environment. This phenomenon of adjusting to align overt rhythmicity is called 'entrainment' to an

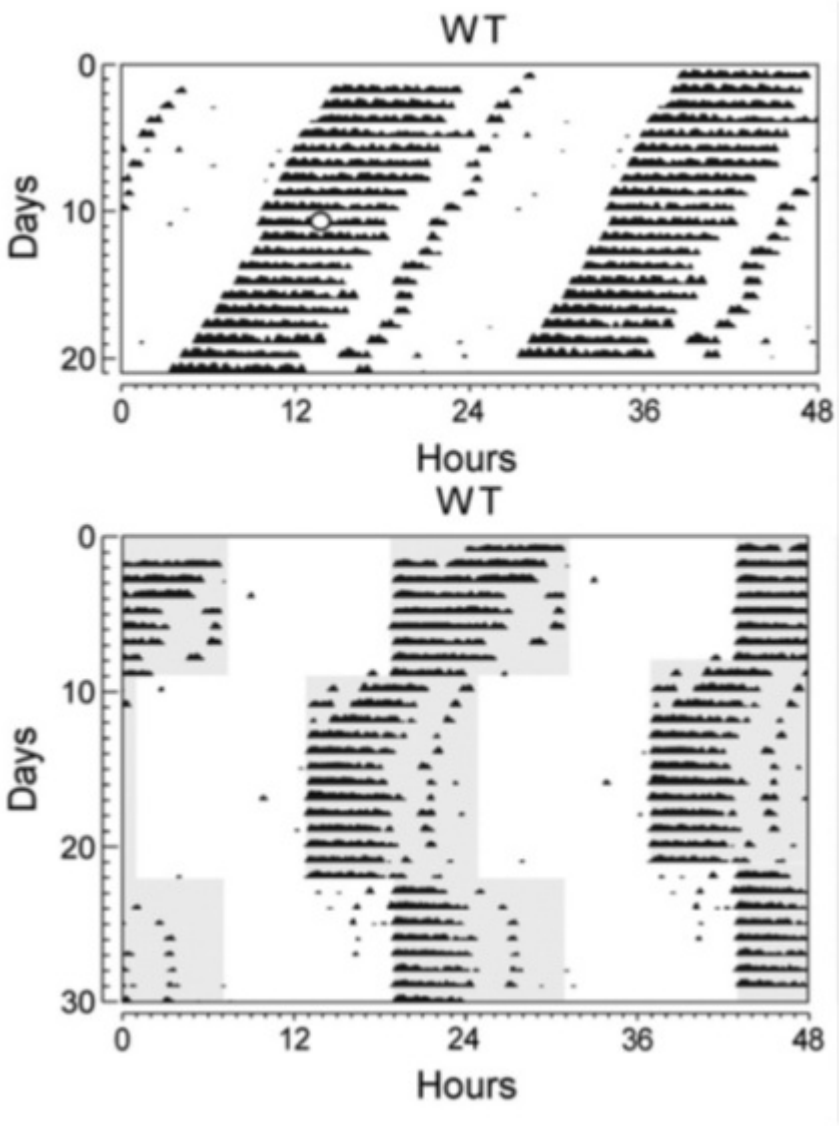

Figure 1. Free-running and entrainment characteristics of circadian rhythms. Representative locomotor behavior (wheel running) activity of WT mice maintained in (A) constant darkness (DD), or (B) 12h: 12h LD cycle, with shaded regions representing the dark phase. The actograms are double-plotted. Reprinted from Experimental Neurology, 232 /1, Takashi Kudo, Dawn H. Loh, Danny Truong, Yingfei Wu, Christopher S. Colwell, Circadian dysfunction in a mouse model of Parkinson's disease, 66-75, Copyright (2011), with permission from Elsevier.

environmental cycle. **3**) The last universal feature of circadian rhythms is that the period of the rhythm remains stable even at various different (but ambient) temperatures. This property is called temperature compensation. Most biochemical reactions are temperature-dependent and typically exhibit a Q_{10} value of 2-3; that is, the rate of a reaction increases 2-3 folds for every 10-degree rise in temperature. However, since organisms are exposed to varying temperatures during the course of a day and over the course of a year, it is vital that the circadian clocks are buffered against variations in temperature to allow them to accurately predict and entrain to environmental time. The period of circadian rhythms are hence 'temperature compensated' and exhibit Q_{10} values of ~1.1, which are significantly less than what would be expected in the absence of any compensation.

Functional Organization of Circadian Timekeeping Systems

Circadian clocks have been described in many diverse species from distinct taxa. All clocks however share a common overall organization; they all contain three parts: input pathway(s), central oscillator(s) and output pathway(s). The input pathway(s) transmit environmental information (e.g. light intensity, temperature, osmotic balance, etc.) to the central oscillator. The central oscillator is the timekeeping mechanism, composed of molecular and biochemical interactions between clock-components that give rise to self-sustained rhythmic oscillations. The central oscillator can be entrained to environmental cycles via the input pathways, but can also free-run in the absence of external input. The output pathways are responsible for translating information from the

central oscillator to generate overt circadian rhythms in gene expression, physiology, metabolism and behavior in an organism.

Circadian Rhythms in Mammals

Hierarchical organization: The mammalian circadian timekeeping system is thought to have a hierarchical organization with the suprachiasmatic nuclei (SCN) of the anterior hypothalamus functioning as the master pacemaker responsible for temporal synchronization of various cell-autonomous oscillators present in the central nervous system and in peripheral tissues. The importance of the SCN was uncovered via initial lesion studies trying to identify location(s) in the brain responsible for generation or maintenance of circadian rhythms. In rats, bilateral electrolytic lesions of the SCN resulted in global loss of circadian rhythms in physiology and behavior, implicating the SCN as the location for generation of circadian rhythms and their entrainment to environmental light-dark cycles (Moore and Eichler, 1972; Stephan and Zucker, 1972). The lesion experiments were later followed up by transplantation studies in which fetal SCN tissue was transplanted into SCN-ablated host animals. Transplantation was found to rescue behavioral circadian rhythmicity in previously arrhythmic animals (Lehman et al., 1987; Silver et al., 1990). Additionally, using *Tau*, a short period mutant strain, Ralph et al. (1990) showed that the free-running period of the restored rhythms was determined by the transplanted SCN tissue. Transplanting fetal SCN from wild-type or a long period mutant (*mCry*$^{-/-}$) into host mice harboring mutations that rendered them otherwise arrhythmic, Sujino et al. (2003) further showed that a functional circadian

clock in the SCN is sufficient to drive some, if not all, circadian rhythms in animals and that the free-running period of those rhythms is determined by the endogenous properties of the SCN. These studies corroborated the role of the SCN as a central pacemaker mediating the maintenance of circadian rhythms in mammals.

Structure and function of the SCN: The suprachiasmatic nuclei are a set of paired structures containing approximately 10,000 neurons each, and are located on either side of the third ventricle in the anterior hypothalamus, superior to the optic chiasm. The SCN receive light input from melanopsin-expressing intrinsically photoreceptive retinal ganglion cells (iPRGCs) via the retinohypothalamic tract (RHT) (Hattar et al., 2002; Do and Yau, 2010). RHT fibers form mono-synaptic connections with retinorecipient cells in the ventral 'core' region of the SCN and this photic communication occurs through the release of neurotransmitters, glutamate and pituitary adenylate cyclase activating peptide (PACAP) (Abrahamson and Moore, 2001; Morin and Allen, 2006). The neurons comprising the core region of the SCN are enriched for the expression of neuropeptides vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP). This ventrolateral core densely projects into the surrounding, dorsomedial 'shell' region of the SCN, which contain neurons enriched for production of the neuropeptide arginine vasopressin (AVP) (Leak et al., 1999; Abrahamson and Moore, 2001). In addition to neurons, the SCN also contains glial cells, which also contain functional circadian clocks that contribute to SCN pacemaking function (Prosser et al., 1994; Michel and Colwell, 2001; Prolo et al., 2005).

Many cellular and molecular processes in the SCN are marked by intrinsic rhythmicity even in the absence of any external input from the retina. Self-sustained oscillations in gene expression, metabolism, neurotransmitter secretion and neuronal firing rate have been observed in the SCN in vivo and in vitro (Schwartz et al., 1980; Groos and Hendriks, 1982; Earnest and Sladek, 1986; Gillette and Reppert, 1987; Bos and Mirmiran, 1990; Newman et al., 1992; Panda et al., 2002; Reppert and Weaver, 2002). Using a transgenic reporter line, Yamazaki and colleagues (2000) showed that circadian rhythms in expression of luciferase, under control of a clock gene (*Per1*) promoter, persist for up to 32 days in the rat SCN in vitro. Our studies similarly indicate that immortalized SCN cells from transgenic mice generate cell-autonomous circadian rhythms in gene expression for up to 7 days in culture (Farnell et al., 2011). Furthermore, endogenous circadian rhythmicity is not just an ensemble property, but is a characteristic of individual cells of the SCN as well. Neurons of the SCN continue to express independently phased rhythms in firing rate even when maintained in dissociated cultures in vitro (Welsh et al., 1995).

Various studies that will be discussed in detail in the succeeding sections have now established that cell-autonomous rhythmicity is not only a property of cells of the SCN, but is actually pervasive in most peripheral tissues as well. What makes the SCN unique, however, is the ability to drive or synchronize rhythms in other cells. The SCN pacemaker function appears to be mediated by secretion of diffusible factors. Silver and colleagues (1996) modified SCN transplantation experiments such that the donor SCN was encapsulated in a permeable membrane to determine whether transplant-host

synaptic connections were necessary to restore circadian rhythmicity in SCN-ablated host animals. The primary observations of this study were that despite the physical barrier between graft and host cells, these SCN transplants still restored the circadian rhythm of locomotor activity, suggesting that some diffusible signal(s) may mediate SCN pacemaker function. Parallel studies by Allen and colleagues (2001) using an immortalized rat SCN cell line (SCN2.2) that retained SCN pacemaking properties (Earnest et al., 1999) provide further evidence for the role of diffusible signals in SCN pacemaker function. In a co-culture environment allowing communication of diffusible factors but not physical contact between different cell types, the capacity of NIH/3T3 fibroblasts to generate rhythms in gene expression and metabolism was dependent on their co-culture with SCN2.2 (and presumably on some SCN-specific signals responsible for conveying this rhythmicity). Although the identities of these diffusible signals are still being investigated, prokineticin 2, TGF-α and cardiotrophin-like cytokine (CLC) have emerged as potential candidates mediating SCN-controlled rhythms in locomotor activity (Kramer et al., 2001; Cheng et al., 2002; Kraves et al., 2006).

Although the presence of diffusible signals has been unequivocally demonstrated, other studies also highlight the requirement of neural connections for effective SCN pacemaking function in generating synchronized physiological and endocrine rhythms (Meyer-Bernstein et al., 1999; Guo et al., 2005; Guo et al., 2006). Using anterograde tracer and retrograde dyes, extensive projections of the SCN have been identified. These projections are predominantly to other parts of the hypothalamus and to some regions of the thalamus. Specifically, these include, the medial preoptic

area, paraventricular nucleus of the thalamus (PVA), paraventricular nucleus of the hypothalamus (PVN), dorsomedial nucleus of the hypothalamus (DMH), medial and lateral subparacentricular zone (sPVZ) among others (Watts et al., 1987; Watts and Swanson, 1987; Kalsbeek et al., 1993; Leak and Moore, 2001). These brain regions in turn are involved in regulation of physiological and behavioral outputs like metabolism, sleep and feeding (reviewed by: Saper et al., 2005; Kalsbeek et al., 2007). Thus, SCN neural projections impinge on different brain centers, potentially modulating the regulation of certain rhythmic behaviors like feeding and sleep.

Molecular clock machinery- the transcriptional-translational feedback loop model: The molecular machinery responsible for the generation of circadian rhythms has been extensively studied over the past two decades. The result is a transcriptional-translational feedback loop (TTFL) model, in which genes comprising the positive elements drive transcription of negative elements that feed-back to inhibit their own transcription. An ancillary feedback loop is thought to stabilize the core loop (Fig. 2). The products of the core clock genes also undergo post-translational modifications that set the speed of the clock such that the period of one oscillatory cycle is approximately 24h (reviewed by, Lowrey and Takahashi, 2011).

The positive factors that drive the molecular machinery are *Clock* and *Bmal1*. Both genes encode basic helix-loop-helix transcription factors that heterodimerize during the day, when CLOCK-BMAL1 activity is at its peak, and drive transcription via E-box (-CACGTG-) enhancer elements in the promoters of their target genes (King et al., 1997; Gekakis et al., 1998; Bunger et al., 2000). The negative elements, *Per1, Per2, Cry1* and

Cry2, which contain E-boxes or E'-boxes in their promoters are transcribed, translated in the cytosol where they form complexes that are then translocated back into the nucleus (Van Der Horst et al., 1999; Vitaterna et al., 1999; Bae et al., 2001; Zheng et al., 2001). Once in the nucleus, the PER-CRY complex inhibits CLOCK-BMAL1 transcriptional activity, in turn shutting off their own transcription. This inhibition is thought to occur via direct CRY-mediated interactions with, and repression of, the CLOCK-BMAL1 heterodimer (Kume et al., 1999; Sato et al., 2006). Additionally, the PER-CRY complex is thought to recruit histone modifying enzymes, like HDAC1, to aid in repression of transcription at clock-specific promoters (Duong et al., 2011).

Genes comprising one of the ancillary loops containing orphan nuclear receptors, *Rora* and *Rev-erbα*, are also transcriptionally activated by CLOCK-BMAL1 heterodimers. Additionally, the *Bmal1* promoter contains RevErbA/ROR-binding elements (ROREs), which are the sites of action of the two *Ror's* and *Rev-erb's*. *Rora* is an activator of *Bmal1* transcription, while *Rev-erbα* is a repressor (Fig. 2) (Preitner et al., 2002; Sato et al., 2004; Akashi and Takumi, 2005).

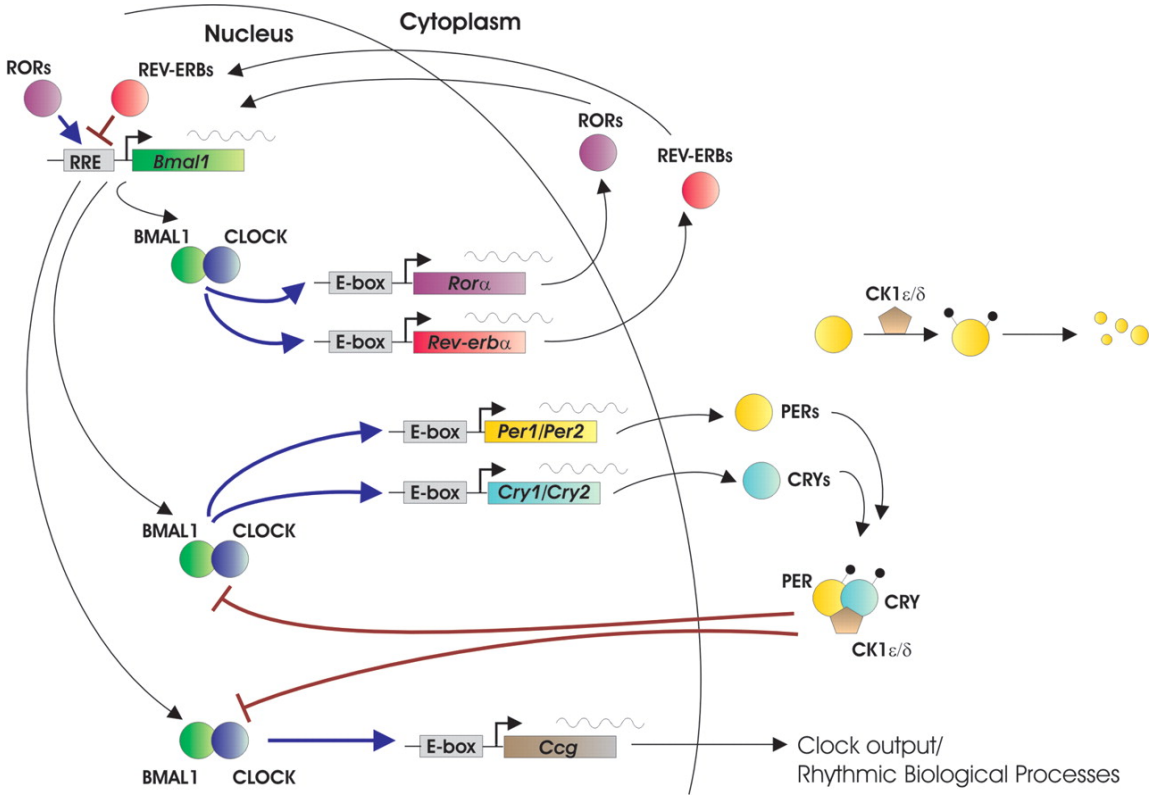

Figure 2. Simplified model of the mammalian circadian transcriptional translational feed-back loop oscillator mechanism. Reprinted from Human Molecular Genetics, 15 (Suppl 2): R271-R277, Caroline H. Ko and Joseph H. Takahashi, (2006), with permission from Oxford University Press.

Post-translational modifications of clock proteins: Successful reconstitution of a cyanobacterial, *Synechococcus elongatus*, clock in vitro without any transcriptional input, provided the first major indication of the potential importance and sufficiency of transcription-independent mechanisms in clock cycling (Nakajima et al., 2005; Tomita et al., 2005). Although these observations are not entirely applicable in mammals, post-transcriptional events important in fine-tuning the molecular clockwork have been discovered (reviewed by, Gallego and Vishrup, 2007). Phosphorylation of the PER proteins by casein kinase ε (*Csnk1e)* was the first such modification identified. The phosphorylation of PER2 affects its proteasomal degradation and PER1 nuclear entry (Vielhaber et al., 2000; Eide et al., 2005). Indeed, the *Tau* mutation first identified in hamsters and resulting in dramatically short (~20h) free-running period of locomotor activity was cloned to the *Csnk1e* locus (Lowrey et al., 2000). Other kinases involved in phosphorylation of clock proteins include casein kinase 2 (*Csnk2a* and *Csnk2b*) which targets components of both the positive limb (BMAL1) and the negative limb (PER2) for phosphorylation (Tamaru et al., 2009; Tsuchiya et al., 2009), glycogen synthase-kinase β, which has also been shown to phosphorylate: **1**) PER2 and promote its nuclear localization, **2**) CRY2 so as to promote its degradation, and **3**) BMAL1and prime it for ubiquitination (Harada et al., 2005; Iitaka et al., 2005; Sahar et al., 2010). The mitogen activated protein kinase (MAPK) and protein kinase c (PKC) are also implicated in phosphorylation of BMAL1 and inhibition of CLOCK-BMAL1 heterodimer activity (Sanada et al., 2002; Robles et al., 2010). Rhythmic phosphorylation is also balanced in part by protein phosphates, PP5 and PP1, which target CK1ε and PER2 respectively for

de-phosphorylation (Gallego et al., 2006; Partch et al., 2006). Focusing on *Bmal1*, the primary clock gene of interest for this dissertation, other post-transcriptional modifications including sumoylation, acetylation, deacetylation and ubiquitination have also been reported. Three SUMO paralogs (SUMO 1, 2 and 3), all target BMAL1 recruiting it for ubiquitination and proteasomal degradation (Cardone et al., 2005; Lee et al., 2008). CLOCK protein, the partner of BMAL1, has acetyl transferase activity and acetylates the 537-lysine residue on BMAL1 (Doi et al., 2006). This is reversed by an NAD+ dependent deacetylase, SIRT1, which binds to CLOCK-BMAL1 and influences circadian gene expression (Hirayama et al., 2007; Nakahata et al., 2008; Bass and Takahashi, 2011).

***Post-transcriptional regulation of the circadian clock*:** Although transcription regulation has been the primary focus of most studies examining the underpinnings of the molecular clock machinery, post-transcriptional mechanisms have garnered increasing interest as an additional regulatory layer (reviewed by, Staiger and Koster, 2011; Cibois et al., 2010). Initial studies in *Drosophila* highlighted that rhythmic *Per* mRNA degradation was regulated at the post-transcriptional level (So and Rosbash, 1997). This was recently also observed for mammalian *Per2* (Woo et al., 2009). AU-rich regions (ARE's) in the 3' untranslated region (UTR) of *Per2* mRNA are targeted by the RNA-binding protein, polypyrimidine tract-binding protein 1 (PTBP1), promoting degradation of *Per2* transcripts. A similar mechanism involving the heterogenous nuclear riboprotein D (hnRNPD) was described in post-transcriptional regulation of *Cry1* mRNA transcript as well (Woo et al., 2010). Some RNA-binding proteins, rather

than causing mRNA degradation, function to stimulate translation instead. One such protein, LARK (RBM4), binds to *cis*- elements in the *Per1* 3' UTR and promotes its translation (Kojima et al., 2007). MicroRNAs (miRNAs) are another important class of post-transcriptional regulators that are known to function as master switches regulating a variety of biological processes, including circadian rhythms. Mechanisms of miRNA biogenesis and action, particularly in modulating circadian rhythms biology, will be discussed in detail in the succeeding sections.

***Peripheral clocks- rhythms and synchronization*:** Cell-autonomous oscillations are not a property only of the SCN but that of all other cells of the body as well. Circadian rhythms in the expression of core clock genes and of other clock-controlled genes have been described in various peripheral tissues (Kita et al, 2002; Panda et al., 2002; Storch et al., 2002; Yamamoto et al., 2004). Interestingly, using transgenic mice expressing a PER2::LUCIFERASE reporter, Yoo et al. (2004) also showed that peripheral tissues continue to express self-sustained rhythms for up to 20-cycles in isolation (out of the body). Importantly, the same report showed that SCN ablation did not abolish rhythms in individual tissues, but it did however cause some loss of synchrony in rhythms among peripheral oscillators, indicating that rather than 'drive' rhythms, the SCN may primarily functions to synchronize clocks in peripheral tissues. Additionally, serum-shock treatment was shown to induce cycling of core clock genes and the clock-controlled gene DBP, in Rat-1 fibroblasts. These rhythms in mRNA expression cycled for 2-3 days and provided preliminary evidence that circadian rhythmicity is a cell-autonomous property of cells even outside the SCN (Balsalobre et

al., 1998). More convincing evidence was provided in studies using *Bmal1-luc* and PER2::LUC reporters to monitor bioluminescence rhythms in individual fibroblasts. Clock gene rhythms in individual cells were observed to persist, even after the damping of ensemble oscillations due to loss of intercellular synchronization (Welsh et al., 2004).

Thus cell-autonomous oscillations are the underlying characteristic of clocks, in the SCN and throughout the body as well. However, mechanisms of interaction between individual clocks to produce ensemble synchronization are yet unclear. The identities of potential signaling factors responsible for this synchronization, either within local environments in the SCN or in peripheral tissues, or systemically between different tissues or organ systems provide exciting avenues for investigation. Based on details provided in the preceding sections, these signals are likely to be diffusible. Given their presence in membrane-bound vesicles in the circulation, and evidence for communication between cells, miRNAs, which act as master switches of gene expression, provide potential candidates capable of communicating temporal information between cells and functioning in synchronization of circadian oscillators within local environments.

Non-coding RNAs- Historical Perspective

The central dogma of biology for decades had been that DNA is transcribed into RNA, which is then translated into proteins. However, it is now clear that the functions of RNA extend well beyond just the transmission of DNA-encoded heritable information to corresponding peptides and proteins. Indications of potential non-coding functions of

RNA's were initially provided by genomic analyses revealing that the number of protein-coding genes had previously been vastly overestimated. Only about 20,000-25,000 protein coding genes, representing approximately 3-5% of the total euchromatin, were identified in the human genome (International Human Genome Sequencing Consortium, 2004). Although the remaining non-protein coding 95-97% of euchromatin was initially considered "junk", high-resolution studies have now revealed that RNAs encoded from these regions are associated with vital regulatory functions that control development, physiology and pathologies (Pennisi, 2012). Indeed, non-coding RNAs have garnered increased attention for their biological importance, and based on ratios of non-coding to genomic DNA, some posit that regulatory RNAs may play vital roles in complexity of organisms (Mattick, 2007). The recent ENCODE project described 8800 small RNA molecules that do not code for any proteins. MicroRNAs (miRNAs) are a sub-type of such non-coding RNAs and provide the primary focus of this dissertation.

Discovery and Biogenesis of MicroRNAs

Discovery: The first studies describing short temporal RNAs (stRNAs), now termed miRNAs, were simultaneously published from the laboratories of Victor Ambros and Gary Ruvkun (Lee et al., 1993; Wightman et al., 1993). In these studies, a small RNA *lin-4* was reported to mediate post-transcriptional regulation via specific sequences in the 3' untranslated region (UTR) of a developmental gene *lin-14* in *C. elegans.* Furthermore, the *lin-14* 3' UTR was necessary and sufficient for this *lin-4* mediated regulation. Seven years later, another report implicated *let-7*, a small 21nt RNA in the

regulation of developmental timing in *C. elegans* by interactions with the 3' UTRs of target genes including *lin-14* (Reinhart et al., 2000). Importantly, while *lin-4* is found only in worms, *let-7* is highly conserved across vertebrates and in some invertebrates as well (Pasquinelli et al., 2000). Since then, interest in miRNAs has exploded and miRNA databases currently estimate presence of more than 2000 human, 1200 mice, 400 fly and 350 worm mature miRNAs, many of which have already been empirically validated (Griffiths-Jones, 2004).

Biogenesis- transcription: miRNAs are found at two locations in the genome- in the introns of genes and in intergenic regions, either independently or as a cluster of multiple miRNAs, presumably transcribed together. Recent estimates indicate that approximately 40-50% of human miRNAs are intronic, with intergenic miRNAs distributed as clusters or independent miRNAs in an approximately 1:1 ratio (Lagos-Quintana et al., 2003; Rodrigues et al., 2004; Saini et al., 2007). Generally, miRNAs are thought to be transcribed by RNA polymerase II to generate a primary-miRNA (pri-miR) transcript with a 5' cap as well as 3' poly(A) tail (Lee et al., 2002; Lee et al., 2004). However, there is some evidence for RNA polymerase III-mediated transcription of miRNA biogenesis as well (Faller and Guo, 2008). Additionally, many miRNAs are expressed in a tissue specific manner and transcriptional regulators have also been shown to induce or inhibit expression of specific miRNAs (reviewed by, Zhang and Zeng, 2010).

Biogenesis- canonical miRNA maturation pathway: Following transcription, miRNAs undergo processing before reaching functional maturity (Fig. 3). While other

mechanisms have also been identified, the canonical miRNA biogenesis pathway involves sequential processing by two RNase III enzymes. In the first step, a primary miRNA (pri-miRNA) transcript, containing characteristic hairpin loop(s), is acted upon the nuclear RNase III endonuclease, DROSHA, along with its partner DGCR8. The DROSHA/DGCR8 microprocessor complex cleaves off the hairpin loop leaving a 5' phosphate and 2-3nt long 3' overhang (Lee et al., 2003; Gregory et al., 2004). This cleavage product is ~70nt long and is designated as a premature miRNA (pre-miRNA). Pre-miRNA(s) are transported out of the nucleus by exportin-5 in a Ran-GTP mediated mechanism (Yi et al., 2003; Lund et al., 2004). Once in the cytoplasm, the pre-miRNA is further cleaved by the cytoplasmic RNase III endonuclease, DICER to give two size-matched single stranded ~19-23nt transcripts (Bernstein et al., 2001; Hutvagner et al., 2001). Thus, each pre-miRNA can potentially code for two mature miRNAs; in some cases both are associated with biological functions. For example, premature miR-142 codes for two functional mature miRNAs miR-142-5p and miR-142-3p. However, in some cases only one strand is functional and the other is degraded. For example, mature miR-152 is generated from the 3' tail of the premature miR-152 stem-loop structure, while the corresponding miRNA generated from the 5' tail is preferentially degraded.

The canonical maturation pathway involving DROSHA and DICER is primarily attributed to biogenesis of clustered or individual intergenic miRNAs. However, ~30-40% of miRNAs are also present in intronic regions and are frequently transcribed as part of the premature mRNA transcript (Rodriguez et al., 2004; Baskerville and Bartel, 2005). Although it was initially assumed that miRNA processing begins after cleavage

of the intron by the splicing machinery, the canonical DROSHA-dependent pathway can also cause cleavage of the hairpin pre-miRNA before splicing events (Kim and Kim, 2007). The splicesome-independent canonical miRNA maturation pathway can thus provide for co-expression of both the miRNA and mRNA transcripts.

Biogenesis- non-canonical miRNA maturation pathways: Intronic miRNAs are also processed in a DROSHA-independent manner, and hence termed the non-canonical pathway. First identified in *Drosophila,* some short hairpin introns were mapped precisely to splice-acceptor and -donor sites. Splicing of these introns generates a functional pre-miRNA independent of DROSHA activity (Fig. 3). The pre-miRNA is exported to the cytoplasm, and cleaved by DICER, yielding mature miRNAs (Okamura et al., 2007; Ruby et al., 2007). Given their intronic origin, these DROSHA-independent, DICER-dependent miRNAs were later termed 'mirtrons' and have now been identified in mammalian and avian species as well (Berezikov et al., 2007; Glazov et al., 2008). Yet other non-canonical DROSHA-independent miRNAs arising out of t-RNAs or endo-shRNAs have also been described (reviewed by, Miyoshi et al., 2010; Yang and Lai, 2011). A recently discovered non-canonical pathway involves DROSHA-dependent, but DICER-independent processing and maturation of miR-451. The primary transcript, *pri-miR-451* is cleaved by DROSHA to generate a *pre-miR-451* that contains a ~18nt duplex stem which is too short to be recognized by DICER for cleavage. This transcript instead is acted upon by AGO2 to generate a longer than average (~30nt) miR-451, which is later trimmed down to a 23nt form (Cifuentes et al., 2010; Yang et al., 2010). It should be noted that miR-451 is conserved only among vertebrates.

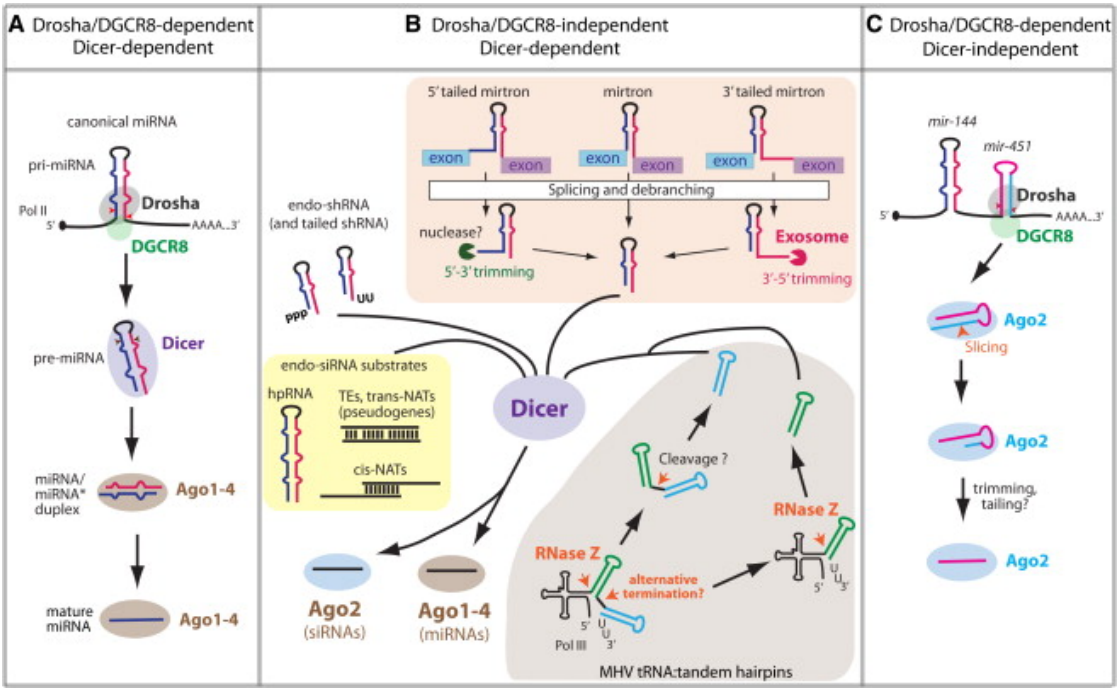

Figure 3. Simplified model of canonical and non-canonical pathways for biogenesis of mature miRNAs in animals. Reprinted from Molecular Cell, 43 /6, Jr-Shiuan Yang and Eric C. Lai, Alternative miRNA Biogenesis Pathways and the Interpretation of Core miRNA Pathway Mutants, 892-903, Copyright (2011), with permission from Elsevier.

Target Recognition and Biological Action of MicroRNAs

RISC loading: Mature miRNAs associate with the RNA-induced silencing complex (RISC) and affect their biological activity. In the cytoplasm, DICER, stabilized by its partner TRBP, associates with Argonaute-2 (AGO2) and forms the RISC loading complex (RLC). In the cytosol, pre-miRNA hairpins associate with the RLC and undergo processing by this complex to generate mature miRNAs (Chendrimada et al., 2005; Gregory et al., 2005; MacRae et al., 2008). After cleavage of the pre-miRNA transcript, DICER and TRBP are thought to dissociate from the RCL allowing for the duplex to unwind and incorporate the mature strand into the RISC. Mechanistic details of whether the unwinding involves a dedicated helicase are unclear. However, AGO2 has been shown to catalyze the process in an ATP-independent fashion (Maniataki et al., 2005). Additionally, AGO2 has also been shown to create single-stranded nicks without DICER involvement, and hence may also catalyze miRNA processing. Thus AGO2 could potentially have a dual-role, as a helicase and an RNase (Diederichs et al., 2007).

Target recognition: Mature miRNAs interact with complementary sequences primarily in the 3' UTRs of their target genes. This interaction is generally characterized by imperfect pairing due to some mismatches in complementarity between the miRNA and its target in the 3' UTR. However, an important factor for miRNA activity is perfect base pairing between nucleotides 2-8 of the miRNA and the target 3' UTR. This region of the miRNA, primarily responsible for target recognition and specificity, is called the 'seed' region and in vitro reporter assays indicate that 7-8mer sites complementary to seed region of candidate miRNAs are sufficient for repression of the target 3' UTRs

(Doench and Sharp, 2004; Brennecke et al., 2005). Some evidence also indicates that seed region complementarity is not always sufficient for repression and that miRNA nucleotides 15-19 may also have significant functional implications as 3' supplementary or 3' compensatory regions (Grimson et al., 2007).

Biological action: Fundamental observations on miRNA function in repressing translation were derived from initial experiments on *C. elegans*. The *lin-4* miRNA was found to inhibit protein synthesis after initiation of translation (Olsen and Ambros, 1999). Additional mechanisms have now been discovered and miRNA-mediated regulation has also been observed at the translation initiation step (reviewed by, Filipowicz et al., 2008). Human *let-7* miRNA inhibits translational initiation in a 5' cap dependent manner. The inhibition was relieved when the 5' cap was replaced by an internal ribosome entry site (IRES), indicating that *let-7* binding to the 3' UTR potentially disrupts eukaryotic initiation factor (eIF) recognition of the 5' cap region (Humphreys et al., 2005; Pillai et al., 2005). Recent studies comparing protein production simultaneously with mRNA expression inferred that in mammalian systems, the predominant mode of miRNAs biological activity involves mRNA destabilization (Baek et al., 2008; Guo et al., 2010). Nevertheless the primary outcome of miRNA-mRNA interactions results in repression of protein levels. There is however some contradictory evidence that implicates miRNAs in up-regulation of their target mRNAs (Vasudevan et al., 2007). In this study, repression by miRNAs was observed during the proliferative phase, but the same miRNAs switched from repressing to activating their

targets upon arrest of the cell cycle. It is yet unknown whether this is a global phenomenon or is specific to the miRNA(s) studied during this specific analysis.

Contribution of MicroRNAs to Circadian Timekeeping

Critical contributions of miRNAs have been demonstrated in a multitude of biological processes including development, cell cycle, metabolism, immune function, to name a few (Ambros, 2004; Bartel, 2004). Recently, miRNA mediated regulation has been described in the circadian timekeeping mechanism as well. In a pioneering study, Cheng et al. (2007) reported that two miRNAs, miR-219 and miR-132, were both rhythmically expressed in the mouse SCN. Knockdown experiments revealed that miR-219 and miR-132 modulate the free-running period of locomotor activity and amplitude of phase-resetting after a light-pulse, respectively. The miRNAs are involved in regulation of cellular excitability and also indirectly modulate the core clock genes *Per1* and *Per2*. Following this initial report, rhythmic miRNA expression was also described in *Drosophila* and in *Arabidopsis* (Yang et al., 2008; Sire et al., 2009). In the chicken retinal photoreceptors a rhythmic miRNA, miR-26a, was shown to regulate the 1c subunit of L-type voltage gated calcium channels providing for potential modulation of core clock outputs (Shi et al., 2009). Another miRNA, miR-122, was shown to modulate amplitude of circadian expression of numerous genes in the mouse liver, including those involved in rhythmic regulation of metabolism (Gatfield et al., 2009). A bona-fide role for miRNAs in regulating clock outputs was recently revealed in studies demonstrating that miR-279 modulates *Drosophila* rest-activity rhythms via targeting the JAK/STAT

pathway ligand unpaired (*Upd*). Knockout of the miRNA however did not affect the core clock gene *Per*, indicating that the miRNA-mediated regulation was downstream of the clock (Luo and Sehgal, 2012). The first evidence of miRNA involvement in core clock mechanisms was derived from the observation that in *Drosophila*, the miRNA, *bantam*, targets *clock* mRNA via multiple canonical sites in the *clock* 3' UTR. Other interesting clock-related targets pulled down during the AGO1 immunoprecipitation experiment were virille (*vri*) and clockworkorange (*cwo*) (Kadener et al., 2009). A study reporting miRNA mediated regulation of a core clock component in mammals involved the miR-192/194 cluster of miRNAs that target the mammalian *Per* genes (Nagel et al., 2009).

Extracellular MicroRNAs

Discovery and significance: While miRNAs perform their primary biological functions intracellularly, an exciting line of investigation has recently focused on the study of miRNAs that are expressed extracellularly. One of the first reports in this area involved retrospective analysis for the presence and expression of diffuse B-cell lymphoma (dBCL) associated miRNAs in serum samples from diseased patients. In this study, miR-155, miR-21 and miR-210 were upregulated in patient sera when compared to healthy controls (Lawrie et al., 2008). A far more extensive study demonstrated that numerous miRNAs were expressed in human plasma (Mitchell et al., 2008). The most salient findings of the study were: **1**) endogenous human miRNAs from donor plasma were stable at room temperature for up to 24h (the length of time examined), **2**) endogenous miRNAs from plasma were resistant to multiple cycles of freeze-thaw (eight

cycles were tested), **3**) exogenous 'naked' (not protected) miRNAs spiked-in to plasma were rapidly degraded (presumably by endogenous RNases in plasma) and **4**) addition of denaturing solution containing RNase inhibitor prevented degradation of spiked-in exogenous miRNAs in plasma. Circulating extracellular miRNAs were also shown to be resistant to exogenous RNaseA mediated digestion, suggesting that this miRNA pool may be enclosed in some protective membrane (Chen et al., 2008; Gilad et al., 2008; Hunter et al., 2008). Expression of extracellular miRNAs has now been observed in many biological fluids including saliva, breast milk, amniotic fluid and urine (Weber et al., 2010). Because miRNAs are associated with many pathological states, profiling of extracellular miRNAs has generated tremendous interest for their potential use as diagnostic and prognostic biomarkers, primarily of cancers, but including other diseases as well (Kosaka et al., 2010a; Wang et al., 2010a; Gao et al., 2011; Mizuno et al., 2011; Zham et al., 2011).

Membrane- or protein- bound extracellular miRNAs: Given that the analyses of extracellular miRNAs are only in their infancy, it is not surprising that there is currently a debate regarding whether circulating miRNAs are vesicle encapsulated or protein-bound. Two recent reports claimed that a majority of extracellular miRNAs did not sediment at ultracentrifugation speeds used to precipitate microvesicle and exosomal content, and instead co-fractionated with protein complexes rather than with vesicles (Fig. 4). Further analyses revealed a role for AGO2 in binding to and stabilization of extracellular miRNAs (Arroyo et al., 2011; Turchinovich et al., 2011). Another study, also using ultracentrifugation, detected the presence of some RNA-binding proteins in

the extracellular environment and concluded that the protein nucleophosmin 1 (NPM1) may be involved in miRNA- binding and stabilization extracellularly (Wang et al., 2010b).

Nevertheless, there is also clear evidence for presence of miRNAs in vesicles like microvesicles and exosomes which have a typical lipid bilayer structure enclosing a central lumen (Fig. 4). Different types of vesicles can be distinguished primarily by their size and intracellular origin (reviewed by, Thery et al., 2009). Microvesicles constitute the largest of the secreted vesicles with diameters of more than 100nm and have been observed to be released from various cell types, including some tumors, neutrophils and dendritic cells. Microvesicles are thought to form at the cell surface by outward budding of the plasma membrane (Chen et al., 2012). In contrast, exosomes are smaller in size and generally between 30-100nm in diameter. Exosomes arise out of multi-vesicular endosomes (MVE's) which are formed when plasma membrane proteins transfer to an early endosome by inward budding. Fusion of the intraluminal components with early endosomes forms multivesicular bodies (MVB's) which are sorted either for lysosomal degradation or for release by plasma membrane fusion. Exosomes are vesicles formed inside MVB's and are released into extracellular environments upon fusion of the MVB with the plasma membrane of the cell (Chen et al., 2012).

Intercellular communication: There is evidence that the miRNA constituents of both microvesicles and exosomes are transferred between cells. Using GFP-expressing embryonic stem cells, Yuan et al. (2009) recently showed that microvesicles dock with and transfer fluorescent signal to recipient cells. Additionally, exposure of mouse

embryonic fibroblasts to miRNA containing ES-cell culture derived microvesicles resulted in an increase in corresponding intracellular miRNA expression in the recipient cells. Considering that exosomal content can be sorted before assembly into MVB's, more robust evidence exists regarding the involvement of this vesicle sub-type in communication of extracellular miRNAs. In a pioneering study, Valadi and colleagues (2007) demonstrated that human and mouse mast cell derived exosomes shuttle cargo between cells. The salient findings of their study were: **1**) exosomal RNA was devoid of 18s and 28s rRNA species and thus preferentially enriched for small RNA's (< 200nt long), **2**) although exosomes expressed ~8% all of the mRNA species expressed in cells, there was no correlation between cellular RNA and exosomal RNA expression (i.e., exosomal RNA was not merely an outcome of cellular RNA distribution), suggesting active sorting mechanism(s) may regulate packaging of RNAs into exosomes, **3**) similar to mRNA expression, exosomes contained miRNAs, but some miRNA species had higher extracellular (in exosomes) expression compared to their intracellular levels (in donor cells), **4**) MC9 derived exosomes exhibited specificity, in delivering their cargo only to MC9 recipient cells but not to CD4 cells, thus implying that exosomes may mediate specificity of signaling as well. These findings were further extended by mechanistic analysis of the miRNA secretion pathway demonstrating that miRNA release was regulated by the enzyme neutral sphingomyelinase 2 (nSMase2), which is responsible for biosynthesis of the sphingolipid, ceramide (Kosaka et al., 2010b). Inhibition of this ceramide-dependent pathway by a pharmacological drug or by siRNA-mediated knockdown of *nSMase2* reduced miRNA secretion, while overexpression of

nSMase2 had a stimulatory effect. Importantly, exosomes were also found to transfer their miRNA cargo from donor to recipient cells, with the transferred miRNAs exhibiting biological activity in the recipient cells (Kosaka et al., 2010b). Recent studies of antigen-presenting cells (APC) provide further indication that miRNA release is regulated by *nSMase2* via a ceramide-dependent pathway (Fig. 4) (Mittelbrunn et al., 2011).

How extracellular vesicles transfer their cargo to recipient cells is largely unknown. Considering vesicles, especially microvesicles, contain cell surface markers unique to their parental cells, it is possible that receptor-ligand interactions may play a role in endocytosis or membrane docking. Certainly preferential uptake of microvesicles has been reported previously (Cocucci et al., 2009; Simons and Raposo, 2009). However, the mechanistic details of vesicle specificity and uptake are yet unknown.

Other than microvesicles and exosomes, high-density lipoproteins (HDL) have been implicated in the trafficking of extracellular miRNAs. In a recent study, HDL-bound miRNAs were detected in plasma and their export out of cells was regulated by *nSMase2*. Additionally, the results indicate that scavenger receptor class B type I is involved in mediating uptake of the HDL-bound miRNAs into recipient cells, where the miRNAs retained their biological activity (Vickers et al., 2011).

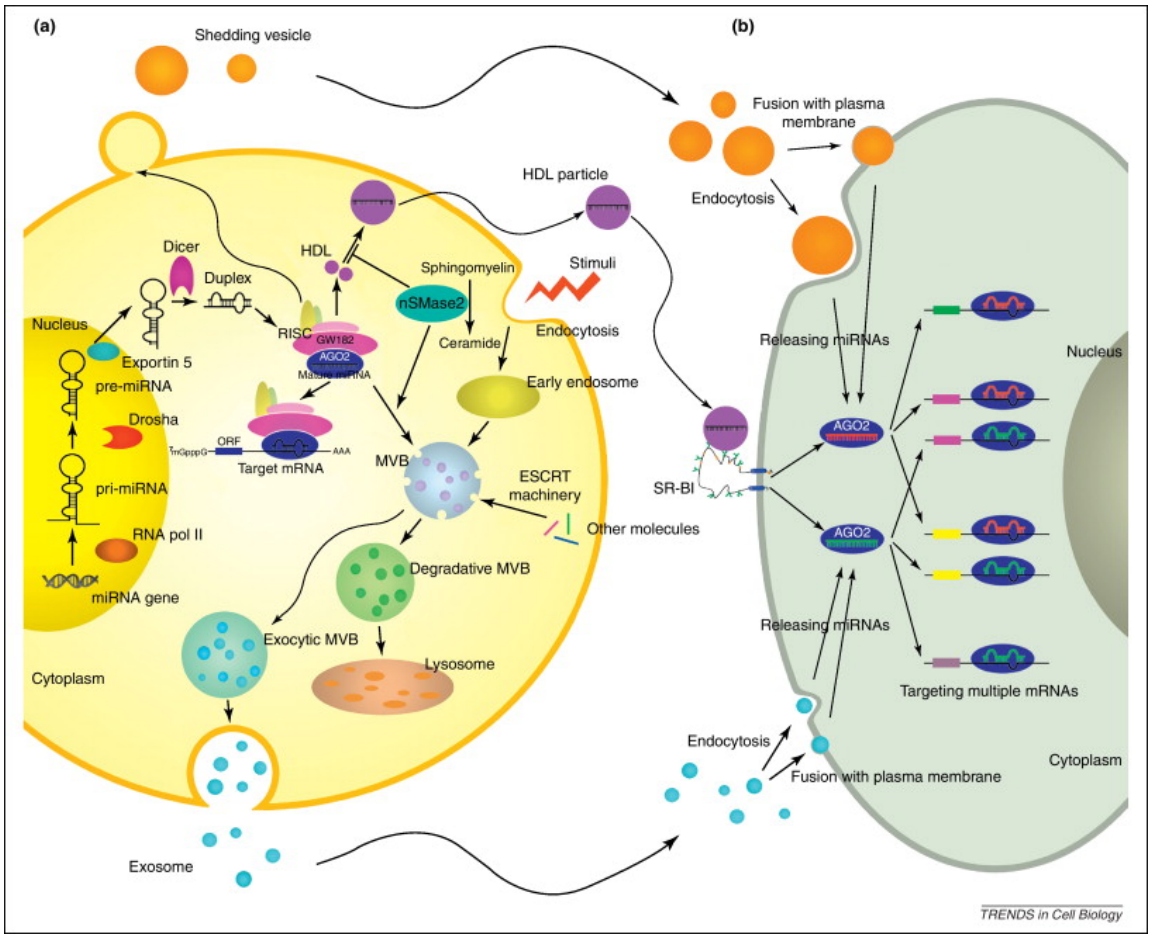

Figure 4. Simplified model of various pathways for secretion and uptake of extracellular miRNAs. Reprinted from Trends in Cell Biology, 22 /3, Xi Chen, Hongwei Liang, Junfeng Zhang, Ke Zen and Chen-Yu Zhang, Secreted microRNAs: a new form of intercellular communication, 125-132, Copyright (2012), with permission from Elsevier.

Summary and Specific Aims

Circadian clocks regulate rhythms in molecular, behavioral and physiological processes in animals. The suprachiasmatic nuclei (SCN) of the anterior hypothalamus function as the master pacemaker of circadian rhythms in mammals. The gears of the clock have now been dissected, resulting in the identification of 'core' and 'regulatory' elements of the clock. Current evidence indicates that rhythmic transcription is not the sole requirement for generation of circadian rhythms, but post-transcriptional and post-translational processes are also critical for robust rhythmicity.

MicroRNAs (miRNAs) are small non-coding RNAs that bind to 3'UTR's and regulate translation or stability of target mRNAs. Although miRNAs have been implicated in regulating the timing of numerous biological events, their relative contribution to the hierarchical organization of the circadian timekeeping system in mammals is unclear. The central hypothesis of my dissertation research is that miRNAs are important cis- and trans-acting regulators of core clock components in the circadian timekeeping mechanism. To test this hypothesis, my dissertation experiments first examined the role of intracellular miRNAs in regulation of the core molecular clock gene, *Bmal1*, in the SCN pacemaker and in peripheral oscillators. Because recent studies indicate that miRNAs are not just expressed intracellularly, but are also packaged in vesicles, transported extracellularly and communicated locally to other cells (Valadi et al., 2007; Kosaka et al., 2010b), I have also conducted studies examining the role of extracellular miRNAs in communication of temporal information between autonomous circadian clocks in peripheral cell types.

Specific Aim 1: Are miRNAs involved in regulation of core clock genes and circadian rhythmicity in the SCN? Comparisons of rhythmic transcriptome and proteome indicate that about ~50-89% of rhythmically-regulated proteins have corresponding mRNA transcripts with non-rhythmic profiles (Panda et al., 2002; Storch et al., 2002; Reddy et al., 2006; Deery et al., 2009). This observation suggests that post-transcriptional and/or post-translational processes may play a significant role in regulating circadian rhythmicity. Because miRNAs function as post-transcriptional regulators modulating the timing of various biological events (Ambros, 2004; Poethig, 2009), the objective of this aim is to test whether: **1**) specific miRNAs target the 3'UTR's of clock genes, **2**) clock-gene-targeting miRNAs are expressed rhythmically in the SCN, and **3**) miRNAs regulate circadian rhythmicity in SCN cells.

Specific Aim 2: Are clock-gene targeting miRNAs expressed extracellularly? Recent studies have indicated that miRNAs, encapsulated in vesicles, are present in the circulation and that their levels change in different pathological and physiological states (Hunter et al., 2008; Mitchell et al., 2008). Because rhythmicity is a prevalent property among core and regulatory elements of the circadian clockworks in most cells and tissues throughout the body, the objective of this aim is to test whether: **1**) specific miRNAs with clock genes as predicted targets are expressed in serum, **2**) their expression is marked by daily fluctuations in vivo, and **3**) rhythmic miRNAs affect 3′ UTR activity of their target clock gene(s). Although serum could predominantly be associated with systemic signals, it could also be considered a local environment for hematopoietic and vasculature-associated cells. Thus, serum miRNAs could function

locally within these cell types, or globally as systemic cues between peripheral organs. Additionally, it is unlikely that the SCN would be contributing to miRNAs circulating in serum. Serum miRNAs are more likely to be contributed by peripheral, rather than central nervous system oscillators and, as such, may be important indicators or modulators of circadian rhythms in peripheral oscillator populations.

Specific Aim 3: Are extracellular miRNAs involved in temporal synchronization of circadian rhythmicity in peripheral oscillators? Endogenous rhythmicity is a characteristic not only of the SCN, but also of peripheral tissues (Yamazaki et al., 2000; Yoo et al., 2004). It remains unknown what factors contribute to synchronization of rhythms within or between different peripheral tissues. Because secreted vesicles may mediate exchange of molecular signals like miRNAs between cells (Valadi et al., 2007; Yuan et al., 2009), to the objective of this aim is to test whether: **1**) miRNAs modulate clock gene protein levels and alter rhythms in core components of the molecular clockworks in a peripheral oscillator model, **2**) overexpression of miRNAs increases their extracellular abundance in culture medium, **3**) exposure to miRNA containing conditioned medium from donor cells increases intracellular levels of the corresponding miRNAs in recipient cells, **4**) the internalized miRNAs are functional in recipient cells, and **5**) disruption of vesicular communication using exocytosis and endocytosis inhibitors affects ensemble circadian rhythms.

This dissertation describes experiments associated with these specific aims as well as the results and conclusions obtained thereof.

CHAPTER II

ROLE OF miR-142-3p IN THE POST-TRANSCRIPTIONAL REGULATION OF THE CLOCK GENE *Bmal1* IN THE MOUSE SCN

Introduction

In mammals, the suprachiasmatic nuclei (SCN) of the anterior hypothalamus function as the master pacemaker mediating the generation and light-dark entrainment of circadian rhythms (Turek, 1985; Ralph et al., 1990). In addition to coordinating circadian rhythmicity in other brain regions and peripheral tissues, the SCN also generates ensemble and cell-autonomous circadian oscillations in many of its cellular and molecular processes independent of external input (Schwartz et al., 1980; Groos and Hendriks, 1982; Shibata et al., 1982; Boos and Mirmiran, 1990; Panda et al., 2002). These endogenous oscillations are especially prevalent in the expression of genes comprising the molecular clockworks and thus vital to the circadian oscillator and pacemaker functions of the SCN. Brain, muscle ARNT-like protein 1 (*Bmal1*), circadian locomotor output cycles kaput (*Clock*), *Period* (*Per1*& *Per*2), the Cryptochrome *(Cry1 & Cry2)* genes, and *Rev-erbα* are rhythmically regulated in interlocked feedback loops that comprise the molecular clockworks common to both SCN and peripheral cells.

While post-translational processes including phosphorylation, ubiquitination, sumoylation, and acetylation have garnered the most attention for their role in the regulation of the molecular clockworks (Lee et al., 2001; Gallego and Vishrup, 2007), recent studies suggest that post-transcriptional mechanisms may also play a role in the

modification of clock proteins and oscillatory regulation of components in the circadian feedback loops (Kojima et al., 2011; Staiger and Koster, 2011). In this regard, microRNAs (miRNAs) have been implicated in the post-transcriptional regulation of the mammalian circadian clock mechanism. Mature miRNAs are small non-coding RNAs, usually 19-25 nucleotides in length, that interact with miRNA-recognition elements (MRE) in the 3' untranslated regions (UTR) of target genes to induce mRNA de-stabilization and/or translational repression (Lee et al., 2003; Bernstein et al., 2001; Bartel, 2004; He and Hannon, 2004). Evidence for miRNA function in SCN clock control of circadian rhythms is derived from observations indicating that in the SCN, miR-219 and miR-132 exhibit circadian rhythms of expression and antagonism of these miRNAs respectively alters the circadian period and light-induced phase shifts of the mouse activity rhythm (Cheng et al., 2007). In addition, miRNAs have been linked to circadian clock function in peripheral tissues as antisense inhibition of miR-122 expression induces post-transcriptional perturbations in the circadian expression of many genes involved in hepatic lipid and cholesterol metabolism (Gatfield et al., 2009).

Despite the increasing evidence that miRNAs are involved in the regulation of mammalian circadian rhythms, little is known about the role of specific miRNAs as modulators of essential components of the molecular clockworks. In *Drosophila*, the miRNA, *bantam*, has been shown to regulate *clock* translation via interactions with multiple target sites within the *clock* 3' UTR (Kadener et al., 2009). The primary objective of the present study was to determine whether specific miRNAs contribute to the post-transcriptional regulation of *Bmal1*. Because previous studies indicate that miR-

142-3p and miR-494 may act synergistically and modulate *Bmal1* 3' UTR activity (Shende et al., 2011), we examined the role of these miRNAs in the post-transcriptional regulation of the molecular clock by determining whether: **1**) miR-142-3p is rhythmically expressed in the SCN in vivo and in an immortalized SCN cell line; and **2**) miRNA overexpression affects endogenous BMAL1 protein rhythms in SCN cells.

Materials and Methods

Animals and SCN tissue collection: Experimental subjects were 40 male C57BL/6J mice at 6-8 weeks of age (JAX Mice & Services, Bar Harbor, ME). Animals were maintained in the vivarium at Texas A&M University System Health Science Center under a standard 12h light: 12h dark cycle (LD 12:12; lights-on at 0600h). Five animals were housed per cage and access to food and water was provided *ad libitum*. Periodic animal care was performed at random times. All procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University.

To determine whether miR-142-3p expression fluctuates rhythmically in the SCN in vivo, mice were maintained in LD 12:12 for 3 weeks prior to experimental analysis and then exposed to constant darkness (DD) beginning at lights-off in the LD 12:12 cycle (1800h). Beginning 15 hours later (0900h or circadian time [CT] 3), animals were sacrificed at 4h intervals (n=5) for 24h by decapitation using an infrared viewer (FJW Optical Systems, Palatine, IL). SCN tissue was immediately dissected as described

previously (Earnest and Sladek, 1986; Liang et al., 1998). All tissue samples were frozen in liquid nitrogen and stored at -80°C until further processing.

Cell culture: For in vitro analysis, immortalized SCN cell lines generated from *mPer2*^{Luc} knockin and from *Per1*^{ldc} / *Per2*^{ldc} mice were used to profile the temporal pattern of miR-142-3p expression. These cell lines were maintained and propagated as described previously (Farnell et al., 2011). Briefly, cells were grown on laminin-coated 60mm dishes (Corning, Inc.) in Minimal Essential Medium (MEM; Invitrogen, Inc.) containing 10% Fetal Bovine Serum (FBS), 3000µg/ml glucose, and 292µg/ml L-glutamine. The medium was replaced every 48h and cultures were split 1:3 to 1:5 every 3-4 days. Prior to experimentation, cells were expanded onto laminin-coated 6-well plates (BD Biosciences, Inc.). Approximately 24h after plating, the medium was changed so as to reduce the FBS concentration to 5% and on the following day cells were rinsed with calcium-magnesium free (CMF) buffer and then cultured in serum-free Neurobasal medium containing B27 supplement (1X, Invitrogen, Inc.). Cultures (n=5) were harvested at 4h intervals for 36h by trypsinization and cell pellets were flash frozen in liquid nitrogen. All samples were stored at -80°C until subsequent analysis of miRNA or mRNA content.

RNA extraction and Real-time PCR: cellular RNA was later extracted from individual SCN tissue samples and individual wells of *mPer2*^{Luc} and *Per1*^{ldc}/*Per2*^{ldc} SCN cells using miRNeasy kit (Qiagen, Inc.) according to the manufacturer's protocols. Total RNA was estimated using Nanodrop ND2000 (Thermo Scientific). Quantitative real-time PCR analysis for miR-142-3p was conducted using Taqman microRNA assays

(Applied Biosystems) as described previously (Shende et al., 2011). Briefly, miR-142-3p from individual samples was reverse transcribed using Taqman MicroRNA Reverse Transcription Kit and the cDNA equivalent of 1.5ng of total RNA was PCR amplified in an ABI PRISM 7500 Fast sequence detection system using the following standard conditions: **1**) heating at 95°C for 10 min, and **2**) amplification over 40 cycles at 95°C for 15 sec and 60°C for 1 min. As an endogenous control, U6 snRNA was also amplified from the same samples using identical parameters. Using the comparative C_T method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (PE-ABI), the relative abundance of miR-142-3p was calculated by normalization first to corresponding U6 snRNA levels in each sample and then to a calibrator consisting of pooled cDNA from multiple samples over the entire time series.

Relative quantification of *Bmal1* mRNA abundance in all samples was performed using SYBR-Green real-time PCR technology (ABI) as described previously (Allen et al., 2004; Farnell et al., 2008). To generate single-strand cDNAs, total RNA (1μg) from individual samples was reverse transcribed using random hexamers and Superscript III reverse transcriptase Kit (Invitrogen, Inc.). Real-time PCR analysis of was performed on duplicate aliquots using the cDNA equivalent of 1ng of total RNA for each sample. The PCR cycling conditions were: **1**) serial heating at 50°C for 2min and 95°C for 10min, **2**) amplification over 40 cycles at 95°C for 15 sec and 60°C for 1 min, and **3**) dissociation at 95°C for 15 sec, 60°C for 1min, 95°C for 15 sec and 60°C for 15sec. To provide an endogenous control, cyclophilin A (*Ppia*) was amplified with the cDNA

equivalent of 1ng total RNA from the same samples. The comparative C_T method was similarly applied to determine the relative expression of *mBmal1* mRNA.

The following primers were used for the real-time PCR analysis:

mBmal1 forward: 5'- CCAAGAAAGTATGGACACAGACAAA -3';

mBmal1 reverse: 5'- GCATTCTTGATCCTTCCTTGGT -3';

mCyclophilin forward: 5'- TGTGCCAGGGTGGTGACTT -3';

mCyclophilin reverse: 5'- TCAAATTTCTCTCCGTAGATGGACTT -3'.

Western Blotting: The effects of miR-142-3p overexpression on endogenous levels of mBMAL1 protein were examined in cultures of *mPer2*^{Luc} SCN cells that were derived from a single passage. Cells were expanded on laminin-coated 60-mm dishes (Corning, Inc.) maintained at 37°C and 5% CO2 in MEM (Invitrogen, Inc.) supplemented with 3000 ug/mL D-glucose, 292 ug/mL L-glutamine and 10% Fetal Bovine Serum (Hyclone). Prior to experimentation, *mPer2*^{Luc} SCN cells were seeded onto laminin-coated 6-well plates, and 24h later cultures were transfected with pEZX-MR04 miRNA expression vector for miR-142-3p (final conc. = 4.0ug per well) using Lipofectamine 2000 (Invitrogen, Inc.) according to the manufacturer's guidelines. Control cultures were similarly transfected with a pEZX-MR04 vector encoding a scrambled, non-targeting control sequence. Following transfection for 5h, cells were rinsed and cultured in growth medium supplemented with 5% FBS. On the following day, cells were rinsed with calcium-magnesium free (CMF) buffer and then maintained in serum-free Neurobasal medium containing B27 supplement (1X, Invitrogen, Inc.). Beginning 12h later, control and miR-142-3p-transfected cultures (n=5) were harvested

at 4h intervals for 24h by trypsinization and cell pellets were flash frozen in liquid nitrogen. All samples were stored at -80°C until subsequent processing. The pellets were sonicated in mammalian protein extraction reagent (MPER; Pierce, Inc.) supplemented with protease inhibitor cocktail (PMSF), and sample protein content in cell homogenates was measured using the bicinchoninic acid method (BCA Protein Assay Kit; Thermo Scientific Pierce). Each sample was loaded at ~20μg protein lysate per lane onto a 10% Tris-Glycine gel and electrophoresis was performed using an X-blot Mini-cell II apparatus (Invitrogen, Inc.). Following separation at 125V for 2h, proteins were transferred onto 0.45μm nitrocellulose membranes (Invitrogen) and blocked at room temperature for 1 hour with 5% skimmed milk in Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 (TBS-T). With interceding rinses in TBS-T, membranes were probed overnight at 4^{o}C with rabbit anti-BMAL1 (1:250; Abcam, Inc.) or a mouse monoclonal antibody against β-ACTIN (1:2000; Sigma Inc.) followed by a 1-hour incubation with horse-radish preoxidase (HRP)-conjugated donkey anti-rabbit IgG (1:1000; Jackson Laboratories) or goat anti-mouse IgG (1:2000; Jackson Laboratories). Immunoreactive signal for BMAL1 was generated using enhanced chemiluminescence (ECL) reagent (Thermo Scientific Pierce) and luminescence for size-appropriate bands were detected using a FluorChem Gel imaging system (Alpha Innotech Corp.). To control for differences in protein content between samples, signal intensity measurements for BMAL1 were normalized to the values for β-ACTIN in each sample. Densitometric analyses for immunoreactive bands were performed using NIH ImageJ software.

Statistical analyses: Time-dependent fluctuations in mmu-miR-142-3p and *Bmal1* mRNA expression were identified by cosine curve-fitting analysis using GraphPad Prism software. With α=0.05, waveform fittings with p-values $\leq$ 0.01 were considered statistically significant and therefore characterized by circadian variation. For waveforms characterized as rhythmic, peak values and those observed during the preceding or succeeding minimum were analyzed *post hoc* for statistical differences using independent t-tests. BMAL1 protein expression was first analysed by one-way analysis of variance (ANOVA). Paired comparisons between peak values and those observed during the preceding or succeeding minimum were analyzed *post hoc* for statistical differences using the Newman-Keuls sequential range test. The α-value was set at 0.05 for these *post hoc* analyses. Independent t-tests were performed on normalized luciferase bioluminescence data to determine the significance of miRNA-mediated repression of luciferase-reported *Bmal1* 3' UTR activity. The α-value was set at 0.05 for all independent t-tests.

Results

The circadian clock regulates rhythmic expression of mature miR-142-3p: Given our previous findings implicating miR-142-3p and miR-494 as potent repressors of the *Bmal1* 3' UTR, we investigated potential clock-related mechanisms regulating expression of these miRNAs. Upstream regions of the miRNA loci were examined for evidence of E-box or CRE-box elements, which are known to drive rhythmic transcription under control of the circadian clock and light respectively. The presence of

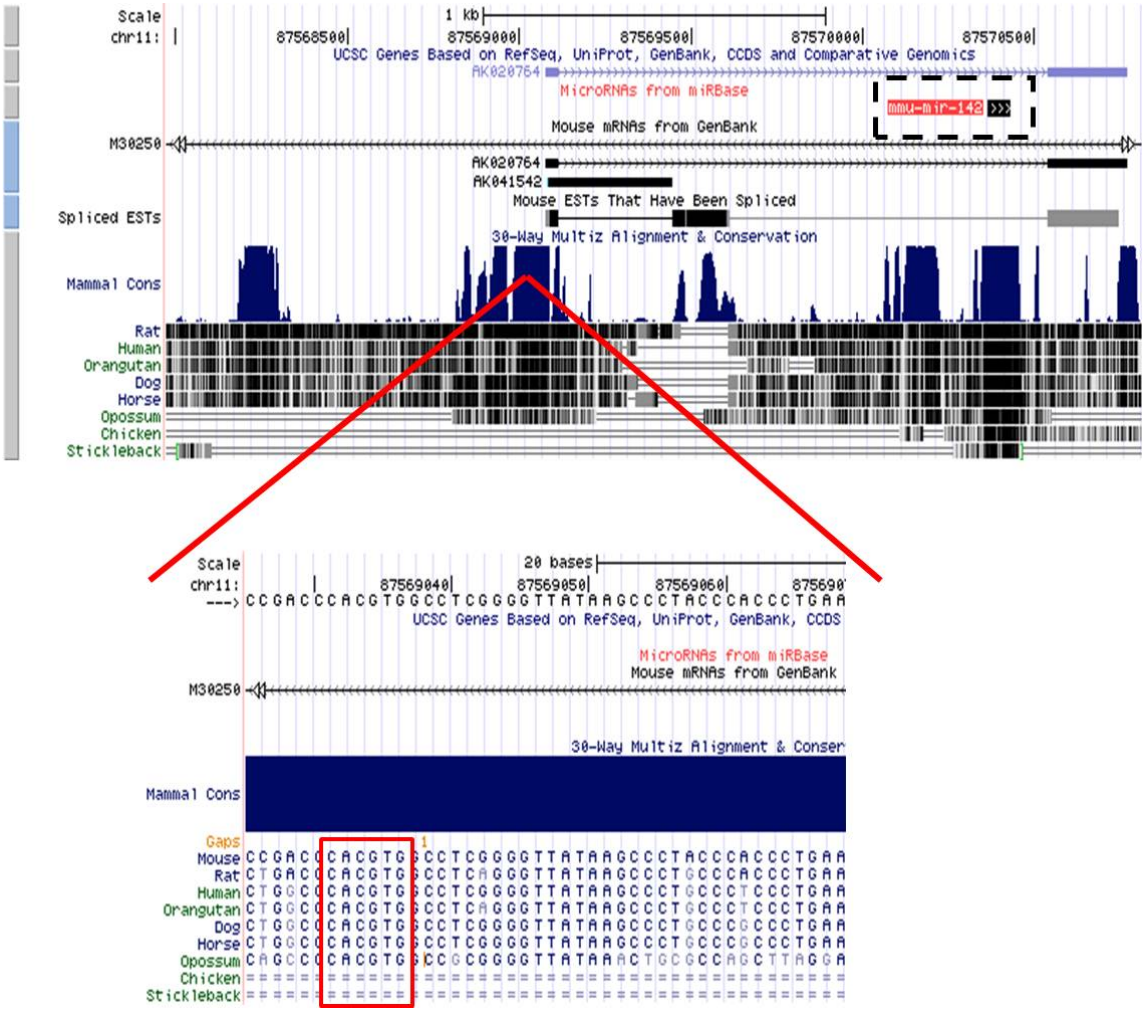

Figure 5. Prediction of canonical E-box element upstream of the mouse miR-142 locus. Diagrammatic representation of a well conserved '-CACGTG-' sequence located from bases 87569031-87569036, upstream of the miR-142 locus on the plus strand of mouse chromosome 11.

a canonical, CACGTG, E-box element was identified ~1.5Kb upstream of the miR-142 locus and analysis of syntenic regions indicated that this element is highly conserved among mammalian lineages (Fig. 5). Since miR-494 is located in a cluster of 21 miRNAs spanning ~10-kb of the mouse chromosome 12, regulatory elements contributing specifically to miR-494 transcription could not be identified.

The presence of an E-box indicated that miR-142 locus may be under transcriptional control by CLOCK-BMAL1. We next examined the temporal profiles of mature miR-142-3p expression in the SCN in vivo. Rhythmic fluctuations were observed in the relative expression of miR-142-3p (normalized to U6 snRNA; $p<0.01$) in the mouse SCN in DD (Fig. 6A). SCN expression of miR-142-3p peaked at CT 3 during the early subjective day and was significantly, and about 2-fold higher than trough levels observed ~12h later around early subjective night at CT 15 ($p<0.001$). Analysis of *Bmal1* expression in the same samples corroborated previous reports examining circadian variation in SCN content of *Bmal1* mRNA (normalized to *Cyclophilin*; $p<0.0001$) with expression peaking during the late subjective night (CT 23) and reaching a nadir during the mid-subjective day around CT 7 (Fig. 6A) (Honma et al., 1998; Maywood et al., 2003). The peak in *Bmal1* expression was significantly greater than the corresponding trough levels ($p<0.001$). Additionally, *Bmal1* mRNA levels in the mouse SCN peaked ~4h in advance of the crest in miR-142-3p expression, suggestive of *Bmal1* involvement in the activation of miR-142-3p transcription.

To determine whether miR-142-3p expression is also rhythmically regulated in vitro, studies were conducted in *mPer2*^{Luc} SCN cells because this immortalized line has

been shown to retain the endogenous rhythm-generating properties of the SCN (Farnell et al., 2011). Similar to the temporal profile for miR-142-3p expression in vivo, levels of mature miR-142-3p were rhythmic (normalized to U6 snRNA, $p<0.0001$) in cultured *mPer2*^{Luc} SCN cells (Fig. 6B**).** The amplitude of rhythmic miR-142-3p expression was robust, with 3- to 4-fold differences between peak and trough values. The maxima observed at 12h and 36h were significantly greater than the nadir attained at 20h ($p<0.05$). *Bmal1* mRNA expression was also rhythmic (normalized to *Cyclophilin*; $p<0.0001$) in the same *mPer2*^{Luc} SCN cultures. The circadian peaks in *Bmal1* mRNA expression observed at 0h and 24h were ~2-fold greater than the preceding and succeeding troughs observed at 8h and 36h. The observed *Bmal1* mRNA rhythm was antiphasic to the circadian profile in miR-142-3p expression from the same samples (Fig. 6B).

To verify that the observed rhythms in miR-142-3p expression were under control of the circadian clock, the temporal profile of miR-142-3p levels were analyzed in an SCN cell line derived from mice with targeted disruption of *Per1* and *Per2* genes (*Per1*^{ldc} */Per2*^{ldc} SCN) because: **1**) these mutant mice exhibit an arrhythmic behavioral phenotype (Bae et al., 2001), and **2**) our previous findings indicate that circadian pacemaking properties are similarly abolished in this cell line (Farnell et al., 2011). In *Per1*^{ldc} */Per2*^{ldc} SCN cells, both *Bmal1* and miR-142-3p expression remained largely at constant levels and showed no significant evidence of circadian rhythmicity ($p>0.05$) (Fig. 6C), indicating that the observed oscillations in miR-142-3p expression were indeed regulated by the circadian clock.

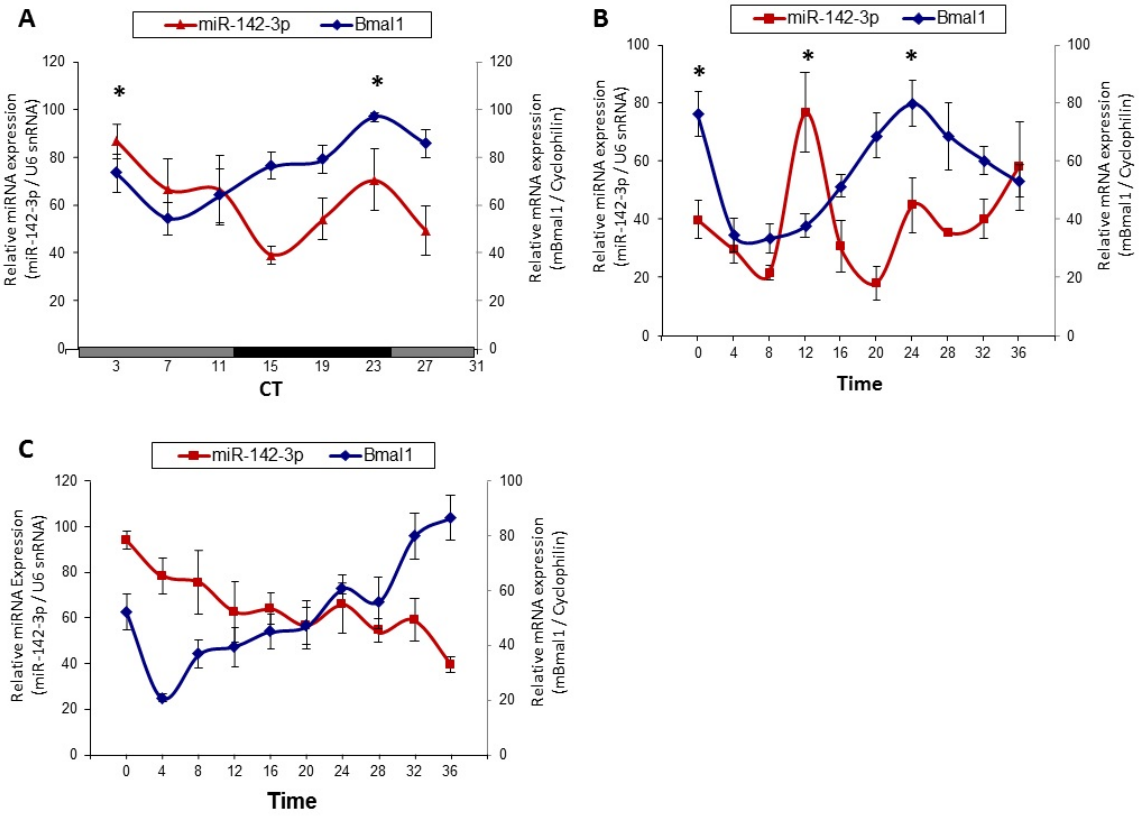

Figure 6. Temporal patterns of miR-142-3p and *Bmal1* in mouse SCN tissue and immortalized SCN cell lines. Symbols denote real-time PCR determinations of miR-142-3p (red traces) normalized to U6 snRNA or *Bmal1* (blue traces) normalized to *Cyclophilin* (mean ± SEM) collected at 4-hour intervals (n = 4-5). The plotted values correspond to the miR-142-3p and *Bmal1* ratios in SCN tissue from C57BL/6J mice during exposure to constant darkness (DD) (**A**), *mPer2*^{Luc} SCN cell line (**B**), and in SCN *Per1*^{ldc}/*Per2*^{ldc} cell line (**C**) and are represented as a percentage of the maximal value obtained for each gene. Asterisks indicate time points during which peak values for gene expression were significantly greater (p<0.05) than those observed during preceding or succeeding minima.

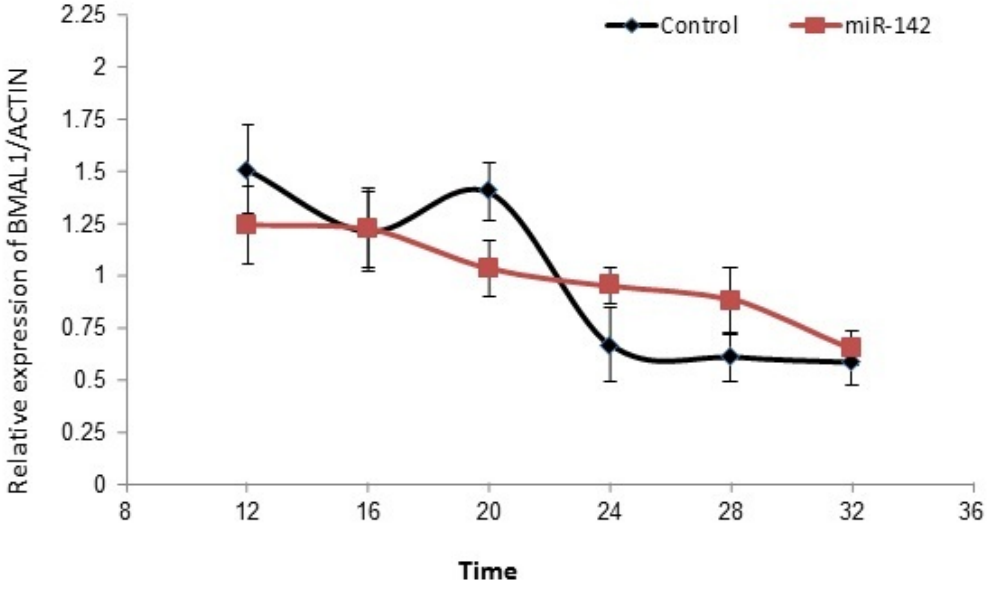

Figure 7. Temporal patterns of BMAL1 protein in immortalized mouse SCN cell line. Symbols denote optical densitometry determinations of BMAL1 protein expression (mean ± SEM) collected at 4-hour intervals (n = 4-5) from pEZX-MR04 miR-142 expression vector (red trace) or pEZX-MR04 control miRNA expression vector (black trace) transfected *mPer2*^{Luc} SCN cells. The plotted values correspond to ratios of BMAL1 protein normalized to β-*ACTIN* expression in each sample. Asterisks indicate time points during which peak BMAL1 expression was significantly greater (p<0.05) than those observed during succeeding minima or during the corresponding time point in miR-142-3p transfected samples.

miR-142-3p disrupts BMAL1 protein rhythms* in vitro*: To investigate functional implications of miR-142-3p in the regulation of circadian rhythms, we next determined whether overexpression of miR-142-3p affects ensemble circadian rhythms of BMAL1 protein expression in cultured *mPer2*^{Luc} SCN cells. Endogenous BMAL1 protein levels were profiled over 24-hours to assess the effect(s) of constitutive overexpression of miR-142-3p (Fig. 7). In control miRNA transfected *mPer2*^{Luc} SCN cultures, we observed robust rhythm in BMAL1 expression ($p<0.001$). The phase of BMAL1 protein was comparable to that of its mRNA with a peak protein expression, occurring around 20h after the final media change, being significantly ($p<0.01$) and ~3-fold higher than the succeeding trough observed 8h later. In comparison, the BMAL1 expression rhythm in miR-142-3p-transfected *mPer2*^{Luc} SCN cultures was abolished ($p>0.05$). The rhythm disruption was accompanied by a steadily decreased over time (Fig. 7). At the time point (20h) associated with peak expression in control samples, corresponding levels of BMAL1 protein expression were significantly lower ($p<0.05$) in miR-142-3p transfected cultures.

Discussion

MiRNAs have recently garnered increased attention with regard to their contribution to regulation of circadian rhythms. Using diverse model systems, most studies have focused on miRNAs that are clock-controlled and function in the regulation of circadian output pathways. The primary observations of these studies suggest that miRNAs may play a role in the modulation of physiological and behavioral rhythms

(Cheng et al., 2007; Gatfield et al., 2009; Kadener et al., 2009; Shi et al., 2009). In addition, there is some evidence indicating that clock-controlled miRNAs may also modulate clock input pathways. In this regard, the findings of Cheng et al. (2007) demonstrate that light-regulated and clock-controlled miRNAs in the mouse SCN are capable of altering membrane excitability and hence, may modulate input to the SCN circadian clock. However, it is yet unclear whether miRNAs regulate specific clock genes and contribute to the feedback modulation of core elements of the circadian clockworks. This is the first study identifying miR-142-3p as a clock-controlled miRNA that can modulate *Bmal1* expression in the mouse SCN. Specifically, our findings indicate that miR-142-3p is expressed rhythmically in the mouse SCN in vivo and in an immortalized SCN cell line. Site-directed mutagenesis indicated that miR-142-3p targets the *Bmal1* 3' UTR at two locations. We further show that constitutive overexpression of miR-142-3p abolishes rhythms in BMAL1 protein accumulation in an SCN cell line**.**

Because rhythmic expression is a pervasive characteristic of core and ancillary elements involved in the circadian clock mechanism, we used bioinformatics analyses to identify evidence of rhythmic transcription of two putative *Bmal1* targeting miRNAs, miR-142-3p and miR-494. E-box (CANNTG) elements are the sites of action for the CLOCK-BMAL1 heterodimer complex which results in rhythmic transcription of target genes (Shigeyoshi et al., 1997). In the present study, a conserved, canonical E-box element was identified ~1.5kb upstream of the miR-142 locus, indicating that this miRNA may be subject to circadian regulation by the core clock machinery. Although we did not verify the necessity of this E-box, a recent report by Tan et al., (2012) utilized

ChIP and luciferase reporter assays to demonstrate that in NIH/3T3 cells CLOCK-BMAL1 heterodimers bind to the E-box element and drive transcription of miR-142-3p.

Temporal analysis identified rhythmic expression of mature miR-142-3p in the SCN, both in vivo and in *mPer2*^{Luc} SCN cells in vitro. It is interesting that in the SCN in vivo, the peak phase of circadian miR-142-3p expression occurred during the early subjective day at ~CT3, several hours in advance of the peak in *Per2* mRNA levels at ~CT11 (data not shown). This phase relationship is similar to that observed between mature miR-219 and *Per2* mRNA rhythm in the mouse SCN. Importantly, miR-219 was also previously shown to be a circadian clock-regulated gene (Cheng et al., 2007). A similar phase difference of ~8h between miR-142-3p and *Per2* was also observed in the *Per2*^{Luc} SCN cells in vitro (data not shown). The lack of rhythmic variation in the expression of both, miR-142-3p and *Bmal1*, in *Per1*^{ldc} / *Per2*^{ldc} SCN cultures provides further evidence that miR-142-3p is a bona-fide clock-regulated gene.

The anti-phasic relationship between rhythmic miR-142-3p and *Bmal1* expression is also consistent with its role as a post-transcriptional repressor of *Bmal1*. To address whether the in vitro site-directed mutagenesis results for miR-142-3p-mediated repression of the *Bmal1* 3' UTR extended to its functional activity in repression of endogenous BMAL1 protein levels, miR-142-3p was constitutively overexpressed in mouse *mPer2*^{Luc} SCN cells. Overexpression of miR-142-3p abolished the rhythm in BMAL1 protein expression in these cells, indicating that this miRNA is involved in the regulation of endogenous BMAL1 protein levels. It is interesting that BMAL1 protein levels decline over time in miR-142-3p-transfected cells. This effect may reflect a

combination of protein turnover and inhibition of translation by miR-142-3p due to its continuous production via the transfected pEZX-MR04 miRNA expression vector. Additionally, miRNAs can function as master switches, controlling expression of hundreds of genes (Lewis et al., 2005). In this regard, it is interesting that miR-142-3p has previously been shown to modulate cAMP levels by targeting adenylate cyclase 9 in T-cells (Lewis et al*.,* 2005; Huang et al*.,* 2009). This putative target for modulation by miR-142-3p is intriguing because cAMP, through its function as a second messenger, is involved in regulating the SCN circadian clock (Prosser and Gillette, 1989; O'Neill et al*.,* 2008). Hence, miR-142-3p could potentially influence circadian physiology by regulating not only a core clock gene like *Bmal1* but also circadian outputs like cAMP that may feed-back as input into the clock mechanism to fine-tune and/or sustain clock function.

It is noteworthy that mature miR-142-3p is highly conserved in multiple vertebrate taxa. Also, the first miR-142-3p recognition element on mouse *Bmal1* 3' UTR is conserved only among mammalian lineages while the second MRE is highly conserved across vertebrates. Hence, our data indicate that miR-142-3p might have important functional implications in the regulation of *Bmal1* expression and the core molecular clockworks across multiple taxa. Additionally, because *Bmal1* is expressed in cells and tissues throughout the body, miR-142-3p may also have an important role in circadian timekeeping function in central as well as in peripheral clocks (Yamamoto et al., 2004).

CHAPTER III

EXPRESSION AND RHYTHMIC MODULATION OF CIRCULATING MICRORNAS TARGETING THE CLOCK GENE *Bmal1* IN MICE*

Introduction

MicroRNAs are small non-coding RNAs that have been identified in many different organisms ranging from *Drosophila* to humans (Bartel, 2004) and implicated in the regulation of a wide array of biological processes. Mature miRNAs are small RNA molecules, typically 19-25 nucleotides long, derived from sequential RNase III-dependent cleavages of longer transcripts (Bernstein et al., 2001; Lee et al., 2003). In the cytoplasm, mature miRNAs associate with components of the RNA-induced Silencing Complex (RISC) and interact with miRNA-recognition elements (MRE's) in the 3' UTRs of target mRNAs. Mismatches or gaps in the base-pairing interactions between the miRNA-mRNA duplex result in translational repression and/or mRNA de-stabilization (Hutvagner and Zamore, 2002; He and Hannon, 2004). In humans, it has been estimated that the number of unique miRNAs exceeds 1000 (Perera and Ray, 2007) and that 20-30% of the transcriptome is subject to miRNA-targeted regulation (Lewis et al., 2005; Xie et al., 2005).

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*Reprinted from "Shende VR, Goldrick MM, Ramani S and Earnest DJ (2011) Expression and rhythmic modulation of circulating microRNAs targeting the clock gene Bmal1 in mice. PLoS ONE **6**:e22586". Creative Commons Attribution License (CCAL).

Although miRNAs target and regulate specific mRNA transcripts via intracellular mechanisms, recent evidence for their presence in vesicles circulating in the blood of humans (Mitchell et al., 2008) raises the possibility that miRNAs may also function as extracellular or secreted regulatory signals that mediate communication between cells (Thery et al., 2009). In accord with their role in modulating the transcriptome and proteome, miRNAs play an integral role in important biological processes like development, metabolism and cancer biology.

Recent studies have also implicated miRNAs in the regulation of the circadian timekeeping mechanism in the mammalian suprachiasmatic nuclei (SCN). Brain, muscle ARNT-like protein 1 (*Bmal1*), circadian locomotor output cycles kaput (*Clock*), as well as the Period (*Per1* and *Per2*) and Cryptochrome (*Cry*) genes comprise the "gears" of the molecular clockworks common to both the SCN and peripheral tissues (Shearman et al., 1997; Zylka et al., 1998; Yamazaki et al., 2000). Interactions between these clock components form positive- and negative-feedback loops in which gene transcription is rhythmically regulated by their protein products, with exception of *Clock*. For example, rhythmic increases in *Bmal1* transcription and the formation of CLOCK-BMAL1 heterodimers positively regulate the rhythmic transcription of the *Per* and *Cry* genes (Takahashi et al., 2008). In turn, the increases in PER and CRY proteins lead to the formation of heterodimers that interact with the CLOCK-BMAL1 complex and negatively feedback on their own transcription. CLOCK-BMAL1 complexes also mediate the regulation of clock-controlled outputs that provide for the rhythmic programming of downstream processes. MiRNA function in SCN-mediated regulation

of circadian rhythms is supported by observations indicating that miR-219 and miR-132 are rhythmically expressed in the SCN and that antagonism of these miRNAs within the SCN region respectively increases the circadian period of behavioral rhythmicity and attenuates circadian photoentrainment (Cheng et al., 2007). Other applications of mouse, *Drosophila* and chicken models provide further evidence for the role of miRNAs in the regulation of circadian rhythms in gene expression or behavior (Gatfield et al., 2009; Kadener et al., 2009; Shi et al., 2009). However, evidence for the role of specific miRNAs as bona fide modulators of core clock genes is limited.

Because rhythmicity is a prevalent property among core and regulatory elements of the circadian clockworks in most cells and tissues throughout the body, we explored the possible timekeeping function of miRNAs in the periphery by initially determining whether specific miRNAs with clock genes as predicted targets are expressed in serum and whether their expression is marked by daily fluctuations. Our analysis focused on miRNAs that are predicted to target the 3' UTR of *Bmal1* mRNA because this clock gene is unique among core clock elements as knockout of *Bmal1* alone produces complete loss of circadian rhythmicity (Bunger et al., 2000). Specifically, experiments were conducted to explore miRNA function in the circadian clockworks by: **1**) first identifying miRNAs predicted to target core and ancillary clock genes; **2**) determining whether expression of specific miRNAs that target *Bmal1* oscillate in mouse serum in vivo; **3**) examining the effects of candidate miRNA overexpression on *Bmal1* 3' UTR activity; and **4**) determining whether mutagenesis of specific target sites abates miRNA-induced repression.

Materials and Methods

Experiment 1- Temporal Profiling of miRNA levels in mouse serum: Temporal profiling was performed to identify potential rhythms in circulating miRNA expression.

Animals: Experimental subjects were 20 male Balb/C mice at 8–10 weeks of age. All animals were born and reared in the animal facility at BIOO Scientific under a standard 12h light:12h dark photoperiod (LD 12:12; lights-on at 0700 hr). Prior to experimental analysis, animals were housed 2-3 per cage. Access to food and water was provided *ad libitum* and periodic animal care was performed at random times.

Ethics Statement: All animal procedures used in this study were conducted in compliance with protocol B002 as approved by the Institutional Animal Care and Use Committee at BIOO Scientific Corp.

Blood collection and fractionation: To determine whether miRNAs are expressed and fluctuate rhythmically in the serum, blood samples were collected at 4 hour intervals from mice maintained in a LD 12:12 cycle. At each timepoint, blood was collected by cardiac puncture from 3-4 mice that were anesthetized with 2,2,2-tribromoethanol (250 mg/kg, intraperitoneal; Sigma) and sacrificed by cervical dislocation. Sampling procedures during the dark phase of the LD 12:12 cycle (Zeitgeber Time [ZT] 12-24) were conducted under dim red light (Kodak filter GBX-2). Blood was allowed to clot at room temperature for 10-15 minutes. Adhesions between the clot and collection tube were gently detached by "rimming the clot" to minimize hemolysis and then samples were centrifuged for 10 minutes in a swinging bucket microcentrifuge (Eppendorf) at 3,000 x g to separate cellular and non-cellular fractions. Immediately following

centrifugation, the serum layer was carefully aspirated off, mixed with three equivalent volumes of TRI reagent (Ambion) and stored at -20°C until further processing.

To fractionate the white blood cells (WBCs), the clot was disaggregated in phosphate buffered saline (PBS) and the fluid layer containing suspended WBCs and red blood cells (RBCs) was collected. Following centrifugation for 30 seconds at 3,000 x g, the cell pellet was resuspended in distilled water for ~10 sec to lyse RBCs by osmotic shock, and then mixed with 10X PBS to restore physiological ionic strength and prevent WBC lysis. The WBC pellet was recovered by centrifugation at 3,000 x g for 40 sec, lysed in 1 ml BiooPure RNA Isolation Reagent (BIOO Scientific, Austin, TX) and stored at -20°C until further processing.

RNA extractions: Total RNA was subsequently extracted from the serum and WBC lysates according to manufacturer's protocols, with the exception that 50ug of linear acrylamide was added as a co-precipitant to the aqueous phase before addition of isopropanol. This modification enhances recovery of the small amounts of nucleic acids (Bartram et al., 2009). RNA samples were suspended in 50ul 0.1mM EDTA and dissolved by heating for 5 min at 65°C. Total RNA was estimated using Nanodrop ND2000 (Thermo Scientific).

Real-time PCR: Quantitative real-time PCR analysis was conducted using Taqman microRNA assays (Applied Biosystems). RNA from individual samples was first reverse transcribed using target-specific stem-loop primers and Taqman MicroRNA Reverse Transcription Kit. All assays were performed according to manufacturer's protocols, using 20ng of total RNA as input with the exception that 90ng input RNA was

used for reverse transcription of miR-494. For analysis of miRNA expression, the cDNA equivalent of 2ng of total RNA was PCR amplified in an ABI PRISM 7500 Fast sequence detection system using the following standard conditions: **1**) heating at 95°C for 10 min, and **2**) amplification over 40 cycles at 95°C for 15 sec and 60°C for 1 min. This analysis was conducted concurrently on duplicate aliquots of RT product from each sample. miR-16 was also amplified from the same samples using identical parameters to control for differences in sample RNA content and reverse-transcription efficiencies because: **1**) this miRNA has provided a good standard for normalization and comparisons of relative abundance in previous studies (Venturini et al., 2007; Kroh et al., 2010); and **2**) ANOVA analysis indicates that miR-16 levels in the serum exhibit no significant variation (p = 0.19) over the 24-hour time course for sampling (data not shown). Using the comparative C_T method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (PE-ABI), the relative abundance for a given miRNA was calculated by normalization first to corresponding miR-16 levels in each sample and then to a calibrator consisting of pooled cDNA from multiple samples over the entire time series. All TaqMan miRNA assays used in this study exhibited PCR efficiencies of 95-101.6%.

Relative quantification of 18s rRNA abundance was performed on some serum and WBC samples using SYBR-Green real-time PCR technology (ABI) as described previously (Allen et al., 2004; Farnell et al., 2008). To generate single-strand cDNAs, total RNA (250ng) from individual samples was reverse transcribed using random hexamers and Superscript III reverse transcriptase (Invitrogen). 18s rRNA was PCR

amplified using the cDNA equivalent of 2.5ng of total RNA. PCR analysis was performed on duplicate aliquots of each sample using the ABI PRISM 7500 Fast sequence detection system and the following conditions: **1**) serial heating at 50°C for 2 min and 95°C for 10 min, **2**) amplification over 40 cycles at 95°C for 15 sec and 60°C for 1 min, and **3**) dissociation at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Relative differences in 18s rRNA abundance were established by comparing serum and WBC determinations to a standard curve that was generated using pooled cDNA from all samples. The following primers were used for the real-time PCR analysis: m18s rRNA forward: 5'-ATGGCCGTTCTTAGTTGGTG -3'; m18s rRNA reverse: 5'-CGCTGAGCCAGTCAGTGTAG -3'.

To estimate the number of copies of representative miRNAs in serum samples, synthetic single-stranded RNA oligonucleotides encoding the mature miRNA sequences for miR-16 and miR-152 were purchased from Integrated DNA Technologies, Inc. Mature miRNA sequence information was procured from miRBASE (release 16.0; Sept. 2010). These synthetic miRNAs were used in a dilution series ranging from 1 molecule/µl to 10^{10} molecules/µl to generate standard curves for quantification of molecules of miR-16 and miR-152. Standard curves derived from concentrations yielding Ct values within the linear range were used to estimate number of copies of miR-16 and miR-152 in the input RNA from ZT7 serum samples that were simultaneously reverse-transcribed, PCR-amplified and analyzed on the same plate. Absolute copy number of miRNAs per microliter of serum was extrapolated using

known information on the amount of input RNA (10ng) and total extracted RNA (1500-4500ng) relative to the specific volume collected (200-300µl) for each serum sample.

Experiment 2- MiRNA regulation of Bmal1 3' UTR activity: Timekeeping function of circulating miRNAs was investigated by 3' UTR reporter activity assays.

Bmal1 3' UTR luciferase reporter construct: miTarget™ miRNA Target Sequence 3' UTR Expression Clone for *Bmal1* was purchased from Genecopoeia. This expression clone contains *Bmal1* (Accession: NM_007489.3) 3' UTR sequence inserted in the pEZX-MT01 vector downstream of a firefly luciferase gene under the control of an SV40 enhancer generating a chimeric transcript that consists of the luciferase coding and *Bmal1* 3' UTR sequences. The pEZX-MT01 vector also contains the *Renilla* luciferase gene under the control of a CMV promoter to provide for dual analysis of firefly and *Renilla* luciferase activities in individual samples and to normalize firefly luciferase signal intensities and account for potential differences in transfection efficiencies across control and experimental cultures. To determine the specificity of miRNA interactions with the *Bmal1* 3' UTR, similar analyses were performed using miRNA Target clone control vector (Genecopoeia; CmiT000001-MT01) which consists of the pEZX-MT01 vector without a 3' UTR tagged to the firefly coding sequence. miTarget™ miRNA Target Sequence 3' UTR Expression Clone encoding the *cKit* 3' UTR (Accession*:* NM_021099.2; generously provided by Dr. Rajesh Miranda, Texas A&M University Health Science Center) was also used as an additional control. Based on the results from Targetscan analysis, the *cKit* 3' UTR is predicted to contain a target

site for miR-494, but not for miR-142-3p or miR-152. The *cKit* 3' UTR also contains a predicted target site for miR-142-5p, the antisense transcript of miR-142-3p.

Transformed *E. coli* cells (Genecopoeia) were grown on kanamycin (final conc. = 50ug/ml) containing imMedia agar plates (Invitrogen). A single isolated colony was propagated in imMedia Kan^+ (final conc. = 50ug/ml) liquid medium and plasmid was extracted using EndoFree Plasmid Maxi kit (Qiagen). The extracted pEZX-MT01 *Bmal1* 3' UTR expression plasmid was sequenced to verify expression and accuracy of the *Bmal1* 3' UTR sequence using the following primers: Forward: 5'-GATCCGCGAGATCCTGAT-3'; Reverse: 5'-TTGGCGTTACTATGGGAACAT-3'. Similar procedures were followed for isolation of the pEZX-MT01 control vector and the pEZX-MT01 *cKit* 3' UTR expression vector.

Cell culture and transfections: Human embryonic kidney cells (HEK 293) at passage 12-15 were used for experimental analysis of miRNA regulation of *Bmal1* 3' UTR activity. Cells were seeded on 60-mm cell culture dishes (Corning) and maintained at 37°C and 5% CO_2 in Dulbecco's minimum essential medium (DMEM; Invitrogen) without antibiotics and supplemented with 10% Fetal Bovine Serum (FBS; HyClone, Logan, UT) and 292ug/mL L-glutamine. Medium was changed at 48-hour intervals and confluent cultures were split 1:4 or 1:5 every 3-4 days. Prior to experimentation, cells were seeded onto 24-well plates in DMEM supplemented with 5% FBS. 24 hours later, co-transfection of verified plasmid DNA clones (0.4ug) with either individual pre-miRs, or paired combinations of pre-miR constructs for miR-494, miR-152, or miR-142-3p (final conc. 33nM/well; ABI) was performed using Lipofectamine 2000 (Invitrogen)

according to the manufacturer's protocols. To compare basal luciferase reporter activity across different pEZX-MT01 vectors and evaluate the potential influence of endogenous miRNAs, parallel analysis was performed on HEK 293 cells in which either the pEZX-MT01 control vector or the *Bmal1* 3' UTR vector was co-transfected with a non-targeting control miRNA. Following incubation with transfection reagents for 5 hours, the medium was replaced and 48 hours later, lysates of HEK293 cultures from all treatment groups (n=4) were collected using Passive Lysis Buffer (Promega). Lysate samples were stored at -20°C and later firefly luciferase activity was analyzed relative to *Renilla* luciferase activity in the same sample using dual-luciferase reporter assay system (Promega). Luminescence was measured in counts per second using a LumiCount microplate luminometer (AL10000; Packard Bioscience).

Bmal1 3′ UTR site-directed mutagenesis: miTarget™ miRNA Target Sequence 3′ UTR Expression Clone containing *Bmal1* 3' UTR sequence (Accession: NM_007489.3) inserted in the pEZX-MT01 vector was purchased from Genecopoeia, Inc. The plasmid was propagated using methods established in our previous study (Shende et al., 2011). Deletions in miR-142-3p binding sites on the *Bmal1* 3' UTR were generated using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene Inc.) according to the manufacturer's protocols. Briefly, the full-length *mBmal1* 3' UTR was PCR mutagenized using specific primers so as to delete nucleotides 1-7 complementary to miR-142-3p seed region. After *Dnp1*-mediated degradation of parental plasmid DNA, the mutagenized plasmid was transformed into XL10-Gold Ultracompetent cells (Stratagene, Inc.) and transformants were selected on kanamycin-containing (final conc.

= 50ug/mL) imMedia agar plates (Invitrogen, Inc.). A single colony was isolated and propagated in imMedia Kan^+ liquid medium (final conc. = 50ug/mL). The plasmid was extracted using HiSpeed Plasmid Midi Kit (Qiagen, Inc.) and then sequenced to verify the targeted deletion (*Bmal1 c.1_7del)*. The same methods were also used to delete nucleotides 335-357 corresponding to a second miR-142-3p target site on the full-length *Bmal1* 3' UTR. This plasmid (*Bmal1 c.335_357del*) was then subjected to a second round of mutagenesis to provide for additional deletion of nucleotides 1-7. The plasmid with targeted deletions of both miR-142-3p target sites on the *mBmal1* 3' UTR (*Bmal1 c.1_7del + c.335_357del*) was propagated and sequenced to verify these deletions as described above. The miRNA 3′ UTR target control vector (Genecopoeia; CmiT000001-MT01), consisting of the pEZX-MT01 vector backbone without any 3' UTR sequence, was used to determine the specificity of miRNA interactions with the full-length and mutagenized *mBmal1* vectors. miR-142-3p-mediated regulation of *mBmal1* 3' UTR was analyzed in human embryonic kidney (HEK293) cells using established methods (Shende *et. al.,* 2011). Briefly, cells were seeded onto 24-well plates (Corning, Inc.) and 24h later were co-transfected with miR-142 expression vector (miExpress Precursor miRNA expression clone; Genecopoeia, Inc., MmiR3437-MR04) and with either the target control, *mBmal1, Bmal1 c.1_7del, Bmal1 c.335_357del* or *Bmal1 c.1_7del + c.335_357del* miRNA 3' UTR clone. As an additional control for specificity of the deletion, cells were also co-transfected with miR-494 expression vector and either the target control, *mBmal1* or *Bmal1 c.1_7del + c.335_357del* miRNA 3' UTR clone. Following transfection for 5 hours, cells were rinsed and the medium was replaced.

Forty-eight hours later, cell lysates of HEK293 cultures from all treatment groups (n= 4) were prepared using Passive Lysis Buffer (Promega, Inc.). Firefly and *Renilla* luciferase activity from individual samples was then analyzed using the dual-luciferase reporter assay system (Promega, Inc.). Luminescence was measured on Synergy2 microplate luminometer (Biotek Inc.) and reported as the ratios of firefly luciferase signal normalized to *Renilla* luciferase activity in the same sample.

Statistical analyses: The temporal patterns of miRNA expression in serum were examined for evidence of circadian variation using statistical analyses that have been used previously for this purpose (Allen et al., 2001; Menger et al., 2005; Menger et al., 2007). Time-dependent fluctuations in miR-494, miR-152 and miR-142-3p expression were first identified by one-way analysis of variance (ANOVA). Paired comparisons between peak values and those observed during the preceding or succeeding minimum were analyzed *post hoc* for statistical differences using the Newman-Keuls sequential range test. The α-value was set at 0.05 for these *post hoc* analyses. Independent *t*-tests were performed on serum and WBC 18s and miRNA comparisons, and on normalized luminescence data to determine the significance of pre-miR and control-miR treatment on luciferase-reported *Bmal1* 3' UTR activity. The α-value was set at 0.01 for independent *t*-tests.

Results

Experiment 1- Temporal Profiling of miRNA levels in mouse serum: Three target prediction databases (microcosm (Griffiths-Jones et al., 2006; Griffiths-Jones et

al., 2008), Targetscan (Lewis et al., 2005) and MiRanda (John et al., 2004)) were used to identify potential miRNAs targeting mammalian clock genes. Because *Bmal1* is the only clock gene in which null mutation produces arrhythmicity (Bunger et al., 2000), we focused on a subset of miRNAs that express consensus recognition sequences for the 3' UTR of either *Bmal1* or for other genes that regulate *Bmal1* expression in the molecular feedback loops comprising the circadian clockworks (Table 1). The *Bmal1* 3' UTR was predicted to contain miR-152, miR-142-3p and miR-494 target sites that are located at nucleotide positions 88-108, 335-357 and 473-495, respectively. It is interesting that in addition to their putative targeting of the *Bmal1* 3' UTR, miR-152 and miR-494 were also predicted to interact with its primary partner *Clock* or with transcriptional activators, retinoic acid-related orphan receptors alpha and beta (*Rorα, Rorβ*).

Table1. Application of target prediction programs to identify candidate miRNAs expected to target *Bmal1* or other genes that regulate *Bmal1* expression.

	MicroCosm	**TargetScan**	**MiRanda**
mmu-miR-142-3p	*Bmal1*	*Bmal1*	*Bmal1*
mmu-miR-152	*Bmal1, Rorβ*	-	*Bmal1, Rorα, Rorβ*
mmu-miR-494	*Bmal1, Rorβ*	*Bmal1, Clock, Rorβ*	*Bmal1, Per2, Rorβ*
mmu-miR-135b	-	-	*Rorα, Rorβ*
mmu-miR-135a	-	-	*Rorα, Rorβ*
mmu-miR-34c	*Reverbα*	-	*Per2*

We next determined whether any of the identified miRNAs predicted to target *Bmal1* or other components of the clock feedback loops were expressed in serum. All of the candidate miRNAs were detected in serum during the daytime of LD 12:12 cycle (ZT 7), but their circulating levels spanned a wide range. miR-142-3p, miR-152, miR-494, miR-135b, miR-135a and miR-34c were found in descending order of abundance in the serum (Fig. 8A). Consistent with previous observations on its circulating levels in humans (Mitchell et al., 2008), miR-16 was highly abundant relative to other miRNAs detected in mouse serum. Quantitative analysis of miR-16 and miR-152 levels revealed that the estimated concentrations of these miRNAs were 408,000-749,000 and 3,400-6,800 copies/µl serum (Fig. 8B), respectively. Using this information as a relative index of abundance, miR-142-3p, miR-152 and miR-494 appear to represent species of moderate to low expression in serum. To gauge the relationship between serum and cellular miRNA levels, we also analyzed the WBC fractions of individual blood samples for expression of specific miRNAs predicted to target *Bmal1*.

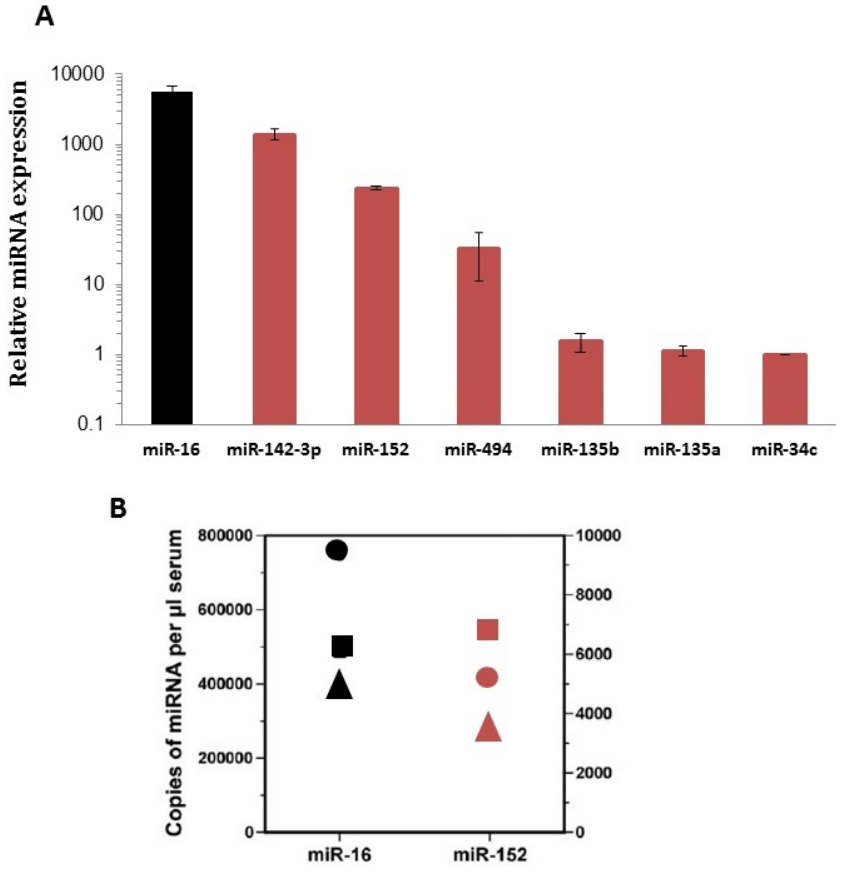

Figure 8. Comparison of circulating miRNAs predicted to target Bmal1 or other genes regulating Bmal1 expression with a highly abundant miRNA in serum. **(A)** Relative expression of miR-16, miR-142-3p, miR-152, miR-494, miR-135b, miR-135a and miR-34c in serum samples collected from mice at ZT 7 (n=3). Bars denote real-time PCR determinations of serum miRNA levels (mean ± SEM) and the values are plotted using a logarithmic scale in comparison with the average for miR-34c expression. **(B)** Quantification of miR-16 and miR-152 expression in serum collected from mice at ZT 7 (n=3). Symbols denote determinations (in duplicate) of the number of copies/µl serum in each sample that were extrapolated by comparing the Ct values for experimental samples with standard curves consisting of a dilution series of known quantities of synthetic miR-16 and miR-152 analyzed on the same plate.

These components of blood exhibited variable differences in miRNA levels such that miR-142-3p and miR-494 expression were lower (77-fold and 1.5-fold, respectively) and miR-152 was higher (25-fold) in serum than in WBCs (Fig. 9A). The differential expression of miR-142-3p in WBCs is not surprising because this miRNA is highly abundant in hematopoietic cell lineages (Merkerova et al., 2008). Because recent findings indicate that despite their abundant expression in the cytoplasm 18s and 28s rRNA are absent in RNA extracted from circulating exosomes (Valadi et al., 2007), 18s rRNA levels were analyzed in serum and WBC fractions of blood samples collected from mice (n=3-4) at ZT3 and ZT7 to confirm that the detected small RNAs reflect serum expression, rather than artifact associated with cellular lysis during sample preparation. Consistent with the observations of Valadi et al. (2007), serum levels of 18s rRNA were negligible and at the limits of detection with real-time PCR analysis. Relative expression levels of 18s rRNA in the serum were 26,000-79,000-fold lower than those found in WBC fractions (Fig. 9B), indicating that the observed miRNA signals in serum are derived from extracellular or vesicle-encapsulated RNA, and not from lysed or intact leukocytes or other cells, in the blood samples.

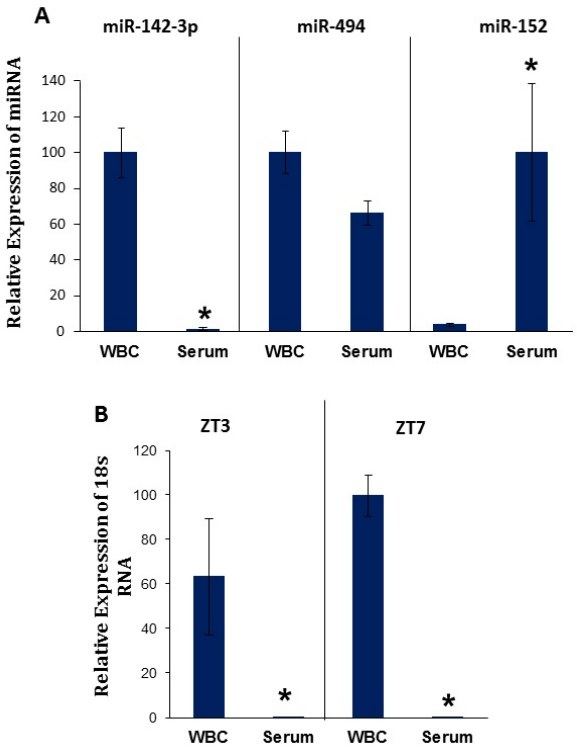

Figure 9. Comparison of miRNA and 18s rRNA levels in the serum and corresponding white blood cell (WBC) fractions. (**A**) Bars denote real-time PCR determinations of serum and WBC miRNA levels (mean ± SEM) in blood samples collected from mice (n=3) at ZT 7. The plotted values correspond to the ratios of fraction-specific miR-142-3p, miR-494 and miR-152 signal and are represented as a percentage of the maximal value obtained among the serum and WBC fractions. Asterisks denote comparisons in which the relative expression of miRNA signal in the WBC fraction was significantly different ($p < 0.05$) from that observed in serum samples. (**B**) Bars denote real-time PCR determinations of 18s rRNA levels (mean ± SEM) in serum and WBC fractions of blood samples collected from mice (n=3-4) at ZT 3 and ZT 7. The plotted values are represented as a percentage of the average for the WBC fraction at ZT 7. Asterisks denote time-specific comparisons (ZT 3 and ZT 7) of 18s rRNA signal in which relative expression in the WBC group was significantly greater ($p < 0.01$) than that observed in serum samples.

Daily profiles of miRNA expression in serum were assessed to determine whether circulating levels of mature miRNAs that are predicted to target mouse *Bmal1* or other genes regulating this key component of the molecular clockworks, miR-494, miR-152 and miR-142-3p, oscillate rhythmically. In mice exposed to LD 12:12, diurnal fluctuations were observed in the relative expression of miR-494 and miR-152 (normalized to miR-16) in the serum (Fig. 10). The rhythm in circulating levels of miR-494 was marked by a bimodal pattern in which the first peak in serum expression occurred around mid-day at ZT 7 and was followed by a secondary peak during the night around ZT 19. For the diurnal oscillation in miR-494 expression, the bimodal peaks in serum levels at ZT 7 and ZT 19 were significantly ($p < 0.05$) and about 2- to 5-fold greater than those observed during the preceding and succeeding minima. Similar to the temporal profile for miR-494 expression, serum levels of miR-152 were characterized by diurnal variation with bimodal peaks (Fig. 10). The first zenith in miR-152 expression occurred again at ZT 7 and was followed by a secondary peak in mature miRNA levels at ZT 15. The amplitude of rhythmic miR-152 expression in the serum was robust, with 2- to 8-fold differences between peak and trough values. The bimodal peaks in circulating levels of miR-152 at ZT7 and ZT15 were significantly greater ($p < 0.05$) than the preceding and succeeding minima. In contrast to other tested miRNAs, miR-142-3p levels in the serum exhibited no evidence of diurnal fluctuations (Fig. 10). In comparison with miR-494 and miR-152, serum levels of miR-142-3p were relatively high with no significant variation over time ($p = 0.377$).

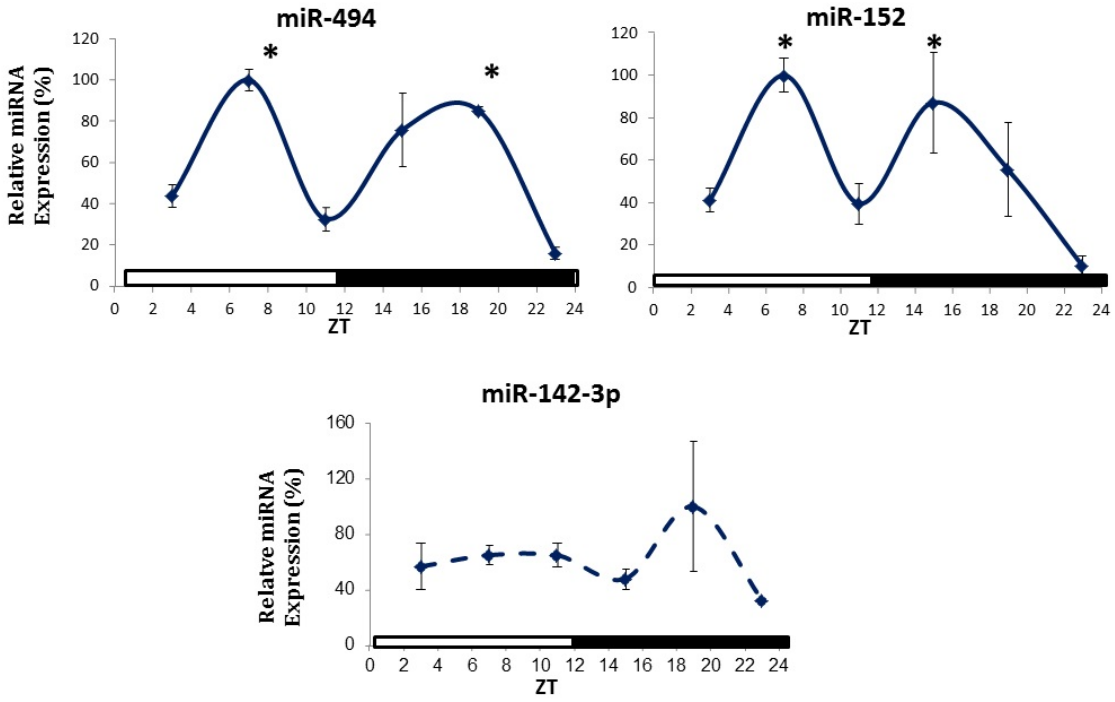

Figure 10. Temporal patterns of miR-494, miR-152 and miR-142-3p expression in mouse serum. Symbols denote real-time PCR determinations of miRNA levels (mean ± SEM) in serum collected at 4-hour intervals from mice (n=3-4) during exposure to a LD 12:12 cycle. The plotted values correspond to the ratios of miR-494 (top left), miR-152 (top right) and miR-142-3p (bottom) signal normalized to miR-16 levels in each sample and are represented as a percentage of the maximal value obtained for each miRNA. Asterisks indicate time points during which peak values for serum expression of a given miRNA were significantly greater ($p < 0.05$) than those observed during preceding and succeeding minima.

Experiment 2- miRNA regulation of Bmal1 3' UTR activity: Since the observed circadian fluctuations in serum levels of miRNAs predicted to target *Bmal1* is suggestive of their involvement in circadian timekeeping mechanisms, we used an in vitro reporter assay to examine the effects of miR-494, miR-152 and even miR-142-3p on *Bmal1* expression via targeting of the 3' UTR of this clock gene. Bioluminescence was analyzed in HEK293 cells co-transfected with the pEZX-MT01 *Bmal1* 3' UTR expression vector and pre-miR constructs for miR-494, miR-152, or miR-142-3p (n=4). To control for non-specific interactions between pre-miRs and the luciferase reporter in the pEZX-MT01 vector, parallel analysis was performed on HEK293 cells co-transfected with these pre-miR constructs and the pEZX-MT01 control vector which does not contain a 3' UTR tagged to the firefly coding sequence (n=4). In HEK293 cells co-transfected with *Bmal1* 3' UTR expression vector, overexpression of miR-494 or miR-142-3p, but not miR-152, produced significant decreases ($p < 0.01$) in luciferase-mediated bioluminescence relative to that found in cells transfected with the control vector (Fig. 11A). Treatment with pre-miR constructs for miR-494 or miR-142-3p repressed *Bmal1* 3' UTR-mediated bioluminescence by about 35% and 60%, respectively, in comparison with control transfections. Transfection with a non-targeting miRNA had no significant effect on luciferase-mediated bioluminescence in *Bmal1* 3' UTR-expressing cells relative to that found in cells transfected with the control vector, suggesting that basal reporter activity is similar between the *Bmal1* 3' UTR and control vectors and that the observed repression of the *Bmal1* 3' UTR is specific for miR-494 and miR-142-3p.

Pre-miR interactions with the 3' UTR of a non-clock gene containing predicted target sites for miR-494 but not miR-152 or miR-142-3p were explored by analyzing bioluminescence from HEK293 cells co-transfected with the pEZX-MT01 vector expressing the *cKit* 3' UTR and pre-miR constructs for these miRNAs (n=4). Consistent with target prediction data indicating that the *cKit* 3' UTR contains a predicted target site for miR-494, overexpression of miR-494 in pEZX-MT01 *cKit* 3' UTR-transfected cells yielded a significant reduction ($p < 0.01$) in luciferase-reported bioluminescence relative to that observed in cells co-transfected with this pre-miR and the control vector (Fig. 11A). In response to treatment with pre-miR constructs for miR-494, *cKit* 3' UTR-mediated bioluminescence was repressed by about 30% in comparison with luciferase reporter expression in cells co-transfected with the control vector. Importantly, treatment with pre-miR constructs for miR-152 had no significant effect in repressing luciferase-mediated bioluminescence in *cKit* 3' UTR-expressing cells. In fact, overexpression of miR-152 in pEZX-MT01 *cKit* 3' UTR-transfected cells produced a small increase in bioluminescence relative to that found in control vector-transfected cells. Interestingly, treatment with pre-miR constructs for miR-142-3p caused a significant increase in *cKit* 3' UTR-mediated bioluminescence relative to the luciferase activity of the control vector. The basis for this inductive effect of miR-142-3p on *cKit* 3' UTR activity is unknown but the *cKit* 3' UTR is predicted to contain a target site for miR-142-5p and overexpression of its antisense transcript, miR-142-3p, may effectively reverse any basal repression derived from interactions of endogenous miR-142-5p with the *cKit* 3' UTR.

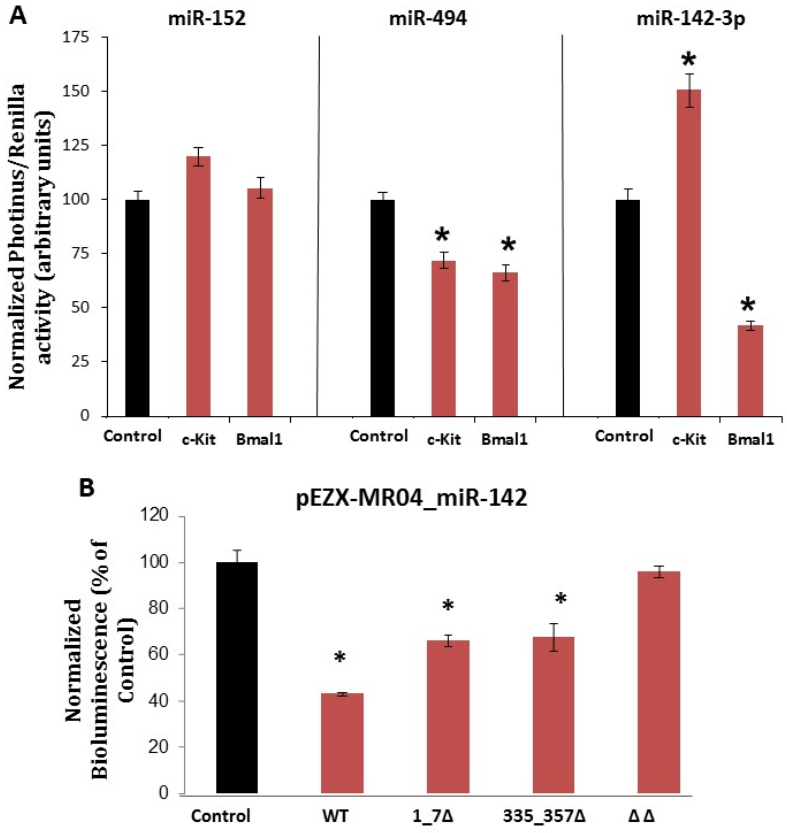

Figure 11. Independent and combinatorial effects of miR-494, miR-152 and miR-142-3p overexpression on *Bmal1* 3' UTR activity. Bars denote mean (±SEM) determinations of luciferase bioluminescence for each treatment group (n=4). The plotted values correspond to the ratios of firefly luciferase signal normalized to *Renilla* luciferase activity in the same sample and are represented as a percentage of the average signal for control vector transfectants. (**A**) Normalized bioluminescence from HEK293 cells expressing the pEZX-MT01 control vector, or pEZX-MT01 vector containing either *cKit* 3' UTR or *Bmal1* 3' UTR in response to treatment with pre-miR constructs (33nM) for miR-494, miR-152 and miR-142-3p. (**B**) Normalized bioluminescence from HEK293 cells expressing the pEZX-MT01 control vector or pEZX-MT01 vector containing the *Bmal1* 3' UTR in response to treatment with paired combinations of these pre-miR constructs (33nM; miR-494+miR-152, miR-494+miR-142-3p, miR-152+miR-142-3p).

Because previous studies indicate that two different miRNAs can act in concert to simultaneously repress translation of a single mRNA (Doench and Sharp, 2004), we next examined the combinatorial effects of miR-494, miR-152 and miR-142-3p overexpression in repressing *Bmal1* 3' UTR activity. For all of the tested miRNA combinations (miR-494+miR-152, miR-494+miR-142-3p, miR-152+miR-142-3p), luciferase-reported bioluminescence was significantly reduced ($p < 0.01$) in *Bmal1* 3' UTR-expressing cells relative to that control vector transfectants (Fig. 11B). Combinatorial effects in repressing luciferase-reported *Bmal1* 3' UTR activity were lowest in response to miR-494 and miR-152 overexpression (~21%) and were highest in cells treated with pre-miR constructs for miR-494 and miR-142-3p (~75%). In conjunction with the observed differences in the repressive effects of individual miRNAs, these results suggest that among the tested transcripts, miR-142-3p is the most potent repressor of *Bmal1* 3'UTR-mediated activity. Moreover, the potentiated repression observed in response to combined treatment with pre-miR constructs for miR-142-3p and miR-494 is compatible with previous evidence for the synergistic regulation of the 3' UTR for a single gene by two or more miRNA species (Doench and Sharp, 2004). Based on the independent and combinatorial effects of miR-494 on *Bmal1* 3' UTR activity, our results support the possibility that oscillations in serum levels of this miRNA may contribute to local rhythms in the post-transcriptional repression of endogenous *Bmal1* in the periphery.

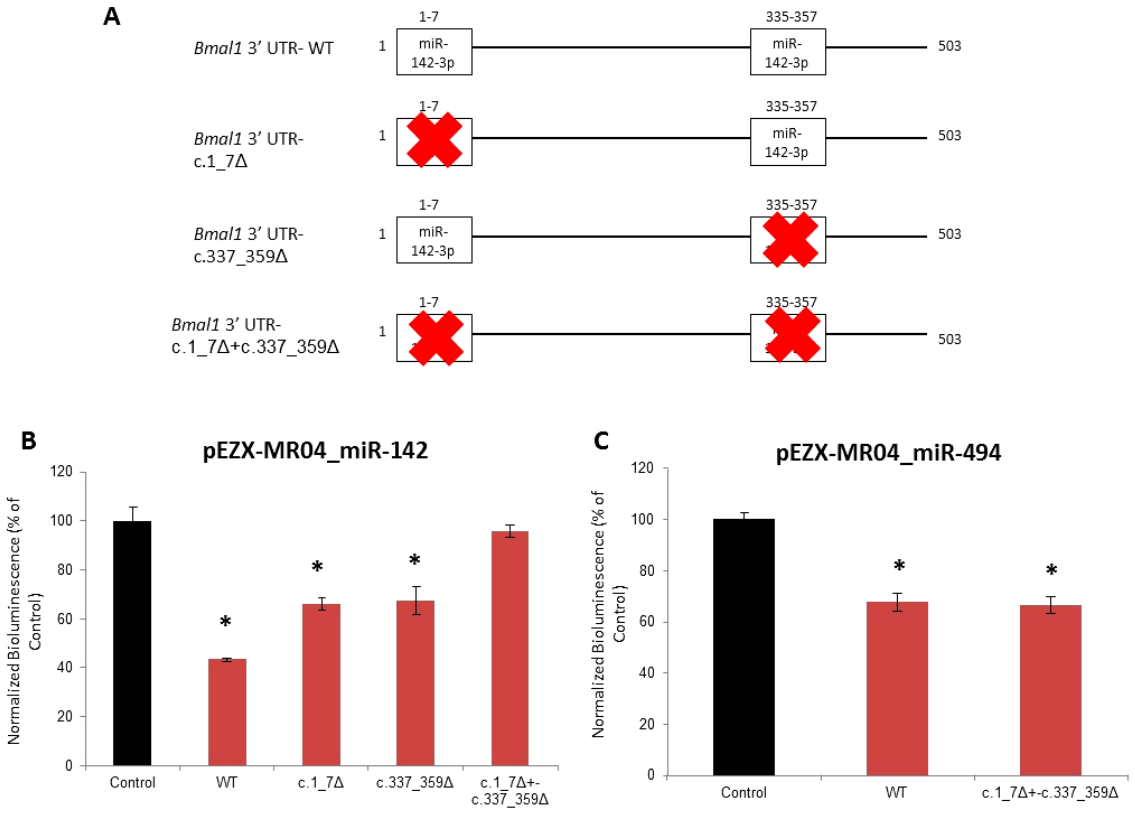

Figure 12. Effects of miR-142-3p on luciferase reporter activity of *Bmal1* wild-type or miR-142-3p MRE mutagenized 3′ UTR constructs. (**A**) Diagrammatic representation of WT and mutagenized *Bmal1* 3' UTR constructs. (**B & C**) The plotted values correspond to the ratios (mean ± SEM) of firefly luciferase signal normalized to *Renilla* luciferase activity in the same sample and are represented as a percentage of the average signal for control vector transfectants. Asterisks indicate observations where normalized bioluminescence values for treatment were significantly different from those observed for control. (**B**) Normalized bioluminescence from HEK293 cells expressing the pEZX-MT01 control vector, or pEZX-MT01 vector containing either wild-type or mutagenized *Bmal1* 3′ UTR in response to treatment with pEZX-MR04 miRNA expression clone for miR-142-3p. (**C**) Normalized bioluminescence from HEK293 cells expressing the pEZX-MT01 control vector or pEZX-MT01 vector containing the *Bmal1* 3′ UTR in response to treatment with pEZX-MR04 miRNA expression clone for miR-494.

Based on target prediction algorithms, the repression produced by miR-142-3p is predicted to occur via two consensus binding sites located at positions 1-7 and 335-357 on the *Bmal1* 3' UTR. To validate specificity and relative contribution of the two predicted target sites, independent and combinatorial site-directed mutagenesis of these loci was performed and an in vitro dual-luciferase reporter assay system was used to examine resultant effects on *Bmal1* 3' UTR activity as described previously (Shende et al., 2011). Bioluminescence was analyzed in HEK293 cells co-transfected with pEZX-MR04 miR-142 expression vector construct and pEZX-MT01 *Bmal1* 3′ UTR construct for either full-length *Bmal1* (WT) or mutants with targeted deletions of nucleotides 1-7 (*c.1_7del)*, nucleotides 335-357 (*c.335_357del*) or both loci (*c.1_7del* + *c.335_357del*) (Fig. 12A). Consistent with its effects observed in our previous study (Shende et al., 2011), miR-142-3p overexpression in HEK293 cells co-transfected with the WT *Bmal1* 3′ UTR expression vector produced significant decreases ($p<0.01$) of ~60% in luciferase-reported bioluminescence relative to that found in cells transfected with the control vector. In comparison, overexpression of miR-142-3p in HEK293 cells co-transfected with either the *c.1_7del* or *c.335_357del Bmal1* 3′ UTR expression vector produced significant ($p<0.05$) but diminished (~30%) reduction in luciferase-reported bioluminescence relative to that found in cells transfected with the control vector. Deletion of both 3' UTR target sites (*c.1_7del* + *c.335_357del*) completely abolished miR-142-3p-mediated repression and there was no significant difference in bioluminescence from cells transfected with control vector or the *c.1_7del* + *c.335_357del* construct ($p=0.33$) (Fig. 12B). Our results thus validate miR-142-3p target

predictions and indicate that both sites contribute equally to the action of miR-142-3p in the repression of the *Bmal1* 3' UTR.

In addition to miR-142-3p, miR-494 also represses the *Bmal1* 3'UTR activity by potentially interacting with nucleotides 473-495. Consequently, the effects of miR-494 on *Bmal1* 3'UTR activity were analyzed to verify that the loss of repression observed with the *Bmal1 c.1_7del + c.335_357del* construct was specific to the action of miR-142-3p. In HEK cells co-transfected with this mutant 3' UTR construct, overexpression of miR-494 caused significant reduction ($p<0.01$) of ~35% in luciferase-reported bioluminescence relative to cells co-transfected with the control vector (Fig. 12C). Importantly, this repression of *Bmal1* 3' UTR activity was similar to that observed for the WT construct, suggesting that the two analyzed loci are specific targets for the action of miR-142-3p.

Discussion

Circulating levels of many humoral factors, including insulin, adrenalin, leptin, ghrelin and corticosterol, are marked by robust circadian rhythmicity (Tasaka et al., 1980; De Boer and Van der Gugten, 1987). These hormones and neuroendocrine factors are thought to play a role in the hierarchical organization of the mammalian circadian system with regard to the coordination of rhythmicity between peripheral and CNS oscillators (Hommel et al., 2006; LeSauter et al., 2009). Although there is now increasing evidence for the presence of circulating miRNAs and their potential implications as biomarkers of pathological and physiological states (Gilad et al., 2008;

Hunter et al., 2008; Mitchell et al., 2008), the current study provides primary evidence indicating that levels of some circulating miRNA species are subject to rhythmic regulation as well. Specifically, our findings indicate that several miRNAs predicted to target core clock genes are also expressed in mouse serum, and that circulating levels of miR-494 and miR-152 are distinguished by diurnal oscillations. Similar to other diurnal and circadian rhythms in various processes including arterial pressure, neurotransmitter receptors, and circulating levels of hormones (Lucas et al., 1980; Wirz-Justice et al., 1987; Li et al., 1999), serum levels of miR-494 and miR-152 oscillate with bimodal patterns. Hughes et al. (2009) identified similar 12h harmonics of expression of multiple genes in vivo. The harmonics were lost ex vivo, and only a single circadian peak was observed. Thus, some bi-modal (12h harmonic) rhythms, including those observed in circulating miRNAs may reflect the independent contribution of two uncoupled oscillators representing system driven and oscillator dependent rhythms. It is noteworthy that despite the similarity in the phase of their rhythmic profiles with bimodal peaks occurring near mid-day and during the night, miR-494 and miR-152 are transcribed at different loci in the mouse genome; both are transcribed from a single locus, on chromosome 11 for miR-152 and on chromosome 12 for miR-494. Another distinction is that miR-152 is an intronic miRNA, while miR-494 is part of a large cluster.

The mechanism responsible for the rhythmic variations in serum levels of miR-494 and miR-152 is unknown. Importantly, comparative analysis of 18s rRNA levels in serum and corresponding WBC samples suggests that the miRNA expression and rhythmic profiles detected in serum are not contamination or artifact derived from lysed

or intact cells in the circulation. The observed rhythms in circulating levels of miR-494 and miR-152 may correspond to temporal variation in the intracellular production, packaging or endocytic trafficking of these miRNAs and/or their uptake by specific target cells. Alternatively, it is possible that the differential stability of some circulating miRNAs over time may contribute to these serum oscillations.

Recent evidence suggests that miRNAs may act as molecular switches regulating the timing of various biological events (Bartel and Chen, 2004). Thus, the present implications for miRNAs in circadian timekeeping seem to represent a logical extension of their known functions. At present, the precise role of miRNAs in either the molecular clockworks or the hierarchical organization of mammalian circadian oscillators is unclear. Several reports indicate that miRNAs may modulate some aspects of circadian pacemaker function and output rhythms (Cheng et al., 2007; Gatfield et al., 2009). Using in vitro analysis of luciferase-reported *Bmal1* 3' UTR activity to examine the effects of miRNA overexpression, miR-494 and miR-142-3p were identified as potential post-transcriptional repressors of *Bmal1*, for the first time implicating specific miRNAs in the regulation of this integral molecular component of the mammalian circadian clock. We also validated specific sites in the 3' UTR responsible for miR-142-3p action. Nucleotides 1-7, which are conserved in the *Bmal1* 3' UTR among mammalian lineages, are 100% complementary to the seed region (nucleotides 2-8) of miR-142-3p (Lewis et al*.,* 2005). The activity of a miRNAs is primarily thought to be dependent on complementarity between 3' UTR elements of target mRNA and the seed region of the candidate miRNA. Indeed, in vitro reporter assays indicate that 7-8mer sites

complementary to seed region of candidate miRNAs are sufficient for miRNA-mediated repression of the target 3' UTRs (Doench and Sharp, 2004; Brennecke et al., 2005; Lai et al., 2005). In corroboration with this presumed importance of seed region interactions, deletion of the first seven nucleotides in the *Bmal1* 3' UTR abated miR-142-3p mediated repression by ~50%, indicating that miR-142-3p targets this specific sequence. Additionally, we also examined miR-142-3p interactions with a highly conserved, canonical miRNA recognition element (MRE) located from positions 335-357 of the *mBmal1* 3' UTR. This site contains a 8mer element perfectly complementary to the seed region of miR-142-3p. In addition to the importance of the seed region, positions 13-19 of mature miRNA strands may also aid in the biological activity of miRNAs by functioning as 3' supplementary or 3' compensatory regions (Brennecke et al., 2005; Yekta et al., 2004; Grimson et al., 2007). Hence, all 22 nucleotides constituting the canonical MRE were deleted to accurately analyze miR-142-3p interactions with this target site. This deletion also yielded a ~50% reduction in miR-142-3p-mediated repression of *Bmal1* 3' UTR activity compared to non-mutagenized controls. Site-directed deletion of both miR-142-3p target sites, at nucleotides 1-7 and 335-357, completely abolished miR-142-3p mediated repression of the *Bmal1* 3' UTR activity. Comparable miR-494-mediated repression of the full-length (WT) and *c.1_7del* + *c.335-_357del Bmal1* 3' UTR clones verified that the deletions were in sites specifically required for miR-142-3p action. Thus, the mutagenesis analyses for the first time indicate that the *Bmal1* 3' UTR is targeted by miR-142-3p at two distinct locations and that both sites contribute equally to the repression produced by miR-142-3p.

The phase relationship between the diurnal rhythms in circulating levels of miR-494 and *Bmal1* oscillations in the periphery is consistent with the potential function of miR-494 in the post-transcriptional regulation of *Bmal1*. In most peripheral tissues, *Bmal1* accumulation follows a circadian profile in which mRNA levels are high from the middle of the night to early morning, and remain low throughout the rest of the cycle (Storch et al., 2002; Yamamoto et al., 2004). Hepatic BMAL1 protein content oscillates in a similar fashion such that the rhythmic peak occurs late in the subjective night and levels rapidly decline near the middle of the subjective day (Lee et al., 2001). Thus, the observed oscillations in circulating levels of miR-494 and miR-152 exhibit an interesting relationship with reported circadian profiles for *Bmal1* in the periphery, with bimodal peaks of miRNA expression encompassing times around the rising and falling phases of the *Bmal1* mRNA as well as protein rhythm . In conjunction with our evidence for miR-494-mediated repression of the *Bmal1* 3' UTR, the bimodal pattern of these serum oscillations may have some significance for the function of miR-494 and other miRNAs regulating core clock components as local and/or systemic cues that fine-tune the circadian harmonics of intercellular interactions and coordinate rhythmicity between autonomous circadian oscillators in peripheral tissues.

Although both miR-494 and miR-142-3p target the same gene, *Bmal1*, overt rhythmicity in extracellular levels may not be the sole criterion determining their role in the mammalian circadian system. Given the abundance of RNases in serum and evidence for the rapid degradation of naked synthetic miRNAs in plasma (Reddi and Holland, 1976; Mitchell et al., 2008), it seems likely that the endogenous serum miRNAs

observed in our experiments are packaged within various types of protective, membrane-bound particles, such as microvesicles and exosomes. Vesicles released into the circulation in vivo presumably arise from a wide variety of cells derived from different lineages and subtypes at different stages of maturation, and are distinguished by diverse functions. Recent studies indicate that these secreted vesicles mediate the intercellular communication of specific RNA signals (Valadi et al., 2007; Yuan et al., 2009; Pegtel et al., 2010; Kosaka et al., 2010) and that vesicle-transmitted miRNAs are functional in recipient cells (Yuan et al., 2009; Pegtel et al., 2010). Furthermore, different miRNAs are packaged in vesicles and exosomes expressing integral cell membrane proteins derived from their parental cells of origin, and these cell surface markers appear to specify the capture of these particles by certain tissues or cell types. For example, mature dendritic cell derived exosomes that express intercellular adhesion molecule-1 (ICAM1) are captured largely by lymphocyte function-associated antigen-1 (LFA-1) expressing activated T-cells and $CD8^+$ dendritic cells, but not by $CD8^-$ dendritic cells (Segura et al., 2007; Nolte-'t Hoen et al., 2009). Hence, the distinctive functions of different circulating miRNAs in regulating the molecular clockworks or overt circadian rhythms within or between specific tissues may be determined not only by temporal variation in extracellular levels but also by the parental cells from which the miRNA-containing vesicles originate, and by cell-surface receptors on specific target cells that capture these circulating vesicles.

Because a number of processes are subject to circadian regulation in the cardiovascular system, circulating miRNAs may play an important role in the local

and/or systemic coordination of circadian rhythms associated with cardiovascular physiology and pathology. The cardiovascular system is distinctly characterized by circadian regulation of various parameters such as arterial pressure, heart rate, and vascular tone (Guo et al., 2003; Shaw et al., 2009). Furthermore, circadian variation is an important factor in the manifestation of cardiovascular pathology, including myocardial infarction, sudden cardiac death and stroke (Shaw et al., 2009). The role of identified miRNAs with *Bmal1* as a putative target and rhythmic variations in their circulating levels in cardiovascular physiology and disease is currently unknown. However, miR-494 has been recently implicated in cardiac pathophysiological processes because cardiac-specific overexpression of this miRNA reduces myocardial infarction size in response to ischemia/reperfusion-induced cardiac injury (Wang et al., 2010c). In addition, the recent finding that deletion of several secretory vesicle proteins disrupts circadian rhythms of blood pressure and heart rate in the mouse (Kim et al., 2009) may have further implications for the role of extracellular miRNAs in regulating cardiovascular physiology. Nevertheless, our results suggest that extracellular miRNAs may play a role in the regulation of peripheral circadian clocks and that circadian profiling and comparison of different serum miRNAs in various disease models may provide a valuable tool in identifying biomarkers for human cardiovascular pathologies associated with circadian rhythm disturbances.

CHAPTER IV

MICRORNAS AS CIS- AND TRANS- ACTING MODULATORS OF CIRCADIAN RHYTHMS IN PERIPHERAL TISSUES

Introduction

An endogenous cell-autonomous circadian timekeeping mechanism generates rhythmic changes in many physiological and behavioral aspects including body temperature, sleep and wakefulness, food intake and locomotor activity. In mammals, endogenous clocks are thought to have a hierarchical organization, with the oscillator(s) in the suprachiasmatic nuclei (SCN) of the hypothalamus acting as the master pacemaker responsible for temporal synchronization of slave oscillators in other tissues and organ systems (Ralph et al., 1990; Reppert and Weaver, 2002). The SCN has been shown to accomplish this by secretion of diffusible factors and modulation of autonomic nervous inputs to peripheral organs (Silver et al., 1996; Allen et al., 2001; Buijs et al., 2003).

The genes which comprise the core molecular gears of the clock, *clock, bmal1, period(s) and cryptochrome(s)*, interact forming a feedback loop that maintains self-sustained oscillations in core-clock and clock-controlled genes not just in the SCN cells but also in most other cells of the body (Shearman et al., 1997; Balsalobre et al., 1998; Gekakis et al., 1998; Kume et al., 1999; Bunger et al., 2000; Yoo et al., 2004). As a result, even in the absence of SCN specific signals, robust overt rhythmicity in peripheral cells and organs has been reported in vitro and under certain conditions in vivo. Dispersed mammalian fibroblast cultures were shown to be synchronized by a

serum shock and explants of peripheral organs exhibit robust PERIOD2::LUCIFERASE reported circadian bioluminescence rhythms for several days in vitro (Balsalobre et al., 1998; Yoo et al., 2004). Additionally, SCN-independent circadian rhythms in physiology, metabolism and behavior have been reported under restricted feeding and in response to chronic drug treatment (Escobar et al., 1998; Tataroglu et al., 2006). Although hormones and metabolites have been implicated as potential candidates responsible for synchronization of peripheral clocks, it is yet unclear whether local paracrine mechanisms for temporal synchronization exist (Dibner et al., 2010). Given their rhythmic expression in the circulation, membrane-bound microRNAs (miRNAs) might constitute potential signals involved in communication of temporal information within local environments in the periphery (Shende et al., 2011).

MiRNAs are evolutionarily conserved, small non-coding RNAs that are generated by sequential processing of larger, primary and premature transcripts (Bernstein et al., 2001; Lee et al., 2003; Bartel, 2004). Computational algorithms predict that ~20-30% of the mammalian mRNA transcriptome is subject to miRNA-mediated regulation and miRNAs have already been shown to play crucial roles in various biological processes like development, oncogenesis and immune response (Lewis et al., 2005; Lindsay, 2008; Sayed and Abdellatif, 2011). Using mouse, fly and chicken model systems, miRNAs have also been identified as important modulators of circadian clock gene and output rhythms (Cheng et al., 2007; Gatfield et al., 2009; Kadener et al., 2009; Shi et al., 2009; Shende et al., 2011; Luo and Sehgal, 2012). MiRNAs primarily function as post-transcriptional modulators. They associate with components of the RNA induced

silencing complex (RISC) and interact with specific 3' untranslated regions (UTRs) of their target genes to affect translational repression or mRNA destabilization (Hutvagner and Zamore, 2002; He and Hannon, 2004). However, in addition to their presence in the cytosol, membrane-bound miRNAs have also been reported extracellularly, in the circulation in vivo and in conditioned cell culture medium in vitro (Mitchell et al., 2008; Yuan et al., 2009). Importantly, these membrane-bound miRNAs can be communicated from donor to recipient cells, where they remain biologically active (Kosaka et al., 2010; Kogure et al., 2011).

Our previous studies indicate that miR-142-3p and miR-494 target the 3' UTR of the core clock gene *Bmal1* and are expressed extracellularly in the circulation with miR-494 exhibiting a diurnal oscillation with bimodal peaks in serum levels (Shende et al., 2011). To explore the functional implications of these *Bmal1*-targeting miRNAs in the mammalian circadian system, we used serum-shocked fibroblasts to first determine whether miR-142-3p and miR-494 modulate endogenous BMAL1 protein levels and alter rhythms in core components of the molecular clockworks in a peripheral oscillator model. In addition to this analysis of their function in the regulation of clock gene rhythmicity, corollary experiments were conducted to address the possible role of miR-142-3p and miR-494 as extracellular signals that communicate temporal information locally among peripheral oscillators. Specifically, different in vitro models were used to determine whether: **1**) overexpression of miR-142-3p and miR-494 increases their extracellular abundance in culture medium, **2**) exposure to conditioned medium from miR-142-3p- and miR-494-overexpressing cells increases intracellular expression of

these miRNAs in recipient cells, **3**) the internalized miRNAs are functional in recipient cells, and **4**) disruption of vesicular communication using exocytosis and endocytosis inhibitors affects ensemble circadian rhythms.

Materials and Methods

Cell culture and transfections: NIH/3T3 fibroblasts, immortalized *mPer2*^{Luc} fibroblasts and HEK293 cells were grown in Dulbeco's minimum essential medium (DMEM; Invitrogen) supplemented with 292ug/mL L-glutamine, 4.5ug/mL D-glucose and 10% Fetal Bovine Serum (FBS). The medium was replaced every 48h and cultures were split 1:3 to 1:5 every 3-4 days. All transfections were performed with Lipofectamine 2000 (Invitrogen Inc.) according to the manufacturer's guidelines.

Experiment 1- Effects of miR-142-3p and miR-494 overexpression on endogenous BMAL1 protein levels and ensemble circadian rhythms of PER2::LUC expression: NIH/3T3 and immortalized *mPer2*^{Luc} fibroblasts were cultured and maintained as described above. Prior to experimentation, cells were seeded onto 6-well plates in DMEM supplemented with 5% FBS. Approximately 24h later, cells were transfected with either a non-targeting control-miR construct or with a combination of pre-miR constructs for miR-142-3p and miR-494 (final total conc. = 66nM). Following transfection for 5h, control and pre-miR-treated cultures (n=4) were placed in DMEM supplemented with 5% FBS. Forty-eight hours post-transfection, cells were collected by trypsinization, centrifuged, and pellets were flash frozen in liquid nitrogen. All samples were stored at -80^{0}C until further processing.

Analysis of BMAL1 protein levels: The pellets were sonicated in mammalian protein extraction reagent (MPER; Pierce, Inc.) supplemented with protease inhibitor cocktail (PMSF), and protein content in cell homogenates was measured using the bicinchoninic acid method (BCA Protein Assay Kit; Thermo Scientific Pierce). Each sample was loaded at ~50μg protein lysate per lane onto a 10% Tris-Glycine gel and electrophoresis was performed using an X-blot Mini-cell II apparatus (Invitrogen, Inc.). Following separation at 125V for 2h, proteins were transferred onto 0.45μm nitrocellulose membranes (Invitrogen) and blocked at room temperature for 1 hour with 5% skimmed milk in Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 (TBS-T). With interceding rinses in TBS-T, membranes were probed overnight at 4^{o}C with rabbit anti-BMAL1 (1:250; Abcam, Inc.) or a mouse monoclonal antibody against β-actin (1:2000; Sigma Inc.) followed by a 1-h incubation with horse-radish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:1000; Jackson Laboratories) or goat anti-mouse IgG (1:2000; Jackson Laboratories). Immunoreactive signal for BMAL1 was generated using enhanced chemiluminescence (ECL) reagent (Thermo Scientific Pierce) and luminescence for size-appropriate bands were detected using a FluorChem Gel imaging system (Alpha Innotech Corp.). To control for differences in protein content between samples, signal intensity measurements for BMAL1 were normalized to the values for β-actin in each sample. Densitometric analyses for immunoreactive bands were performed using NIH ImageJ software.

Analysis of PER2::LUC rhythms: To examine the function of miRNAs targeting *Bmal1* in the regulation of circadian rhythms, bioluminescence was analyzed from

mPer2^{Luc} fibroblast cultures that were transfected with pre-miR constructs for miR-142-3p and miR-494 as described above. About 24h hours post-transfection, cultures were serum shocked for 2h with DMEM supplemented with 50% horse serum After serum shock treatment, cultures were maintained in DMEM recording media (Sigma-Aldrich) containing 10mM HEPES, 0.03% NaHCO3, N2 supplement (1X; Invitrogen), 4.510g/L glucose, 25 units/ml penicillin, 25µg/ml streptomycin and 0.1mM beetle luciferin (Promega, Madison, WI), and analysis of mPer2Luc bioluminescence was initiated.

Real-time analysis of PER2::LUC bioluminescence: Individual cultures were sealed airtight with sterile glass coverslips (VWR) and sterile silicon grease (Dow Corning, Inc.). The temporal patterns of mPER2::LUC bioluminescence were analyzed using an automated 32-channel luminometer (LumiCycle; Actimetrics, Wilmette, IL) that was maintained within a standard cell culture incubator at 32^0C. Bioluminescence from individual cultures was continuously recorded with a photomultiplier tube for ~70 s at intervals of 10 min for 4–5 days. Due to the transient induction of bioluminescence following the medium change at the initiation of this analysis, the first cycle was excluded from data analysis. Bioluminescence data were analyzed using the Lumicycle Analysis program (Actimetrics). For each raw data set, baseline drift was removed by fitting a polynomial curve with an order equal to the number of recorded cycles. Rhythm parameters (period, phase and damping) were determined from baseline-subtracted data using the damped sine fit and the Levenberg–Marquardt algorithm.

Experiment 2- Export and internalization of miR-142-3p and miR-494 in NIH/3T3 and HEK293 cultures: Timekeeping functions and communication of

extracellular miRNAs was investigated using HEK293 and NIH/3T3 cell culture models of peripheral circadian oscillators.

Export of miR-142-3p and miR-494 into culture medium: Studies examining intercellular communication of miRNAs were performed using NIH/3T3 and HEK293 cells as depicted (Fig. 15). On day 0, donor NIH/3T3 or HEK293 cultures were seeded onto 24-well plates in DMEM supplemented with 5% FBS. Approximately 24h later (day 1), cells were transfected either with CmiR001-MR04 control vector encoding a scrambled precursor miRNA (Genecopoeia) or with a combination of miExpress™ precursor miRNA expression clones for mmu-miR-142 and mmu-miR-494 (n=4; final conc. = 0.8ug of plasmid/well). These expression clones contain the premature miRNA sequence inserted in the pEZX-MR04 vector downstream of an eGFP fluorescent reporter gene under the control of a CMV promoter. Following transfection for 4-5h, cells were rinsed with CMF and then maintained in DMEM supplemented with 5% FBS. Aliquots of the conditioned culture medium were collected at 12h, 24h (day 2) and 36h (day 3) post-transfection, and then flash frozen in liquid nitrogen. All conditioned media samples were stored at -80^{0}C until further processing.

Uptake of miR-142-3p and miR-494 from conditioned medium: For miRNA uptake experiments, recipient cultures were seeded onto 24-well plates on day 2 of the experiment. On day 3, the recipient cells were rinsed with CMF and exposed to conditioned medium collected at 36h post-transfection from CmmiR001-MR04-(scrambled control) or pEZX-MR04 (miR-142 + miR-494)-transfected donor cells. Recipient cells were then collected by trypsinization following 2h, 4h and 8h of

exposure to this scrambled control- or miR142+miR-494-containing conditioned medium (n=4 at each timepoint). Trypsinized cells were pelleted by centrifugation, the cell pellets were flash frozen in liquid nitrogen and stored at -80^{0}C until further processing.

RNA extraction from cells and conditioned culture medium: Total RNA, including the small RNA fraction was extracted using miRNeasy kit (Qiagen) from: **1**) donor cells collected at 36h post-transfection, **2**) donor cell culture conditioned medium collected at 12h, 24hand 36h post-transfection, and **3**) recipient cells collected at 2h, 4h and 8h of exposure to donor cell conditioned medium. Extracted RNA was estimated using Nanodrop ND1000 spectrophotometer (Thermo Scientific), aliquoted and stored at -80^{0}C until further processing.

Quantative Real-time PCR analysis: Quantitative real-time PCR analysis was conducted using Taqman microRNA assays (Applied Biosystems) as described previously (Shende et al., 2011). Briefly, RNA from individual samples was first reverse transcribed using target-specific stem-loop primers and Taqman MicroRNA Reverse Transcription Kit. All assays were performed according to manufacturer's guidelines. For analysis of miRNA expression, the cDNA equivalent of 2ng of total RNA was PCR amplified in an ABI PRISM 7500 Fast sequence detection system. To control for differences in sample RNA content and reverse-transcription efficiencies between samples, miR-16 was also amplified from the same samples using identical parameters. Using the comparative C_T method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (PE-ABI), the relative abundance for a given miRNA was

calculated by normalization first to corresponding miR-16 levels in each sample and then to a calibrator consisting of pooled cDNA from multiple samples comprising the experimental series.

Experiment 3- Effects of disrupting vesicular communication on ensemble circadian rhythms of PER2::LUC expression: Effects of exocytosis and endocytosis inhibitors on PER2::LUC rhythms were examined using *mPer2*^{Luc} fibroblasts that were seeded onto 35mm cell culture dishes (Corning) in DMEM supplemented with 5% FBS. At confluence (~24 hours later), cultures were serum shocked for 2h with DMEM supplemented with 50% adult horse serum. Following the serum shock treatment, PER2::LUC bioluminescence rhythms were monitored for 68-72h in cells (n=4 for control and each treatment) that were maintained in serum-free recording medium containing either vehicle control (DMSO; 5uL/mL), exocytosis inhibitor (Brefeldin-A; conc. 0.15ug/mL), or endocytosis inhibitor (Dynasore; conc. 40uM). Following treatment, both DMSO- and inhibitor drug-treated cells were rinsed with CMF, serum shocked again for 2h, and PER2::LUC bioluminescence rhythms were monitored for another 72h. Bioluminescence data were analyzed as described above.

Statistical analyses: Values obtained for densitometric analyses of normalized BMAL1 protein expression levels and for intracellular and conditioned medium miRNA expression comparisons were analyzed by independent *t*-tests. The α-value was set at 0.05 to identify statistically significant differences between treatment and controls.

Results

Experiment 1- Effects of miR-142-3p and miR-494 overexpression on endogenous BMAL1 protein levels and ensemble circadian rhythms of PER2::LUC expression: Based on the identification of miR-142-3p and miR-494 as potential repressors of *Bmal1* 3' UTR activity in the previous study, we examined the function of these miRNAs in repressing endogenous BMAL1 protein levels. Combinatorial overexpression of miR-142-3p and miR-494 resulted in significant repression ($p<0.05$) of BMAL1 protein levels compared to cells transfected with a non-targeting control (Fig. 13A). In cells transfected with miR-142-3p and miR-494, this repression in BMAL1 protein levels ranged from 18-56% of control values. Because BMAL1 is an integral component of the molecular clock mechanism (Bunger et al., 2000), experiments were conducted to determine whether overexpression of these BMAL1-targeting miRNAs also affects ensemble circadian rhythms in culture. Real-time recording of PER2::LUC bioluminescence from *mPer2*^{Luc} fibroblasts revealed severe disruption of ensemble rhythms in miR-142-3p+miR-494-transfected cultures (Fig. 13B). Compared to control-transfected cells, the period of the PER2::LUC rhythms in miRNA overexpressing cultures was significantly increased ($p<0.001$) by ~7h. Overexpression of miR-142-3p and miR-494 also induced a ~50% reduction in rhythm amplitude and a significant increase ($p<0.001$) in rhythm damping rate relative to control cultures (Fig. 13B & Table 2). Collectively, these results suggest that tonic increases in intracellular levels of miR-142-3p and miR-494 may alter ensemble rhythmicity via their action on *Bmal1*.

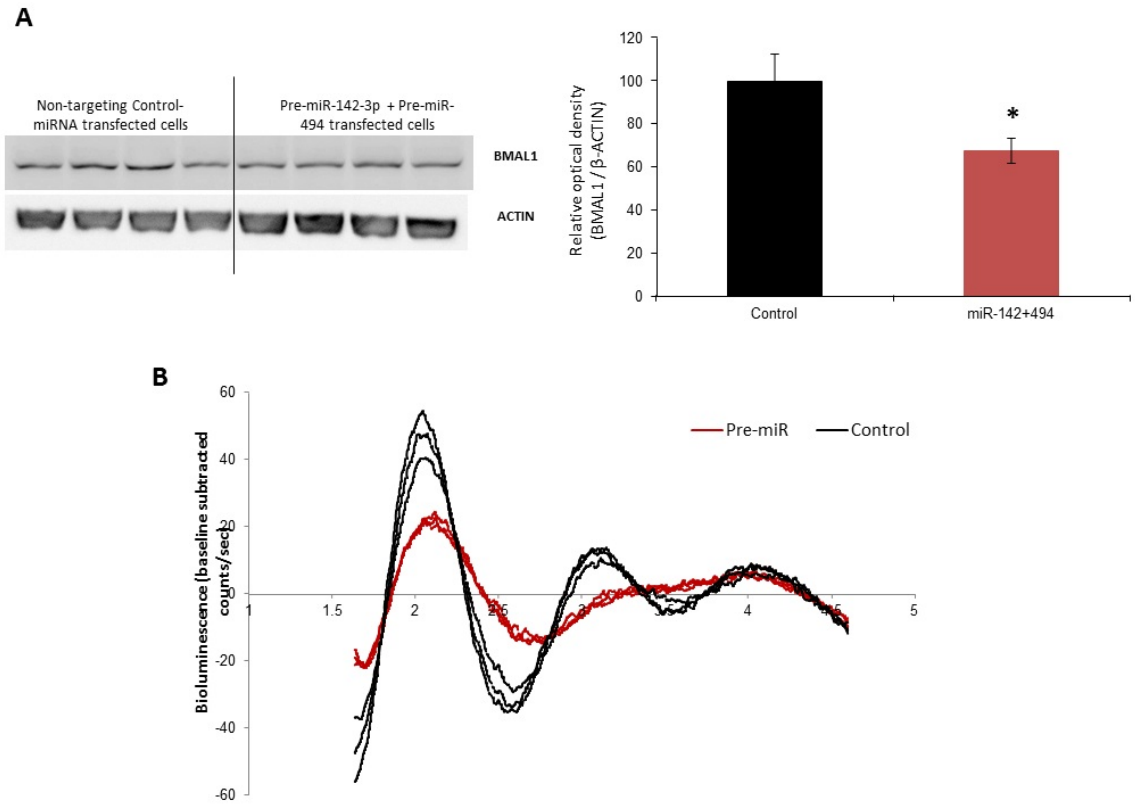

Figure 13. Effects of overexpression of miR-142-3p and miR-494 on BMAL1 protein expression and ensemble PER2::LUC bioluminescence rhythms. (**A**) Representative Western blot results and densitometric analysis of BMAL1 protein levels in scrambled control (black bars) and pre-miR-142-3p+pre-miR-494 (red bars) transfected NIH/3T3 cells (n=4). Plotted values represent the relative optical density (mean ± SEM) and correspond to the ratios of BMAL1/β-ACTIN immunoreactive signal that were adjusted in relation to the average for the control-transfected cells, which was arbitrarily set at 100. Asterisks denote values for relative protein levels in pre-miR-transfected cells that were significantly decreased (p<0.05) in comparison with those observed in controls. (**B**) Photomultiplier-based recordings of ensemble bioluminescence (expressed as detrended baseline-subtracted counts per second) from individual cultures of *mPer2*^{Luc} fibroblast cells transfected with scrambled control (black traces) or pre-miR-142-3p+pre-miR-494 (red traces) constructs (n=3).

Table 2. Cycle parameters for PER2::LUC bioluminescence rhythms following miRNA overexpression.

	Period (mean ± SEM)	**Damping (mean ± SEM)**
Control	24.77 (± 0.22)	1.02 (± 0.02)
miR-142-3p+miR-494	31.84 (± 0.59)*	0.78 (± 0.01)*

Experiment 2- Export and internalization of miR-142-3p and miR-494 in NIH/3T3 and HEK293 cultures: To accurately determine changes in intracellular and extracellular miRNAs after transfection with pEZX-MR04 miRNA expression vectors, we first analyzed the endogenous baseline expression of miR-142-3p and miR-494 in HEK293 and NIH/3T3 cells (Fig. 14A). In both, NIH/3T3 and HEK293 cells, the relative expression of endogenous miR-494 was significantly, ~300-fold and ~65-fold respectively, higher than endogenous expression of miR-142-3p ($p<0.001$). Consequently, transfection with pEZX-MR04-miR-142 miRNA expression vector resulted in ~180-fold and ~1800-fold upregulation of intracellular miR-142-3p levels in miRNA expression vector transfected NIH/3T3 and HEK293 cells respectively compared to control transfectants ($p<0.001$; Fig. 14B & 14C). However, given its higher intracellular abundance, miR-494 was significantly and ~7-fold higher only in pEZX-MR04-miR-494 miRNA expression vector transfected HEK293 cells (*$p<0.01$*) (Fig. 14B). The intracellular levels of miR-494 in NIH/3T3 cells did not exhibit significant changes even after transfection with the miR-494 miRNA expression vector (Fig. 14C).

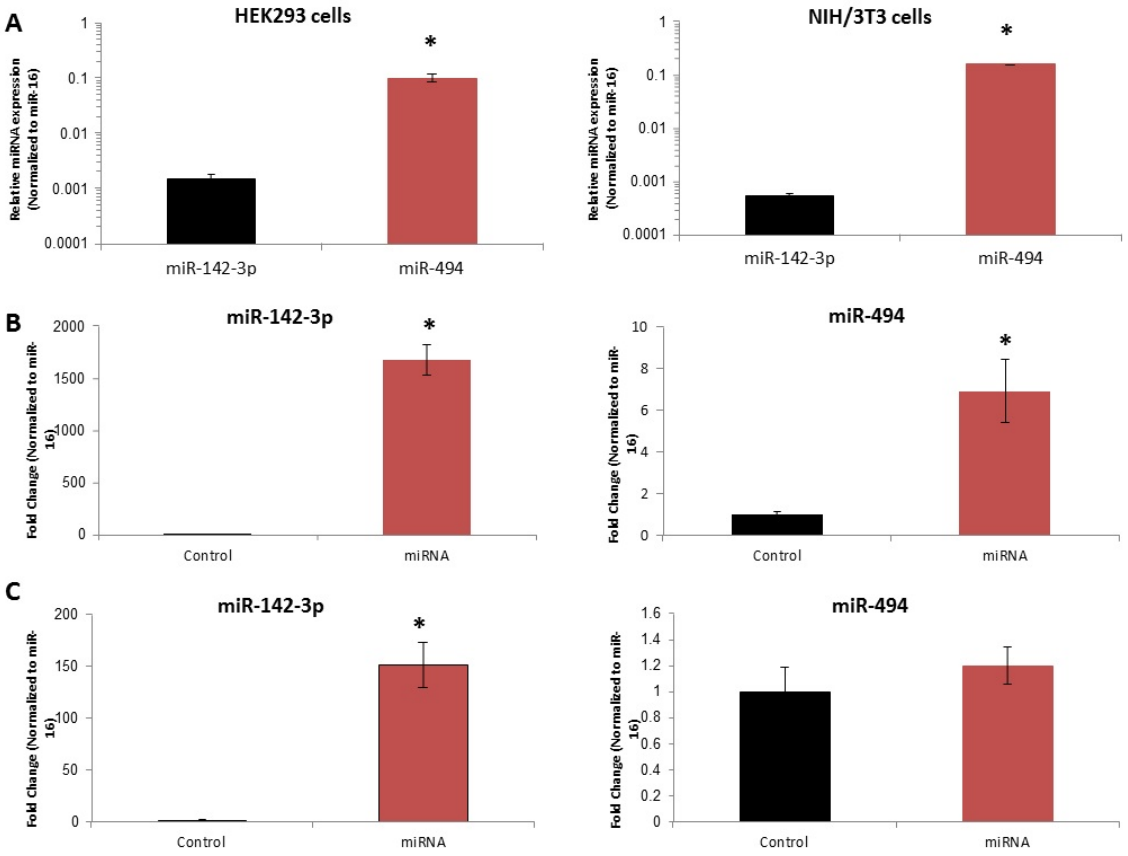

Figure 14. Comparison of intracellular levels of miR-142-3p and miR-494 in HEK293 and NIH/3T3 cells. (**A**) Bars represent real-time PCR determinations (mean ± SEM) of endogenous miR-142-3p (black bars) and miR-494 (red bars) in HEK293 (left panel) and NIH/3T3 (right panel) cultures (n=4). The plotted values correspond to the ratios of intracellular miR-142-3p and miR-494 signal in non-transfected HEK293 and NIH/3T3 cells. Asterisks denote comparisons in which the relative expression of miR-142-3p signal was significantly different (p<0.05) from that of miR-494. (**B & C**) Bars represent real-time PCR determinations (mean ± SEM) of miR-142-3p (left panels) and miR-494 (right panels) in HEK293 (**B**) and NIH/3T3 (**C**) cultures. Plotted values correspond to the ratios of intracellular miRNA signal, in cultures (n=4) transfected with pEZX-MR04-control vector (black bars) or with a combination of pEZX-MR04-miR-142 + pEZX-MR04-miR-494 vectors (red bars), and were adjusted in comparison with the average for miRNA expression in control-transfected cultures that was arbitrarily set as 1.

To assess whether miR-142-3p and miR-494 are expressed extracellularly in vitro in conditioned cell culture medium, HEK293 and NIH/3T3 cells were co-transfected with a combination of pEZX-MR04-miR-142 and pEZX-MR04-miR-494 expression vectors. Conditioned culture medium was collected at timed intervals post-transfection and expression of miR-142-3p and miR-494 relative to miR-16 was estimated as depicted in Fig. 15. At each timepoint, expression of miR-142-3p and miR-494 were significantly higher ($p<0.01$) in conditioned medium from HEK293 cells transfected with the miRNA expression vectors compared to conditioned medium from cells transfected with a vector encoding a non-targeting scrambled control miRNA (Fig. 16A). The extracellular expression of miR-142-3p in conditioned medium from miRNA-transfected cells increased steadily such that levels of miR-142-3p at 24h and 36h were significantly higher ($p<0.01$) than those at 12hr. Peak miR-142-3p expression was achieved at 36h and was ~60-fold higher in conditioned medium from miRNA expression vector- compared to scrambled control- transfected cells. Similar to miR-142-3p, extracellular expression of miR-494 at each timepoint was also significantly greater ($p<0.01$) in conditioned medium from miRNA-transfected cells compared to that from cells transfected with a scrambled control vector (Fig. 16A). Interestingly, the peak in extracellular expression of miR-494 was observed at 24h with ~75-fold increase in conditioned medium from miRNA-transfected cells compared to controls. At 36hr, the miR-494 expression in conditioned medium from miRNA-transfected cells decreased to ~50% of that observed at 24h (Fig. 16A). Extracellular expression of miR-142-3p and miR-494 in conditioned medium from NIH/3T3 cells was similar to that observed with

HEK293 conditioned medium. Specifically, miR-142-3p and miR-494 levels were significantly higher ($p<0.01$) than those found in control-transfected conditioned medium with miR-142-3p increasing over time while peak accumulation of miR-494 occurring earlier, at 12h (Fig. 16B).

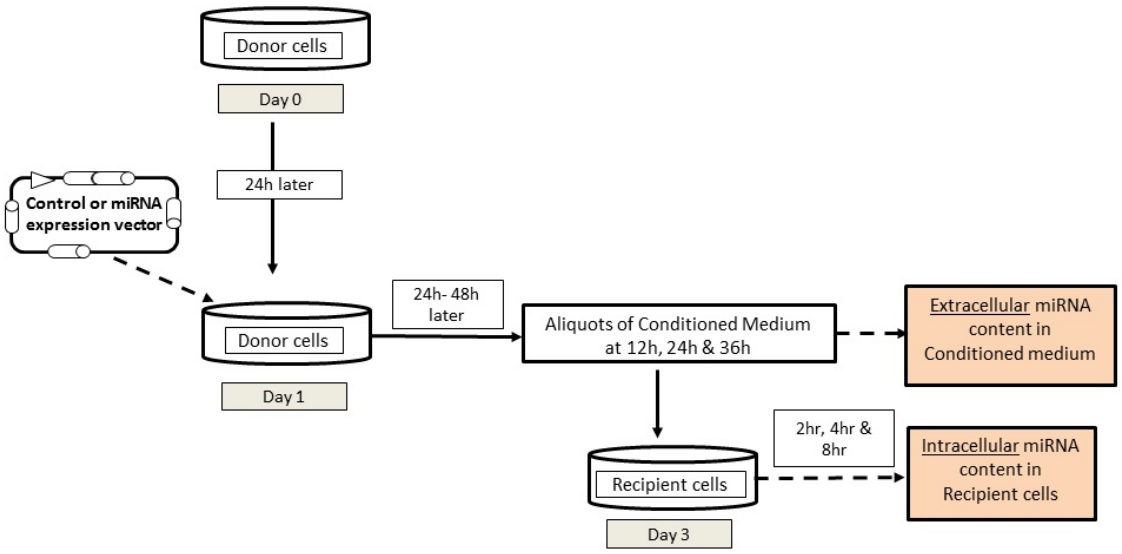

Figure 15. Experimental paradigm for miRNA communication experiments. Diagrammatic representation of the paradigm used for experiments testing miRNA export into and internalization from conditioned culture medium.

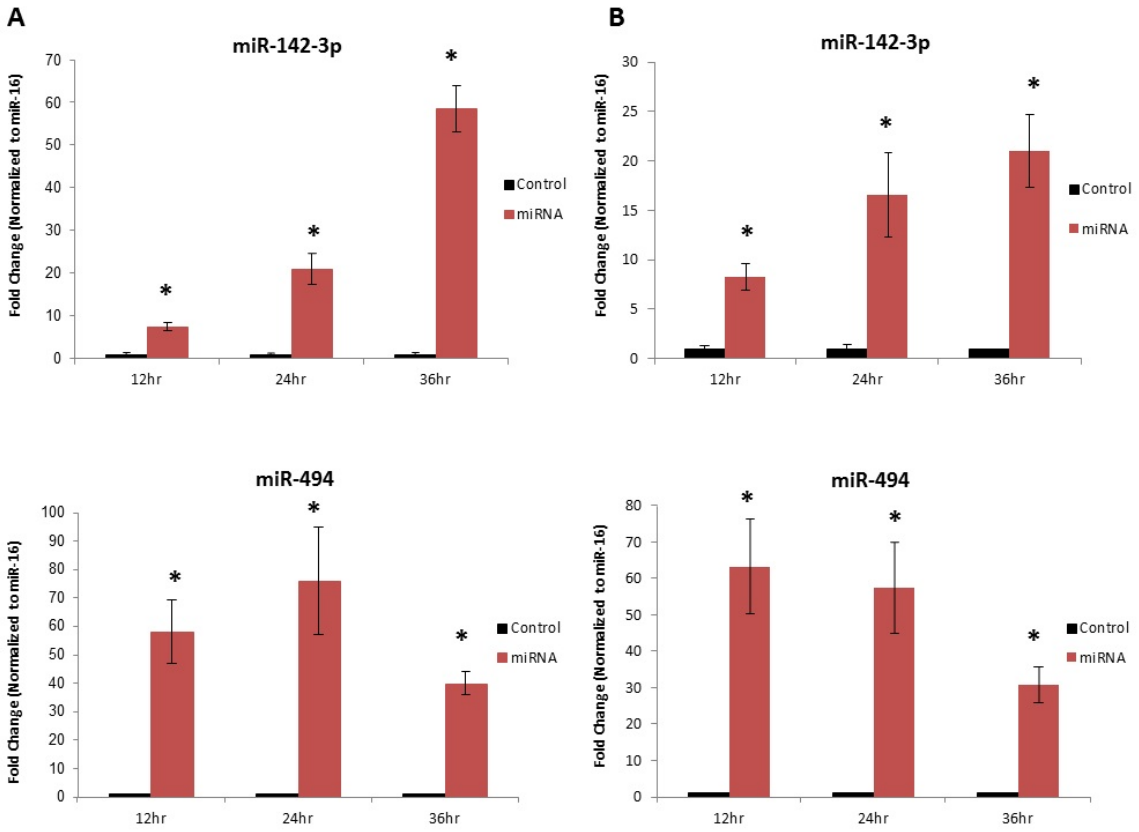

Figure 16. Comparison of conditioned medium levels of miR-142-3p and miR-494 in HEK293 and NIH/3T3 cells. Bars represent real-time PCR determinations (mean ± SEM) of miR-142-3p (top panels) and miR-494 (bottom panels) at 12h, 24h and 36h in conditioned medium from HEK293 (**A**) and NIH/3T3 (**B**) cultures. Plotted values correspond to the ratios of miRNA signal in conditioned medium from cultures (n=4) transfected with pEZX-MR04-control vector (black bars) or with a combination of pEZX-MR04-miR-142 + pEZX-MR04-miR-494 vectors (red bars), and were adjusted in comparison with the average for miRNA expression in control-transfected cultures at each timepoint that was arbitrarily set as 1. Asterisks denote comparisons in which the relative expression of miRNA signal in control-transfected cultures was significantly different (p<0.05) from that in miR-142+miR-494-transfected cultures.

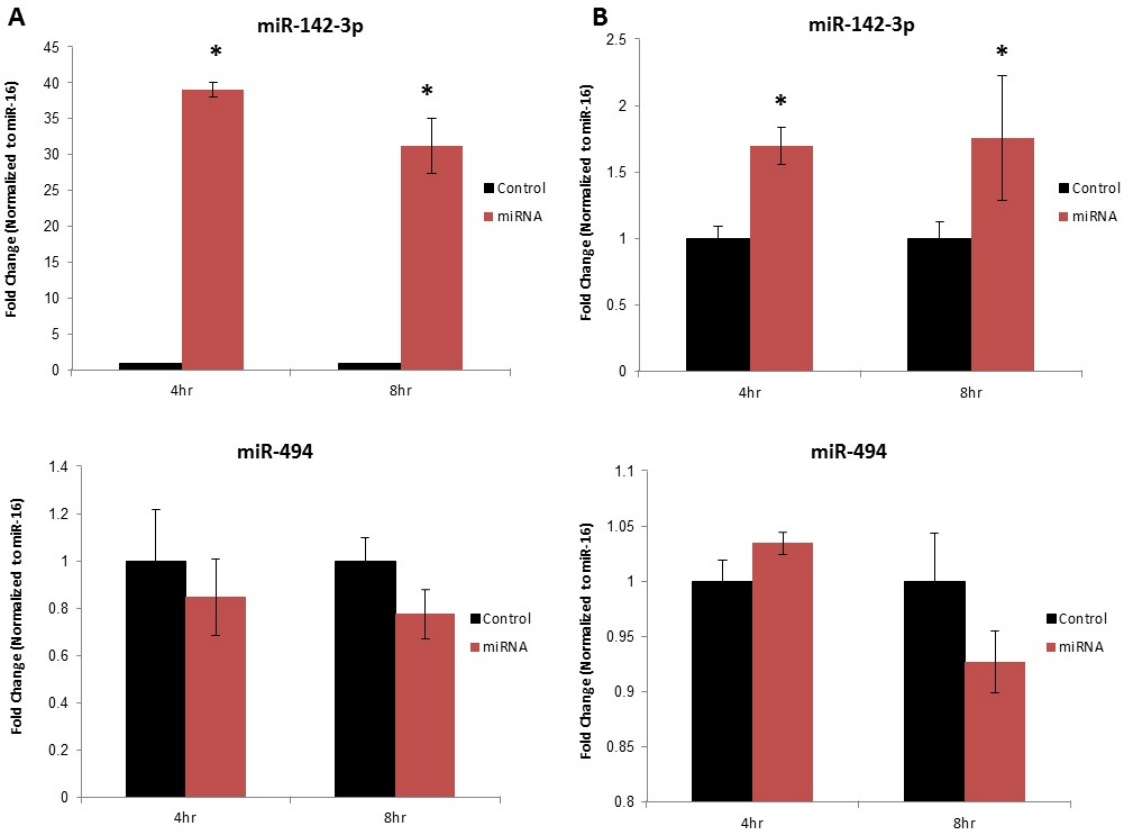

Figure 17. Comparison of intracellular levels of miR-142-3p and miR-494 in HEK293 and NIH/3T3 cells exposed to conditioned culture medium. Bars represent real-time PCR determinations (mean $\pm$ SEM) of miR-142-3p (top panels) and miR-494 (bottom panels) in HEK293 (**A**) and NIH/3T3 (**B**) cultures after 4h and 8h of exposure to conditioned medium from control- or miR-142+miR-494-transfectd cultures. Plotted values correspond to the ratios of intracellular miRNA signal, in cultures (n=4) exposed to conditioned medium from control-transfected (black bars) or from miR-142+miR-494-transfected (red bars) cultures, and were adjusted in comparison with the average for miRNA expression in control-transfected cultures that was arbitrarily set as 1. Asterisks denote comparisons in which the relative expression of miRNA signal in cultures exposed to control-transfected conditioned medium was significantly different ($p<0.05$) from that in cultures exposed to miR-142+miR-494-transfected conditioned medium.

Whether the extracellular miRNAs in conditioned culture medium were biologically active, was analyzed by determining whether the miRNAs could be internalized by recipient cells. Exposure to miRNA-containing conditioned medium produced significant increases ($p<0.001$) in miR-142-3p levels in the recipient HEK293 cells. Compared to control miR-containing conditioned medium, intracellular levels of miR-142-3p were elevated by 40- and 35-fold after 4h and 8h of exposure to miRNA conditioned medium, respectively (Fig. 17A). Relative to controls, recipient NIH/3T3 cells also exhibited modest but significant elevation ($p<0.05$) in intracellular miR-142-3p levels at 4h and 8h of exposure to miRNA conditioned medium (Fig. 17B). Given the high endogenous expression of miR-494, it is not surprising that we did not observe significant elevation in intracellular miR-494 levels in either HEK293 or NIH/3T3 recipient cells after exposure to miRNA-conditioned medium.

To further validate vesicle mediated communication of miRNA signals between donor and recipient cells, recipient cells were treated with Dynasore, an endocytosis inhibitor for the duration of their exposure to conditioned medium from control or miRNA transfected donor cells. Inhibition of endocytosis significantly abated increases in intracellular miR-142-3p even after exposure to miRNA-conditioned medium (Fig. 18A). There was no significant difference in intracellular miR-142-3p levels between recipient cells exposed for 8h either to control- or to miRNA- conditioned medium. For further verification, the experimental paradigm was modified such that immediately following transfection with either control or miRNA expression vector, donor cells were treated with Brefeldin-A (BFA), an exocytosis inhibitor and conditioned medium was

collected as described above. Exposure of recipient cells to BFA-treated control- or miRNA- conditioned medium for 8h did not result in significant differences in intracellular miR-142-3p levels between recipient cells exposed either to control or to miRNA-conditioned medium (Fig. 18A). Thus both, inhibition of exocytosis in donor cells, and inhibition of endocytosis in recipient cells had similar effects in inhibiting communication of extracellular miRNAs between donor and recipient cells (Fig. 18A). We next investigated whether the communicated miR-142-3p retained its biological activity in recipient cells by examining the effects of exposure to miRNA-containing conditioned medium on the *Bmal1* 3' UTR activity in recipient HEK293 cells (Fig. 18B). Exposure to miRNA-containing conditioned medium for 8h significantly reduced ($p<0.05$) luciferase-reported bioluminescence in *Bmal1* 3′ UTR-expressing cells relative to that in control vector transfectants, implicating that the elevated intracellular miR-142-3p was functional in the recipient cells.

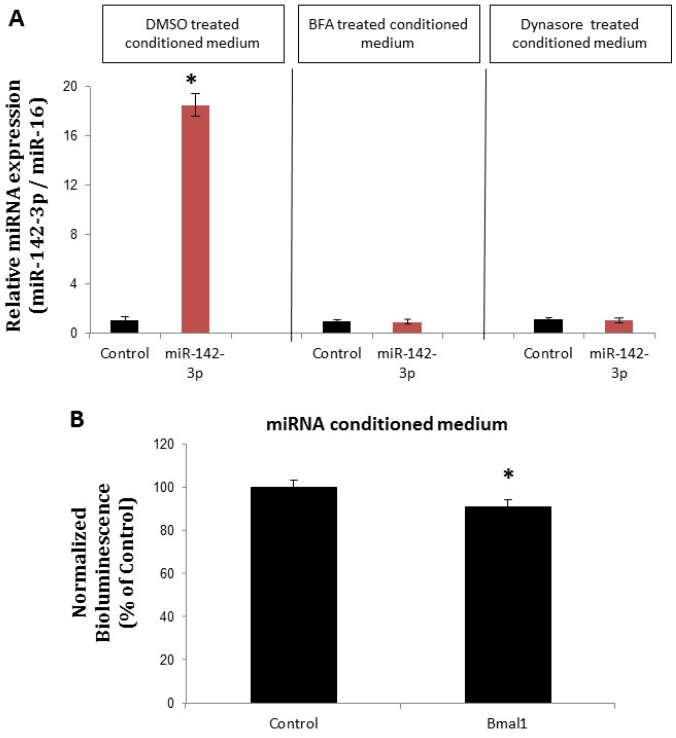

Figure 18. Effects of exocytosis and endocytosis inhibitors on miR-142-3p uptake and functional activity of miR-142-3p in recipient cells. (**A**) Bars represent real-time PCR determinations (mean ± SEM) of miR-142-3p in HEK293 cultures after 8h of exposure to conditioned medium from control-transfected (black bars) or miR-142+miR-494-transfectd (red bars) cultures. Plotted values correspond to the ratios of intracellular miRNA signal, in cultures (n=4) and was adjusted in comparison with the average for miRNA expression in control-transfected cultures that was arbitrarily set as 1. (**B**) Bars denote normalized bioluminescence (mean ± SEM) determinations from HEK293 cells expressing the control or *Bmal1* 3′ UTR vector in response to treatment with miRNA conditioned culture medium for 8h (n = 4). The plotted values correspond to the ratios of firefly luciferase signal normalized to *Renilla* luciferase activity in the same sample and are represented as a percentage of the average signal for control vector transfectants which was arbitrarily set at 100. Asterisks indicates normalized bioluminescence signal in cultures expressing control-vector was significantly different ($p<0.05$) from that in cultures expressing *Bmal1* 3' UTR vector.

Experiment 3- Effects of disrupting vesicular communication on ensemble circadian rhythms of PER2::LUC expression: To assess the potential role of vesicle-mediated communication in the regulation of temporal synchrony among peripheral circadian oscillators, the effects of exocytosis or endocytosis inhibitors on ensemble rhythmicity of *mPer2*^{Luc} fibroblasts were analyzed. During treatment with Brefeldin-A (BFA), the period of PER2::LUC bioluminescence rhythm was significantly lengthened ($p<0.001$). In addition to increasing the period by~7h, BFA induced severe damping of rhythm amplitude such that there was no observable peak in PER2::LUC bioluminescence by the third day of treatment (Fig. 19A and Table 3). Similar to the effects of exocytosis inhibitor, treatment with the endocytosis inhibitor, Dynasore, significantly increased ($p<0.001$) the period of the PER2::LUC rhythm. In Dynasore-treated *mPer2*^{Luc} fibroblasts, circadian period was increased by ~9h relative to that observed in vehicle controls (Fig. 19B and Table 3). However, Dynasore treatment had no effect on the amplitude of the PER2::LUC rhythm. Both drugs are reversible inhibitors, and post-treatment rhythms resembled those observed in vehicle control cultures. Some residual effects of BFA on rhythm amplitude were transiently observed during the 1st day after drug treatment. However, other rhythm parameters such as period and damping rate were comparable during subsequent cycles ($p>0.05$). In Dynasore-treated cultures, post- treatment rhythm amplitude and damping were similar to those observed in control cultures. However, the increase in circadian period persisted after drug washout.

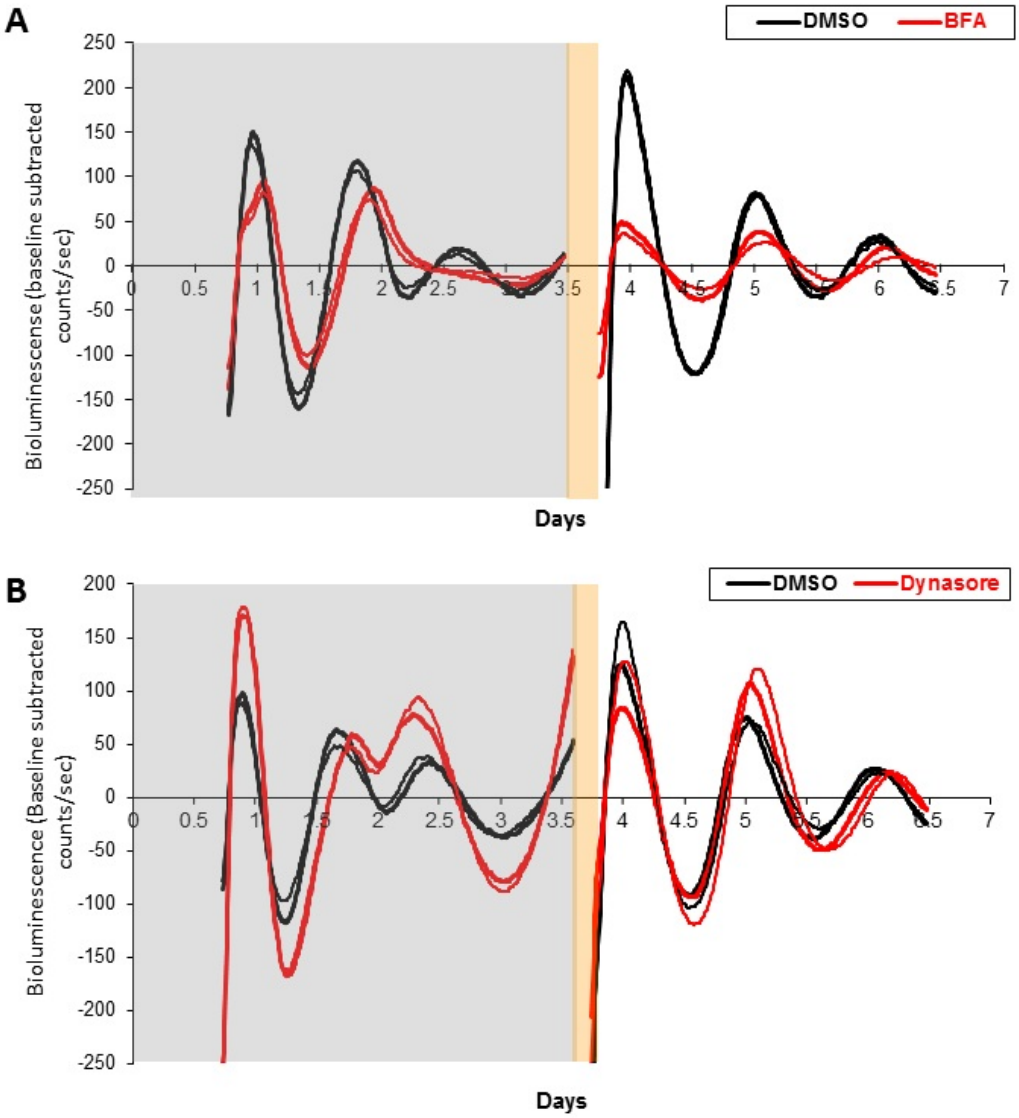

Figure 19. Effects of exocytosis or endocytosis inhibitors on ensemble PER2::LUC bioluminescence rhythms. (**A & B**) Representative individual photomultiplier-based recordings of ensemble bioluminescence (expressed as detrended baseline-subtracted counts per second) from representative cultures of *mPer2*^{Luc} fibroblast cultures (n=4) treated with DMSO vehicle control (black traces), Brefeldin-A (red traces, **A**) or Dynasore (red traces, **B**). Grey shaded region represents drug- or vehicle control-treatment, pink shaded region represents post-treatment serum-shock and non-shaded region represents post-treatment recovery.

Table 3. Cycle parameters for PER2::LUC bioluminescence rhythms following treatment with Brefeldin-A or Dynasore.

	During Treatment (mean ± SEM)		Post-treatment (mean ± SEM)	
	Period	**Damping**	**Period**	**Damping**
DMSO	21.21 (± 0.56)	0.96 (± 0.04)	23.65 (± 0.18)	1.25 (± 0.04)
BFA-0.15ug	30.36 (± 0.85)*	0.95 (± 0.03)	24.94 (± 1.28)	1.51 (± 0.15)
DMSO	21.10 (± 0.17)	1.39 (± 0.11)	26.93 (± 0.17)	2.14 (± 0.03)
DYNA-30uM	30.06 (± 0.28)*	n/a	29.13 (± 0.43)*	2.03 (± 0.19)

Discussion

Numerous studies have independently demonstrated extracellular expression of miRNAs, both in the circulation in vivo and in conditioned medium from cultured cells in vitro (Valadi et al., 2007; Gilad et al., 2008; Hunter et al., 2008; Mitchell et al., 2008; Yuan et al., 2009; Wang et al., 2010b). Because distinctive miRNAs signatures characterize various diseases, much attention has been focused on the use of circulating miRNAs as diagnostic or prognostic biomarkers of pathologies (Chen et al., 2008; Rabinowits et al., 2009; Huang et al., 2010; Wang et al., 2010a). Whether these extracellular miRNAs are by-products of cellular activity and only have diagnostic potential or if they are active moieties with functional and therapeutic importance remain fundamental questions yet to be clearly answered. In this regard, our study examines

whether extracellular miRNAs can potentially communicate temporal information between cells. Specifically, the findings indicate that miR-142-3p and miR-494, which are expressed in the circulation in mouse serum, can function intracellularly to repress endogenous BMAL1 protein and modulate ensemble circadian rhythms of cultured cells. Additionally, miR-142-3p and miR-494 can be secreted into extracellular environments and may be communicated between cells in vitro by vesicular trafficking.

The circadian system is composed of cell-autonomous clocks present in most cells of the body. Efficient functioning and fine-tuning of rhythmic physiological and behavioral outputs depends on two factors; first, the precise functioning of each intracellular core oscillator, and second, effective communication of temporal information among clocks both locally, within in a tissue or organ system, and globally, between various central and peripheral organ systems. Using NIH/3T3 fibroblasts, we provide primary evidence that combinatorial overexpression of miR-142-3p and miR-494 can repress endogenous BMAL1 protein expression by ~33% compared to cells overexpressing a scrambled control miRNA. The protein repression potentially also affects ensemble circadian rhythms of cells in culture since miR-142-3p and miR-494 overexpressing fibroblasts exhibit lengthened period and faster damping of PER2::LUC reported bioluminescence rhythms. Thus, our data implicate miR-142-3p and miR-494 as important post-transcriptional modulators of the intracellular core circadian timekeeping machinery.

Although the master pacemaker in the SCN is thought to effect synchronized behavioral output rhythms by secretion of diffusible factors like prokineticin 2, TGF-α

and cardiotropin-like cytokine (Kramer et al., 2001; Cheng et al., 2002; Kraves et al., 2006), it is unclear if local temporal communication also occurs between or within peripheral clocks. By utilizing pEZX-MR04 miRNA expression vectors, and two cell lines as model systems for peripheral clocks, we analyzed in vitro, whether miRNAs could be expressed extracellularly and could be communicated between cells. It is important to note that in contrast to chemically stabilized miRNA mimics like Pre-miR miRNA overexpression constructs, the pEZX-MR04 miRNA expression vector used during these studies codes for stem-loop premature miRNAs that undergo processing by *Dicer* to yield the mature miRNA. The miRNAs thus generated utilize part of the endogenous miRNA biogenesis machinery and hence potentially faithfully reproduce biogenesis, processing, accessibility and stability characteristics identical to those of corresponding endogenous miRNAs. Compared to conditioned medium from scrambled control miRNA transfected cultures, we detected robust, sustained increases with time in extracellular miR-142-3p expression in conditioned medium collected from both, HEK293 and NIH/3T3 cells transfected with pEZX-MR04-miR-142 miRNA expression vector. It was recently reported in studies involving multiple cell types including HEK293, MCF7, mouse mast and dendritic cells that miR-142-3p is preferentially exported into extracellular environments (Guduric-Fuchs et al., 2012). Thus, it is possible that the sustained increase in extracellular miR-142-3p could either be a result of its continuous intracellular transcription from the pEZX-MR04 vector, or its preferential export out of the cells, or a combination of both. Extracellular expression of miR-494 was also significantly higher (by ~60-70 fold) in conditioned medium from

HEK293 and NIH/3T3 cells transfected with miRNA expression vector compared to corresponding levels in conditioned medium from control miRNA transfected cultures. This was surprising, given that transfection with the pEZX-MR04-miR-494 miRNA expression vector had resulted only in modest increases (~6-fold) in intracellular miR-494 in HEK293 cells, and no difference in intracellular miR-494 levels in NIH/3T3 cells. Our data thus raise the intriguing possibility that specific homeostatic mechanisms regulating intracellular levels of miR-494 might exist, and perturbations of these levels as achieved by transfection with miR-494 expression vector may result in preferential export of excess miRNA molecules out of the cell. Additionally, in both cell types tested, extracellular miR-494 levels exhibited differences between 12h, 24 and 36h after transfection with the miRNA expression vector. In HEK293 cell culture conditioned medium, miR-494 levels at 24h were significantly and ~2-fold higher than those observed at 36h. miR-494 expression in NIH/3T3 conditioned medium at 12h was similarly ~2-fold higher than corresponding levels at 36h. Our previous in vivo data have identified diurnal oscillations in miR-494 expression characterized by 3-4 fold differences in serum levels of this miRNA over an 8-12h period. Although the cause of this reduction over time is unclear, the change in extracellular miR-494 levels observed in vitro may be a result of miRNA re-uptake or of lower stability of extracellular miR-494, or a combination of both.

The observation that exposure to miRNA containing conditioned medium resulted in increased levels and corresponding functional activity of intracellular miR-142-3p in recipient cells suggests that miR-142-3p, and potentially other extracellular

miRNAs, might be communicated between cells. Because extracellular vesicles like exosomes and microvesicles have been shown to transport miRNA cargo between cells (Kosaka et al., 2010; Kogure et al., 2011), we used exocytosis and endocytosis inhibitors to assess the effects of disruption of vesicular communication on miRNA transfer between cells and on ensemble circadian rhythms in culture. Treatment with either inhibitor resulted in significant lengthening of the period of PER2::LUC reported bioluminescence rhythms in fibroblasts. Dynasore disrupts endocytosis by inhibition of *dynamin,* which is required for calthrin-coating of endocytic vesicles. Using s*hibire,* which is a loss of function mutation in *dynamin*, other studies have reported similar increases in the period of circadian free-running behavioral rhythms in *Drosophila* (Kilman et al., 2009; Wulbeck et al., 2009). Dynasore treatment was also reported to cause decreased amplitude of rhythms in mouse SCN slice cultures (Deery et al., 2009). Thus, effects of endocytosis inhibition on ensemble circadian rhythms in culture and in vivo have also been verified by two other independent reports. Although aberrant neurotransmitter release due to disrupted vesicular communication might explain some of the effects observed in SCN cultures or in *Drosophila* mutants, the PER2::LUC fibroblast cell line used in this study is devoid of neurons. Thus, our data suggest that miRNAs, either independently or synergistically with other protein or small molecule components of extracellular vesicles, might facilitate temporal synchronization between cells within local environments in the periphery.

For miRNAs to function as signaling molecules, specificity of communication of candidate miRNAs would be an important consideration. The specificity can potentially

be generated at multiple levels: **1**) biogenesis within the donor cell or tissue type, **2**) vesicular packaging of miRNAs and export out of cells, and **3**) uptake by recipient cells. Some evidence supporting regulation at these different levels already exists. For example, recent studies have described cell-type dependent preferential export of miRNAs such that expression of some miRNAs may be higher in extracellular vesicles than in parental cells (Mittelbrunn et al., 2011; Pigati et al., 2010; Wang et al., 2010b; Guduric-Fuchs et al., 2012). Additionally, there is growing evidence concerning selective packaging of certain miRNAs into vesicles (Zhang et al., 2010; Hergenreider et al., 2012). Although the mechanistic details are yet unclear, cell- or tissue-type dependent vesicle export mechanisms could also account for specificity of miRNA signaling. Extracellular vesicles contain cell-surface markers characteristic of their donor cell types. These cell surface markers can mediate preferential uptake of vesicles by specific donor cells as seen in mature dendritic cell derived exosomes expressing intercellular adhesion molecule-1 (ICAM1) being preferentially captured by lymphocyte function-associated antigen-1 (LFA-1) expressing activated T-cells and $CD8^+$ dendritic cells, but not by $CD8^-$ dendritic cells (Segura et al., 2007; Nolte-'t et al., 2009). Although the specific contribution(s) of each level of regulation to communication of temporal information by extracellular miRNAs is yet unclear, our results provide evidence that extracellular miRNAs may play a role in temporal synchronization of peripheral clocks with local environments in the periphery.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

The circadian timekeeping system is composed of a network of cell-autonomous oscillators that receive environmental information via input pathways, interact among each other via physical connections or diffusible factors and generate rhythmic outputs that function as an ensemble to help an organism anticipate temporally influenced events throughout the day. In mammals, the SCN is considered the seat of the master pacemaker responsible for synchronizing rhythms in central and peripheral oscillators. For accurate functioning, this complex system is regulated at three levels: **1**) the intracellular molecular timekeeping machinery in each cell that contains a clock, **2**) synchronization of cell-autonomous clocks within local environments in the SCN and in peripheral tissue(s), and **3**) synchronization of clocks globally, that is, between SCN and peripheral tissues or among two or more peripheral tissues. This dissertation focuses on two of these fundamental levels, aiming to deduce the role of miRNAs in regulation of the intracellular clock machinery and in communication of temporal information to help generate synchrony among cell-autonomous clocks within local environments.

miRNAs as Ancillary Loops Stabilizing the Intracellular Clockwork

For more than a decade, the core transcriptional-translational feedback loop was considered to be sufficient and necessary for the generation of circadian rhythms in mammals. However, recent studies have encouraged re-evaluation of this view.

Circadian rhythms in redox cycles of the antioxidant proteins, peroxiredoxins, were observed in human red blood cells (RBC's), indicating that non-transcriptional events were sufficient in sustaining some cellular circadian rhythms (O'Neill and Reddy, 2011). Additionally, comparisons of circadian transcriptome and proteome revealed that less than 50% of rhythmic proteins had mRNAs that were also rhythmic, indicating significant post-transcriptional or post-translational regulation of circadian genes (Deery et al., 2009).

In this regard, the data presented here suggests that miRNAs perform a modulatory role by interacting with core clock gene(s) to effectively fine-tune the intracellular clock machinery. The salient observations supporting this position are: **1**) miR-142-3p is expressed rhythmically in the mouse SCN, in vivo, and in an immortalized SCN cell line in vitro, **2**) miR-142-3p targets two locations in the 3' UTR of the core clock gene *Bmal1*, **3**) overexpression of miR-142-3p in SCN cells causes disruption of BMAL1 protein rhythms, and **4**) combinatorial overexpression of miR-142-3p and miR-494 represses BMAL1 protein levels and causes disruption of ensemble circadian rhythms in cultured fibroblasts in vitro.

During the preparation of this dissertation, another study was published implicating miR-142-3p as a target of BMAL1-mediated transcription in NIH/3T3 cells. The authors validated an E-box element upstream of the miR-142 locus as the site of CLOCK-BMAL1 heterodimer binding. On serum-shock, fibroblasts also exhibited rhythmic accumulation of mature miR-142-3p and of *Bmal1* mRNA with phase angles similar to what we have observed in our SCN samples. The same report also

demonstrated miR-142-3p mediated repression of BMAL1 protein levels in HEK293 and in NIH/3T3 cells (Tan et al., 2012). Additionally, a previous study profiling diurnal oscillations in the miRNA transcriptome of liver had indicated that miR-142-5p was rhythmic in the liver with peak expression occurring around ZT8 (Na et al., 2009). Two mature miRNAs, miR-142-3p and miR-142-5p, are both synthesized from the common premature miRNA, pre-miR-142. Based on the data from Tan et al. (2012), indicating that miR-142-3p is rhythmically expressed in serum shocked NIH/3T3 fibroblasts, it is likely that miR-142-3p may also be rhythmic in peripheral tissues, like the liver. Interestingly, the reported peak for miR-142-5p at ZT8 in the mouse liver is ~5h phase delayed compared to our observations that miR-142-3p expression in the SCN peaks around CT3. This delay is consistent with other published reports regarding the phases of molecular rhythms in SCN compared to peripheral tissues (Panda et al., 2002). Although transcriptome analyses have revealed that rhythmic mRNAs in the SCN and peripheral tissues only share minor overlap, essential elements of the molecular clock are rhythmic in almost all tissues tested so far. Thus, rhythmicity of miR-142-3p expression in the SCN and possibly in the liver is consistent with its potential intrinsic involvement in modulating core intracellular clock machinery in both SCN and in peripheral clocks.

How crucial is miRNA-mediated regulation by miR-142-3p or by other miRNAs in general? I posit that miRNAs interact either with core clock genes or other factors influencing core clock genes and give rise to additional feed-back loop(s) that further stabilize the molecular clock (Fig. 20). Support for this assertion comes from theoretical models recently proposed by Liu and Wang (2012), who introduced variables including

miRNA expression and degradation rate (for miR-219 and miR-132) into classical transcription-translation based models, and compared outputs of the new equations with recently published empirical data. Incorporation of miRNAs into classical models did not significantly alter oscillations of *Bmal1*, *Per* and *Cry* mRNA rhythms. However, the models predicted that miR-219 functioned to reduce the period of *Per* mRNA oscillations. Supporting this prediction are observations from Cheng et al. (2007), who recently demonstrated that knockdown of miR-219 in the SCN resulted in lengthening of the free-running period of locomotor activity. Additionally, in vitro assays described in the same report had also indicated that both miR-219 and miR-132, independently enhanced *Per1* transcriptional activation. Thus, these miRNAs positively regulate a component of the negative limb of classical feed-back loop. In comparison, our results implicating miR-142-3p and miR-494 as repressors of *Bmal1* position them as negative regulators of the positive limb, in turn slowing down *Per1* transcriptional activation. Indeed, overexpression of miR-142-3p and miR-494 increases the period of PER2::LUC reported bioluminescence rhythms, an effect in opposition to that predicted for overexpression of miR-219.

Much like effects of kinases and phosphatases, or ROR's and REV-ERB's, this antagonistic relationship between two regulators is not unusual in the circadian system. Both miR-142-3p and miR-219 are rhythmically regulated, clock-controlled miRNAs and may counter-act each other's effects on *Per1* transcription. It is also noteworthy that miR-142-3p directly targets *Bmal1* while the effect of miR-219 on *Per1* is likely indirect, potentially inducing a lag between the two opposing actions.

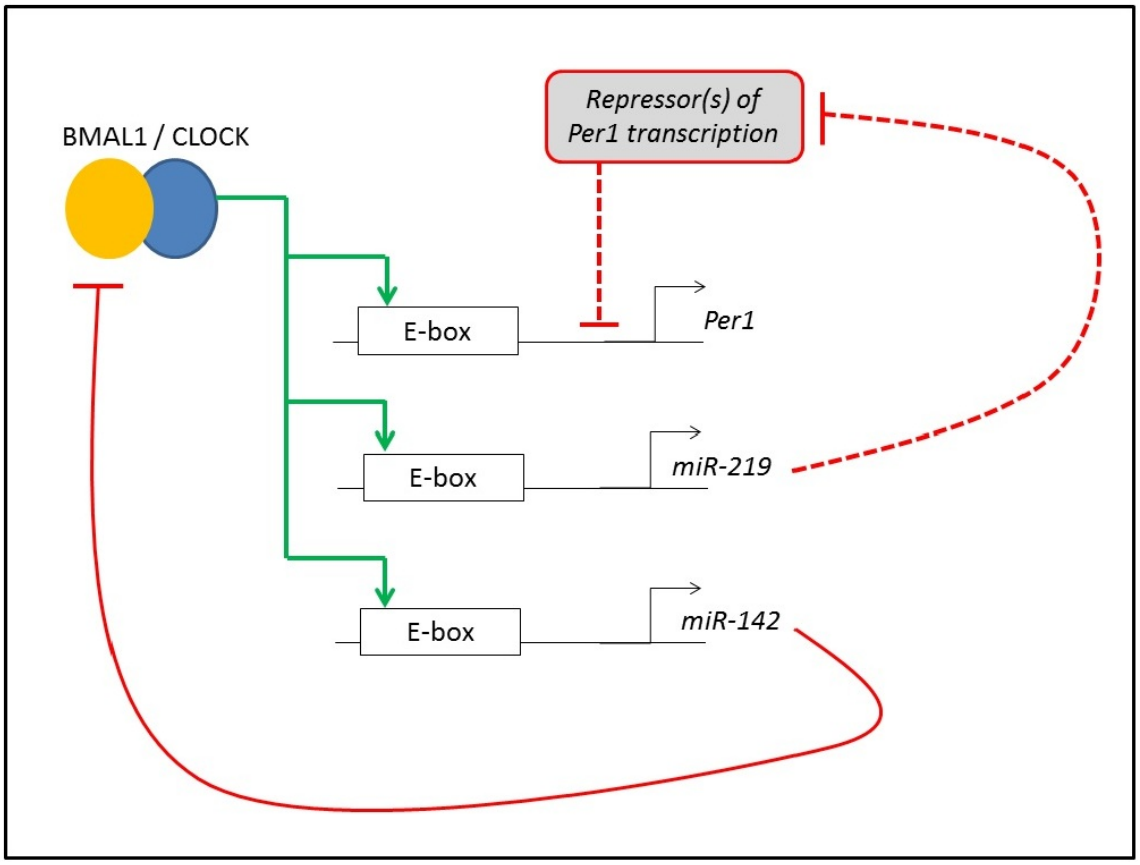

Figure 20: Simplified model of possible mechanisms for miRNA modulation of *Per1* transcription. CLOCK-BMAL1 heterodimers bind to E-box enhancer in miR-142-3p and miR-219 promoters and drive their rhythmic transcription. miR-219 inhibits yet unknown negative regulator(s), consequently enhancing *Per1* transcription and 'speeding up' the oscillatory cycle. In contrast, miR-142-3p feeds back to repress BMAL1 protein, consequently attenuating *Per1* transcription and 'slowing down' the oscillatory cycle. Additionally, by repressing BMAL1, miR-142-3p negatively regulates of its own transcription and of miR-219 as well.

miRNAs as Signaling Molecules in Local Synchronization of Rhythms

The studies described in this dissertation also indicate a role for miRNAs in temporal synchronization of clocks within local environments in peripheral tissues. Primary findings reported in chapters III and IV in support of this are: **1**) extracellular levels of miR-494 are characterized by rhythmic expression in mouse serum, **2**) miR-494, both independently and in synergy with miR-142-3p, targets the *Bmal1* 3' UTR, **3**) combinatorial overexpression of miR-142-3p and miR-494 represses BMAL1 protein levels and disrupts ensemble circadian rhythms of fibroblast cells in vitro, **4**) miR-142-3p and miR-494 are exported out of cells and into the extracellular environment in vitro, **5**) extracellular miR-142-3p is communicated between spatially separated cells in vitro, and **6**) inhibition of vesicular communication between cells disrupts ensemble circadian rhythms in vitro.

We estimated expression of ~6,500 and ~80,000 molecules of miR-152 and miR-16 per milliliter of blood respectively. Intracellular expression of miRNAs has been estimated to range from a few to multiple thousand molecular per cell depending on cell type and the miRNAs under consideration. Thus, given the expression levels in serum, the limited set of circulating miRNAs examined during this dissertation are unlikely to function as systemic signals synchronizing all peripheral oscillators; their expression levels may not be high enough for global function. However, within local environments, as demonstrated by the in vitro experiments, communication of extracellular miRNAs may be relevant or indeed important for maintenance of temporal synchronization between cell-autonomous oscillators.

It is noteworthy that miRNAs, or other small molecule transmitters like ATP or cAMP, have certain advantages as signaling molecules because, **1**) they may be energetically more efficient to produce than proteins, hormones or even small peptides, **2**) they may be easier to transport via vesicles, **3**) small regulatory RNAs like miRNAs can modulate a large number of genes (up to 200 target genes for individual miRNAs: Lewis et al., 2005), **4**) miRNA form multi-turnover complexes, thus magnifying the effects of even small changes in miRNA levels, and **5**) if presence and communication of AGO2-complexed miRNAs in serum is verified, extracellular miRNAs could conceivably also be transmitted between cells as functional units facilitating rapid regulation by the transported miRNA species in recipient cells. Communication of extracellular miRNAs has already been documented in important physiological and pathological phenomenon including vascular disease, immune function, virus infection and hepatocellular carcinoma (Pegtel et al., 2010; Zhang et al., 2010; Kogure et al., 2011; Mittelbrunn et al., 2011).

Diurnal oscillations in circulating miR-494 levels in mouse serum and in vitro data demonstrating miR-142-3p transfer between cells provide primary insight that extracellular miRNAs may also play a role in circadian physiology as well. This system could be regulated at multiple levels and data-mining from previous microarray experiments provides exciting indications that miRNA synthesis and miRNA packaging or secretion may be clock-controlled. Using publically available circadian micro-array database (http://bioinf.itmat.upenn.edu/circa), I have examined whether mature miRNA production, which is an outcome of *Dicer1* activity, may be rhythmic. Interestingly,

Dicer1 mRNA has indications of circadian rhythmicity in skeletal muscle and liver of mice. The mRNA peaks around mid-subjective-day in both tissues and reaches a nadir around mid-subjective-night. More crucial for rhythmic miRNA secretion is their packaging and vesicular release, which was shown by Kosaka et al. (2010) to be under control of a ceramide-dependent pathway. Ceramide synthesis itself is thought to be regulated by the enzyme acidic spingiomyelinase (aSMase) and/or by the enzyme neutral spingiomyelinase (nSMase). Kosaka et al. (2010) demonstrated that both GW4869, an nSMase-inhibitor drug and siRNA mediated RNA-interference of nSMase2 effectively knocked down secretion of miRNAs in HEK293 cells. Thus data from microarray experiments indicating high amplitude rhythms in nSMase transcript abundance in the mouse liver, kidney and adrenal glands are exciting and promising. The nSMase expression peaks during early- to mid- subjective day and reaches trough values during early-subjective night in the liver and during mid- to late- subjective night in kidney and adrenal gland. There is also indication of low amplitude rhythms in nSMase in the mouse SCN. Thus the circadian clock may impinge on miRNA secretion pathway(s) to rhythmically regulate extracellular miRNA-mediated communication and this regulation may be critical for physiology and pathologies, including circadian rhythms disorders, metabolic disorders and even certain cancers.

Remaining Questions and Concluding Remarks

The studies presented in this dissertation are a step towards exploring the contribution of miRNAs to post-transcriptional regulation of the circadian clock. I have

reported some mechanistic details underlying regulation of the miRNA, miR-142-3p, and described how it feeds back to target its activator, *Bmal1*. I have focused on an additional miRNA, miR-494 that also targets *Bmal1*, to study extracellular expression and function of miRNAs. Given that miRNAs are estimated to regulate 30-50% of the transcriptome and between 10-30% of the transcriptome is also under circadian regulation, significant insights into the contribution of miRNAs to rhythmic physiology and behaviors have yet to be determined.

The groundwork laid during this dissertation has however generated some interesting questions. What other miRNAs are rhythmically transcribed in the SCN? How is their expression regulated? What are their targets? How do non-cyclic miRNAs affect the rhythmic transcriptome? Important tools for initial forays into answering some of these questions are already available. BMAL1 ChIP-sequencing experiments have been carried out to identify targets of the CLOCK-BMAL1 heterodimer complex (Hatanaka et al., 2010, Rey et al., 2011). With non-coding genomic regions better annotated than ever, those ChIP-Seq data could be revisited to potentially detect miRNAs or other regulatory RNAs that had previously been missed. Over the past few years, databases of validated miRNA targets have also become available, which are a useful tool for verifying if miRNA regulators of a potential mRNA of interest have already been identified. Concurrently, a method for identification of miRNA targets has also been described in *Drosophila* (Kadener et al., 2009). At various timepoints in a circadian cycle, AGO1 was immunoprecipitated and bound mRNAs were identified by microarray analysis. Because the AGO family of proteins (4 members in mammals) are

primarily responsible for miRISC-mediated transcriptional silencing, this procedure can potentially identify all mRNAs actively associated with repression complexes. This method could perhaps also be coupled to sequencing of the immunoprecipitated complexes to identify both, miRNAs and their corresponding mRNA targets.

Research into the regulatory functions of extracellular miRNAs is rapidly advancing and the studies described in this dissertation provide a glimpse into the role of extracellular miRNAs in communication of temporal information. However, many questions yet remain unanswered. For example, how are miRNAs packaged? Does the circadian machinery contribute to sorting of miRNAs for extracellular release? What organs contribute miRNAs to the circulating miRNA pool? What are the identities of circulating miRNA species, and how many of them are rhythmic? Is miRNA secretion or miRNA uptake rhythmically regulated? Is there targeted communication of miRNA signals between cells? Given how little we know about extracellular miRNAs, these questions are arguably much harder to address. So how and where do we begin? An ideal system for such analyses would allow one to disassociate peripheral rhythms from SCN influence, and then determine the potential contributions of circulating miRNAs to synchronization of peripheral clocks. In this regard, one of the best characterized paradigms for studying peripheral rhythms independent of SCN influence, is that of restricted feeding. Rhythms in peripheral tissues in SCN lesioned animals under restricted feeding conditions have already been well documented (Honma et al., 1988). Using SCN-lesioned, wild-type and tissue-specific conditional *Dicer* knockout mice, it could be possible to compare the rhythmic mRNA transcriptome of various peripheral

tissues as well the rhythmic circulating miRNA'ome under ad-lib or restricted feeding paradigms. This could help answer some potential questions including, **1**) what is the relative contribution of individual organs (e.g. the liver) to the circulating miRNA'ome, **2**) how does the circulating miRNA'ome vary between ad-lib fed, and restricted feeding conditions, and **3**) is there an effect of miRNA knockdown in one tissue on identities and/or phases of cycling genes in other peripheral tissues. With regard to the potential influence of the circadian clock on rhythmic packaging and secretion of miRNAs, the extracellular miRNA'ome of wild-type and arrhythmic clock mutant mice could be profiled to identify candidate circulating miRNAs that are rhythmically regulated. Additionally, an interesting question that remains to be addressed in vivo is whether miRNAs are communicated between spatially separated cells. This could possibly be investigated using tissue specific expression of 'foreign' miRNAs (i.e., miRNAs lacking any sequence homology with endogenous generated miRNAs.) Analysis of other closely associated tissues for presence of this foreign miRNA may shed light on whether miRNAs are exchanged between cell-types in vivo.

In summary, the research described here represents initial advances and exciting possibilities regarding the role miRNAs could play in regulation of circadian physiology and pathologies by targeting clock genes in SCN pacemaker and peripheral oscillators. Molecular, genetic and cell culture tools available for murine models provide strong foundations and excellent resources for further illuminating the circadian regulatory functions of miRNAs.

REFERENCES

Abrahamson EE, Moore RY (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. Brain Research 916:172-191.

Akashi M, Takumi T (2005) The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nature Structural & Molecular Biology 12:441-448.

Allen G, Rappe J, Earnest DJ, Cassone VM (2001) Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts. The Journal of Neuroscience 21:7937-7943.

Allen GC, Farnell Y, Bell-Pedersen D, Cassone VM, Earnest DJ (2004) Effects of altered Clock gene expression on the pacemaker properties of SCN2.2 cells and oscillatory properties of NIH/3T3 cells. Neuroscience 127:989-999.

Ambros V (2004) The functions of animal microRNAs. Nature 431:350-355.

Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proceedings of the National Academy of Sciences of the United States of America 108:5003-5008.

Aschoff J, Fatranska M, Giedke H, Doerr P, Stamm D, Wisser H (1971) Human circadian rhythms in continuous darkness: entrainment by social cues. Science 171:213-215.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525-536.

Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP (2008) The impact of microRNAs on protein output. Nature 455:64-71.

Balsalobre A, Damiola F, Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93:929-937.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281-297.

Bartel DP, Chen CZ (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nature Reviews Genetics 5:396-400.

Bartram A, Poon C, Neufeld J (2009) Nucleic acid contamination of glycogen used in nucleic acid precipitation and assessment of linear polyacrylamide as an alternative co-precipitant. Biotechniques 47:1019-1022.

Baskerville S, Bartel DP (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11:241-247.

Bass J, Takahashi JS (2011) Circadian rhythms: Redox redux. Nature 469:476-478.

Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ (2005) Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nature Reviews Genetics 6:544-556.

Berezikov E, Chung WJ, Willis J, Cuppen E, Lai EC (2007) Mammalian mirtron genes. Molecular Cell 28:328-336.

Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363-366.

Bos NP, Mirmiran M (1990) Circadian rhythms in spontaneous neuronal discharges of the cultured suprachiasmatic nucleus. Brain Research 511:158-162.

Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of microRNA-target recognition. PLoS Biology 3:e85.

Buijs RM, la Fleur SE, Wortel J, Van Heyningen C, Zuiddam L, Mettenleiter TC, Kalsbeek A, Nagai K, Niijima A (2003) The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. The Journal of Comparative Neurology 464:36-48.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, Bradfield CA (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Cardone L, Hirayama J, Giordano F, Tamaru T, Palvimo JJ, Sassone-Corsi P (2005) Circadian clock control by SUMOylation of BMAL1. Science 309:1390-1394.

Chen X, Liang H, Zhang J, Zen K, Zhang CY (2012) Secreted microRNAs: a new form of intercellular communication. Trends in Cell Biology 22:125-132.

Chen X et al. (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Research 18:997-1006.

Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436:740-744.

Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, Nakazawa T, Shimizu K, Okamura H, Impey S, Obrietan K (2007) microRNA modulation of circadian-clock period and entrainment. Neuron 54:813-829.

Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J, Weaver DR, Leslie FM, Zhou QY (2002) Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature 417:405-410.

Cibois M, Gautier-Courteille C, Legagneux V, Paillard L (2010) Post-transcriptional controls - adding a new layer of regulation to clock gene expression. Trends in Cell Biology 20:533-541.

Cifuentes D, Xue H, Taylor DW, Patnode H, Mishima Y, Cheloufi S, Ma E, Mane S, Hannon GJ, Lawson ND, Wolfe SA, Giraldez AJ (2010) A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. Science 328:1694-1698.

Cocucci E, Racchetti G, Meldolesi J (2009) Shedding microvesicles: artefacts no more. Trends in Cell Biology 19:43-51.

De Boer SF, Van der Gugten J (1987) Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. Physiology & Behavior 40:323-328.

DeCoursey PJ, Walker JK, Smith SA (2000) A circadian pacemaker in free-living chipmunks: essential for survival? Journal of Comparative Physiology A 186:169-180.

DeCoursey PJ, Krulas JR, Mele G, Holley DC (1997) Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure. Physiology & Behavior 62:1099-1108.

Deery MJ, Maywood ES, Chesham JE, Sladek M, Karp NA, Green EW, Charles PD, Reddy AB, Kyriacou CP, Lilley KS, Hastings MH (2009) Proteomic analysis reveals the role of synaptic vesicle cycling in sustaining the suprachiasmatic circadian clock. Current Biology 19:2031-2036.

Dibner C, Schibler U, Albrecht U (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annual Review of Physiology 72:517-549.

Diederichs S, Haber DA (2007) Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell 131:1097-1108.

Do MT, Yau KW (2010) Intrinsically photosensitive retinal ganglion cells. Physiological Reviews 90:1547-1581.

Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AA (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630-633.

Doench JG, Sharp PA (2004) Specificity of microRNA target selection in translational repression. Genes & Development 18:504-511.

Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125:497-508.

Duong HA, Robles MS, Knutti D, Weitz CJ (2011) A molecular mechanism for circadian clock negative feedback. Science 332:1436-1439.

Earnest DJ, Sladek CD (1986) Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants in vitro. Brain Research 382:129-133.

Earnest DJ, Liang FQ, Ratcliff M, Cassone VM (1999) Immortal time: circadian clock properties of rat suprachiasmatic cell lines. Science 283:693-695.

Eide EJ, Woolf MF, Kang H, Woolf P, Hurst W, Camacho F, Vielhaber EL, Giovanni A, Virshup DM (2005) Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. Molecular and Cellular Biology 25:2795-2807.

Escobar C, Diaz-Munoz M, Encinas F, Aguilar-Roblero R (1998) Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. American Journal of Physiology 274:R1309-1316.

Faller M, Guo F (2008) MicroRNA biogenesis: there's more than one way to skin a cat. Biochimica et Biophysica Acta 1779:663-667.

Farnell YF, Shende VR, Neuendorff N, Allen GC, Earnest DJ (2011) Immortalized cell lines for real-time analysis of circadian pacemaker and peripheral oscillator properties. The European Journal of Neuroscience 33:1533-1540.

Farnell YZ, Allen GC, Nahm SS, Neuendorff N, West JR, Chen WJ, Earnest DJ (2008) Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats. Alcoholism, Clinical and Experimental Research 32:544-552.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nature Reviews Genetics 9:102-114.

Gallego M, Virshup DM (2007) Post-translational modifications regulate the ticking of the circadian clock. Nature Reviews Molecular Cell Biology 8:139-148.

Gallego M, Kang H, Virshup DM (2006) Protein phosphatase 1 regulates the stability of the circadian protein PER2. The Biochemical Journal 399:169-175.

Gao W, Liu L, Lu X, Shu Y (2011) Circulating microRNAs: possible prediction biomarkers for personalized therapy of non-small-cell lung carcinoma. Clinical Lung Cancer 12:14-17.

Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F, Ruskeepaa AL, Oresic M, Esau CC, Zdobnov EM, Schibler U (2009) Integration of microRNA miR-122 in hepatic circadian gene expression. Genes & Development 23:1313-1326.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science 280:1564-1569.

Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, Bentwich Z, Hod M, Goren Y, Chajut A (2008) Serum microRNAs are promising novel biomarkers. PLoS ONE 3:e3148.

Gillette MU, Reppert SM (1987) The hypothalamic suprachiasmatic nuclei: circadian patterns of vasopressin secretion and neuronal activity in vitro. Brain Research Bulletin 19:135-139.

Glazov EA, Cottee PA, Barris WC, Moore RJ, Dalrymple BP, Tizard ML (2008) A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Research 18:957-964.

Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123:631-640.

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R (2004) The Microprocessor complex mediates the genesis of microRNAs. Nature 432:235-240.

Griffiths-Jones S (2004) The microRNA Registry. Nucleic Acids Research 32:D109-111.

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. Nucleic Acids Research 36:D154-158.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research 34:D140-144.

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Molecular Cell 27:91-105.

Groos G, Hendriks J (1982) Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro. Neuroscience Letters 34:283-288.

Guduric-Fuchs J, A OC, Camp B, O'Neill CL, Medina RJ, Simpson DA (2012) Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. BMC Genomics 13:357.

Guo H, Brewer JM, Lehman MN, Bittman EL (2006) Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. The Journal of Neuroscience 26:6406-6412.

Guo H, Ingolia NT, Weissman JS, Bartel DP (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466:835-840.

Guo H, Brewer JM, Champhekar A, Harris RB, Bittman EL (2005) Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. Proceedings of the National Academy of Sciences of the United States of America 102:3111-3116.

Guo YF, Stein PK (2003) Circadian rhythm in the cardiovascular system: chronocardiology. American Heart Journal 145:779-786.

Harada Y, Sakai M, Kurabayashi N, Hirota T, Fukada Y (2005) Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta. The Journal of Biological Chemistry 280:31714-31721.

Hatanaka F, Matsubara C, Myung J, Yoritaka T, Kamimura N, Tsutsumi S, Kanai A, Suzuki Y, Sassone-Corsi P, Aburatani H, Sugano S, Takumi T (2010) Genome-wide profiling of the core clock protein BMAL1 targets reveals a strict relationship with metabolism. Molecular and Cellular Biology 30:5636-5648.

Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295:1065-1070.

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nature Reviews Genetics 5:522-531.

Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, Braun T, Urbich C, Boon RA, Dimmeler S (2012) Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nature Cell Biology 14:249-256.

Hirayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, Sassone-Corsi P (2007) CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature 450:1086-1090.

Hommel JD, Trinko R, Sears RM, Georgescu D, Liu ZW, Gao XB, Thurmon JJ, Marinelli M, DiLeone RJ (2006) Leptin receptor signaling in midbrain dopamine neurons regulates feeding. Neuron 51:801-810.

Honma S, Ikeda M, Abe H, Tanahashi Y, Namihira M, Honma K, Nomura M (1998) Circadian oscillation of BMAL1, a partner of a mammalian clock gene Clock, in rat suprachiasmatic nucleus. Biochemical and Biophysical Research Communications 250:83-87.

Huang B, Zhao J, Lei Z, Shen S, Li D, Shen GX, Zhang GM, Feng ZH (2009) miR-142-3p restricts cAMP production in CD4+CD25- T cells and CD4+CD25+ TREG cells by targeting AC9 mRNA. EMBO Reports 10:180-185.

Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X (2010) Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. International Journal of Cancer 127:118-126.

Hughes ME, DiTacchio L, Hayes KR, Vollmers C, Pulivarthy S, Baggs JE, Panda S, Hogenesch JB (2009) Harmonics of circadian gene transcription in mammals. PLoS Genetics 5:e1000442.

Human Genome Sequencing C (2004) Finishing the euchromatic sequence of the human genome. Nature 431:931-945.

Humphreys DT, Westman BJ, Martin DI, Preiss T (2005) MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proceedings of the National Academy of Sciences of the United States of America 102:16961-16966.

Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB (2008) Detection of microRNA expression in human peripheral blood microvesicles. PLoS ONE 3:e3694.

Hutvagner G, Zamore PD (2002) A microRNA in a multiple-turnover RNAi enzyme complex. Science 297:2056-2060.

Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293:834-838.

Iitaka C, Miyazaki K, Akaike T, Ishida N (2005) A role for glycogen synthase kinase-3beta in the mammalian circadian clock. The Journal of Biological Chemistry 280:29397-29402.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human MicroRNA targets. PLoS Biology 2:e363.

Kadener S, Menet JS, Sugino K, Horwich MD, Weissbein U, Nawathean P, Vagin VV, Zamore PD, Nelson SB, Rosbash M (2009) A role for microRNAs in the Drosophila circadian clock. Genes & Development 23:2179-2191.

Kalsbeek A, Teclemariam-Mesbah R, Pevet P (1993) Efferent projections of the suprachiasmatic nucleus in the golden hamster (Mesocricetus auratus). The Journal of Comparative Neurology 332:293-314.

Kalsbeek A, La Fleur S, Van Heijningen C, Buijs RM (2004) Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. The Journal of Neuroscience 24:7604-7613.

Kalsbeek A, Kreier F, Fliers E, Sauerwein HP, Romijn JA, Buijs RM (2007) Minireview: Circadian control of metabolism by the suprachiasmatic nuclei. Endocrinology 148:5635-5639.

Kilman VL, Zhang L, Meissner RA, Burg E, Allada R (2009) Perturbing dynamin reveals potent effects on the Drosophila circadian clock. PLoS ONE 4:e5235.

Kim SM, Power A, Brown TM, Constance CM, Coon SL, Nishimura T, Hirai H, Cai T, Eisner C, Weaver DR, Piggins HD, Klein DC, Schnermann J, Notkins AL (2009) Deletion of the secretory vesicle proteins IA-2 and IA-2beta disrupts circadian rhythms of cardiovascular and physical activity. FASEB J 23:3226-3232.

Kim YK, Kim VN (2007) Processing of intronic microRNAs. The EMBO Journal 26:775-783.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TD, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, Takahashi JS (1997) Positional cloning of the mouse circadian clock gene. Cell 89:641-653.

Kita Y, Shiozawa M, Jin W, Majewski RR, Besharse JC, Greene AS, Jacob HJ (2002) Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. Pharmacogenetics 12:55-65.

Ko CH, Takahashi JS (2006) Molecular components of the mammalian circadian clock. Human Molecular Genetics 15 Spec No 2:R271-277.

Kogure T, Lin WL, Yan IK, Braconi C, Patel T (2011) Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology 54:1237-1248.

Kojima S, Shingle DL, Green CB (2011) Post-transcriptional control of circadian rhythms. Journal of Cell Science 124:311-320.

Kojima S, Matsumoto K, Hirose M, Shimada M, Nagano M, Shigeyoshi Y, Hoshino S, Ui-Tei K, Saigo K, Green CB, Sakaki Y, Tei H (2007) LARK activates posttranscriptional expression of an essential mammalian clock protein, PERIOD1. Proceedings of the National Academy of Sciences of the United States of America 104:1859-1864.

Kosaka N, Iguchi H, Ochiya T (2010a) Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Science 101:2087-2092.

Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T (2010b) Secretory mechanisms and intercellular transfer of microRNAs in living cells. The Journal of Biological Chemistry 285:17442-17452.

Kramer A, Yang FC, Snodgrass P, Li X, Scammell TE, Davis FC, Weitz CJ (2001) Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. Science 294:2511-2515.

Kraves S, Weitz CJ (2006) A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. Nature Neuroscience 9:212-219.

Kudo T, Loh DH, Truong D, Wu Y, Colwell CS (2011) Circadian dysfunction in a mouse model of Parkinson's disease. Experimental Neurology 232:66-75.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98:193-205.

Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T (2003) New microRNAs from mouse and human. RNA 9:175-179.

Lai EC, Tam B, Rubin GM (2005) Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. Genes & Development 19:1067-1080.

Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, Hatton CS, Harris AL (2008) Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. British Journal of Haematology 141:672-675.

Leak RK, Moore RY (2001) Topographic organization of suprachiasmatic nucleus projection neurons. The Journal of Comparative Neurology 433:312-334.

Leak RK, Card JP, Moore RY (1999) Suprachiasmatic pacemaker organization analyzed by viral transynaptic transport. Brain Research 819:23-32.

Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855-867.

Lee J, Lee Y, Lee MJ, Park E, Kang SH, Chung CH, Lee KH, Kim K (2008) Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. Molecular and Cellular Biology 28:6056-6065.

Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843-854.

Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. The EMBO Journal 21:4663-4670.

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN (2004) MicroRNA genes are transcribed by RNA polymerase II. The EMBO Journal 23:4051-4060.

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425:415-419.

Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL (1987) Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. The Journal of Neuroscience 7:1626-1638.

LeSauter J, Hoque N, Weintraub M, Pfaff DW, Silver R (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. Proceedings of the National Academy of Sciences of the United States of America 106:13582-13587.

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15-20.

Li P, Sur SH, Mistlberger RE, Morris M (1999) Circadian blood pressure and heart rate rhythms in mice. American Journal of Physiology 276:R500-504.

Liang FQ, Walline R, Earnest DJ (1998) Circadian rhythm of brain-derived neurotrophic factor in the rat suprachiasmatic nucleus. Neuroscience Letters 242:89-92.

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433:769-773.

Lindsay MA (2008) microRNAs and the immune response. Trends in Immunology 29:343-351.

Liu K, Wang R (2012) MicroRNA-mediated regulation in the mammalian circadian rhythm. Journal of Theoretical Biology 304:103-110.

Lowrey PL, Takahashi JS (2011) Genetics of circadian rhythms in Mammalian model organisms. Advances in Genetics 74:175-230.

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288:483-492.

Lucas LA, Eleftheriou BE (1980) Circadian variation in concentrations of testosterone in the plasma of male mice: a difference between BALB/cBy and C57BL/6By inbred strains. Journal of Endocrinology 87:37-46.

Luo W, Sehgal A (2012) Regulation of circadian behavioral output via a MicroRNA-JAK/STAT circuit. Cell 148:765-779.

MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. Proceedings of the National Academy of Sciences of the United States of America 105:512-517.

Maniataki E, Mourelatos Z (2005) A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes & Development 19:2979-2990.

Mattick JS (2009) Deconstructing the dogma: a new view of the evolution and genetic programming of complex organisms. Annals of the New York Academy of Sciences 1178:29-46.

Maywood ES, O'Brien JA, Hastings MH (2003) Expression of mCLOCK and other circadian clock-relevant proteins in the mouse suprachiasmatic nuclei. Journal of Neuroendocrinology 15:329-334.

Menger GJ, Lu K, Thomas T, Cassone VM, Earnest DJ (2005) Circadian profiling of the transcriptome in immortalized rat SCN cells. Physiological Genomics 21:370-381.

Menger GJ, Allen GC, Neuendorff N, Nahm SS, Thomas TL, Cassone VM, Earnest DJ (2007) Circadian profiling of the transcriptome in NIH/3T3 fibroblasts: comparison with rhythmic gene expression in SCN2.2 cells and the rat SCN. Physiological Genomics 29:280-289.

Merkerova M, Belickova M, Bruchova H (2008) Differential expression of microRNAs in hematopoietic cell lineages. European Journal of Haematology 81:304-310.

Meyer-Bernstein EL, Jetton AE, Matsumoto SI, Markuns JF, Lehman MN, Bittman EL (1999) Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. Endocrinology 140:207-218.

Michel S, Colwell CS (2001) Cellular communication and coupling within the suprachiasmatic nucleus. Chronobiology International 18:579-600.

Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences of the United States of America 105:10513-10518.

Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, Bernad A, Sanchez-Madrid F (2011) Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nature Communications 2:282.

Miyoshi K, Miyoshi T, Siomi H (2010) Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production. Molecular Genetics and Genomics : MGG 284:95-103.

Mizuno H, Nakamura A, Aoki Y, Ito N, Kishi S, Yamamoto K, Sekiguchi M, Takeda S, Hashido K (2011) Identification of muscle-specific microRNAs in serum of muscular dystrophy animal models: promising novel blood-based markers for muscular dystrophy. PLoS ONE 6:e18388.

Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Research 42:201-206.

Morin LP, Allen CN (2006) The circadian visual system, 2005. Brain Research Reviews 51:1-60.

Na YJ, Sung JH, Lee SC, Lee YJ, Choi YJ, Park WY, Shin HS, Kim JH (2009) Comprehensive analysis of microRNA-mRNA co-expression in circadian rhythm. Experimental & Molecular Medicine 41:638-647.

Nagel R, Clijsters L, Agami R (2009) The miRNA-192/194 cluster regulates the Period gene family and the circadian clock. The FEBS Journal 276:5447-5455.

Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP, Sassone-Corsi P (2008) The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 134:329-340.

Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science 308:414-415.

Newman GC, Hospod FE, Patlak CS, Moore RY (1992) Analysis of in vitro glucose utilization in a circadian pacemaker model. The Journal of Neuroscience 12:2015-2021.

Nolte-'t Hoen EN, Buschow SI, Anderton SM, Stoorvogel W, Wauben MH (2009) Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. Blood 113:1977-1981.

O'Neill JS, Reddy AB (2011) Circadian clocks in human red blood cells. Nature 469:498-503.

O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH (2008) cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science 320:949-953.

Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC (2007) The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell 130:89-100.

Olsen PH, Ambros V (1999) The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Developmental Biology 216:671-680.

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH (1998) Resonating circadian clocks enhance fitness in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 95:8660-8664.

Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109:307-320.

Partch CL, Shields KF, Thompson CL, Selby CP, Sancar A (2006) Posttranslational regulation of the mammalian circadian clock by cryptochrome and protein phosphatase 5. Proceedings of the National Academy of Sciences of the United States of America 103:10467-10472.

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408:86-89.

Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, de Gruijl TD, Wurdinger T, Middeldorp JM (2010) Functional delivery of viral miRNAs via exosomes. Proceedings of the National Academy of Sciences of the United States of America 107:6328-6333.

Pennisi E (2012) Genomics. ENCODE project writes eulogy for junk DNA. Science 337:1159, 1161.

Perera RJ, Ray A (2007) MicroRNAs in the search for understanding human diseases. BioDrugs : Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy 21:97-104.

Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM (2010) Selective release of microRNA species from normal and malignant mammary epithelial cells. PLoS ONE 5:e13515.

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309:1573-1576.

Pittendrigh CS (1960) Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symposia on Quantitative Biology 25:159-184.

Poethig RS (2009) Small RNAs and developmental timing in plants. Current Opinion in Genetics & Development 19:374-378.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251-260.

Prolo LM, Takahashi JS, Herzog ED (2005) Circadian rhythm generation and entrainment in astrocytes. The Journal of Neuroscience 25:404-408.

Prosser RA, Gillette MU (1989) The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP. The Journal of Neuroscience 9:1073-1081.

Prosser RA, Edgar DM, Heller HC, Miller JD (1994) A possible glial role in the mammalian circadian clock. Brain Research 643:296-301.

Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH (2009) Exosomal microRNA: a diagnostic marker for lung cancer. Clinical Lung Cancer 10:42-46.

Ralph MR, Foster RG, Davis FC, Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975-978.

Reddi KK, Holland JF (1976) Elevated serum ribonuclease in patients with pancreatic cancer. Proceedings of the National Academy of Sciences of the United States of America 73:2308-2310.

Reddy AB, Karp NA, Maywood ES, Sage EA, Deery M, O'Neill JS, Wong GK, Chesham J, Odell M, Lilley KS, Kyriacou CP, Hastings MH (2006) Circadian orchestration of the hepatic proteome. Current Biology 16:1107-1115.

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:901-906.

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935-941.

Rey G, Cesbron F, Rougemont J, Reinke H, Brunner M, Naef F (2011) Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. PLoS Biology 9:e1000595.

Robles MS, Boyault C, Knutti D, Padmanabhan K, Weitz CJ (2010) Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. Science 327:463-466.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. Genome Research 14:1902-1910.

Ruby JG, Jan CH, Bartel DP (2007) Intronic microRNA precursors that bypass Drosha processing. Nature 448:83-86.

Sahar S, Zocchi L, Kinoshita C, Borrelli E, Sassone-Corsi P (2010) Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation. PLoS ONE 5:e8561.

Saini HK, Griffiths-Jones S, Enright AJ (2007) Genomic analysis of human microRNA transcripts. Proceedings of the National Academy of Sciences of the United States of America 104:17719-17724.

Sanada K, Okano T, Fukada Y (2002) Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. The Journal of Biological Chemistry 277:267-271.

Saper CB, Scammell TE, Lu J (2005) Hypothalamic regulation of sleep and circadian rhythms. Nature 437:1257-1263.

Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, Naik KA, FitzGerald GA, Kay SA, Hogenesch JB (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron 43:527-537.

Sato TK, Yamada RG, Ukai H, Baggs JE, Miraglia LJ, Kobayashi TJ, Welsh DK, Kay SA, Ueda HR, Hogenesch JB (2006) Feedback repression is required for mammalian circadian clock function. Nature Genetics 38:312-319.

Sayed D, Abdellatif M (2011) MicroRNAs in development and disease. Physiological Reviews 91:827-887.

Schwartz WJ, Davidsen LC, Smith CB (1980) In vivo metabolic activity of a putative circadian oscillator, the rat suprachiasmatic nucleus. The Journal of Comparative Neurology 189:157-167.

Segura E, Guerin C, Hogg N, Amigorena S, Thery C (2007) CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo. Journal of Immunology 179:1489-1496.

Shaw E, Tofler GH (2009) Circadian rhythm and cardiovascular disease. Current Atherosclerosis Reports 11:289-295.

Shearman LP, Zylka MJ, Weaver DR, Kolakowski LF, Jr., Reppert SM (1997) Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron 19:1261-1269.

Shende VR, Goldrick MM, Ramani S, Earnest DJ (2011) Expression and rhythmic modulation of circulating microRNAs targeting the clock gene Bmal1 in mice. PLoS ONE 6:e22586.

Shi L, Ko ML, Ko GY (2009) Rhythmic expression of microRNA-26a regulates the L-type voltage-gated calcium channel alpha1C subunit in chicken cone photoreceptors. The Journal of Biological Chemistry 284:25791-25803.

Shibata S, Oomura Y, Kita H, Hattori K (1982) Circadian rhythmic changes of neuronal activity in the suprachiasmatic nucleus of the rat hypothalamic slice. Brain Research 247:154-158.

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros JJ, Dunlap JC, Okamura H (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell 91:1043-1053.

Silver R, LeSauter J, Tresco PA, Lehman MN (1996) A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 382:810-813.

Simons M, Raposo G (2009) Exosomes--vesicular carriers for intercellular communication. Current Opinion in Cell Biology 21:575-581.

Sire C, Moreno AB, Garcia-Chapa M, Lopez-Moya JJ, San Segundo B (2009) Diurnal oscillation in the accumulation of Arabidopsis microRNAs, miR167, miR168, miR171 and miR398. FEBS Letters 583:1039-1044.

So WV, Rosbash M (1997) Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. The EMBO Journal 16:7146-7155.

Staiger D, Koster T (2011) Spotlight on post-transcriptional control in the circadian system. Cellular and Molecular Life Sciences 68:71-83.

Stephan FK, Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proceedings of the National Academy of Sciences of the United States of America 69:1583-1586.

Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH, Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. Nature 417:78-83.

Sujino M, Masumoto KH, Yamaguchi S, van der Horst GT, Okamura H, Inouye ST (2003) Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice. Current Biology 13:664-668.

Takahashi JS, Hong HK, Ko CH, McDearmon EL (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nature Reviews Genetics 9:764-775.

Tamaru T, Hirayama J, Isojima Y, Nagai K, Norioka S, Takamatsu K, Sassone-Corsi P (2009) CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. Nature Structural & Molecular Biology 16:446-448.

Tan X, Zhang P, Zhou L, Yin B, Pan H, Peng X (2012) Clock-controlled mir-142-3p can target its activator, Bmal1. BMC Molecular Biology 13:27.

Tasaka Y, Inoue S, Maruno K, Hirata Y (1980) Twenty-four-hour variations of plasma pancreatic polypeptide, insulin and glucagon in normal human subjects. Endocrinologia Japonica 27:495-498.

Tataroglu O, Davidson AJ, Benvenuto LJ, Menaker M (2006) The methamphetamine-sensitive circadian oscillator (MASCO) in mice. Journal of Biological Rhythms 21:185-194.

Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nature Reviews Immunology 9:581-593.

Tomita J, Nakajima M, Kondo T, Iwasaki H (2005) No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. Science 307:251-254.

Tsuchiya Y, Akashi M, Matsuda M, Goto K, Miyata Y, Node K, Nishida E (2009) Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. Science Signaling 2:ra26.

Turchinovich A, Weiz L, Langheinz A, Burwinkel B (2011) Characterization of extracellular circulating microRNA. Nucleic Acids Research 39:7223-7233.

Turek FW (1985) Circadian neural rhythms in mammals. Annual Review of Physiology 47:49-64.

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature Cell Biology 9:654-659.

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M, de Wit J, Verkerk A, Eker AP, van Leenen D, Buijs R, Bootsma D, Hoeijmakers JH, Yasui A (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627-630.

Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318:1931-1934.

Venturini L, Battmer K, Castoldi M, Schultheis B, Hochhaus A, Muckenthaler MU, Ganser A, Eder M, Scherr M (2007) Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood 109:4399-4405.

Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nature Cell Biology 13:423-433.

Vielhaber E, Eide E, Rivers A, Gao ZH, Virshup DM (2000) Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. Molecular and Cellular Biology 20:4888-4899.

Vitaterna MH, Selby CP, Todo T, Niwa H, Thompson C, Fruechte EM, Hitomi K, Thresher RJ, Ishikawa T, Miyazaki J, Takahashi JS, Sancar A (1999) Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proceedings of the National Academy of Sciences of the United States of America 96:12114-12119.

Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q (2010a) Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. European Heart Journal 31:659-666.

Wang K, Zhang S, Weber J, Baxter D, Galas DJ (2010b) Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Research 38:7248-7259.

Wang X, Zhang X, Ren XP, Chen J, Liu H, Yang J, Medvedovic M, Hu Z, Fan GC (2010c) MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. Circulation 122:1308-1318.

Watts AG, Swanson LW (1987) Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat. The Journal of Comparative Neurology 258:230-252.

Watts AG, Swanson LW, Sanchez-Watts G (1987) Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat. The Journal of Comparative Neurology 258:204-229.

Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K (2010) The microRNA spectrum in 12 body fluids. Clinical Chemistry 56:1733-1741.

Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14:697-706.

Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Current Biology 14:2289-2295.

Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75:855-862.

Wirz-Justice A (1987) Circadian rhythms in mammalian neurotransmitter receptors. Progress in Neurobiology 29:219-259.

Woo KC, Ha DC, Lee KH, Kim DY, Kim TD, Kim KT (2010) Circadian amplitude of cryptochrome 1 is modulated by mRNA stability regulation via cytoplasmic hnRNP D oscillation. Molecular and Cellular Biology 30:197-205.

Woo KC, Kim TD, Lee KH, Kim DY, Kim W, Lee KY, Kim KT (2009) Mouse period 2 mRNA circadian oscillation is modulated by PTB-mediated rhythmic mRNA degradation. Nucleic Acids Research 37:26-37.

Wulbeck C, Grieshaber E, Helfrich-Forster C (2009) Blocking endocytosis in Drosophila's circadian pacemaker neurons interferes with the endogenous clock in a PDF-dependent way. Chronobiology International 26:1307-1322.

Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M (2005) Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434:338-345.

Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T (2004) Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Molecular Biology 5:18.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H (2000) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288:682-685.

Yang JS, Lai EC (2011) Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Molecular Cell 43:892-903.

Yang JS, Maurin T, Robine N, Rasmussen KD, Jeffrey KL, Chandwani R, Papapetrou EP, Sadelain M, O'Carroll D, Lai EC (2010) Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. Proceedings

of the National Academy of Sciences of the United States of America 107:15163-15168.

Yang M, Lee JE, Padgett RW, Edery I (2008) Circadian regulation of a limited set of conserved microRNAs in Drosophila. BMC Genomics 9:83.

Yekta S, Shih IH, Bartel DP (2004) MicroRNA-directed cleavage of HOXB8 mRNA. Science 304:594-596.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proceedings of the National Academy of Sciences of the United States of America 101:5339-5346.

Yuan A, Farber EL, Rapoport AL, Tejada D, Deniskin R, Akhmedov NB, Farber DB (2009) Transfer of microRNAs by embryonic stem cell microvesicles. PLoS ONE 4:e4722.

Zahm AM, Thayu M, Hand NJ, Horner A, Leonard MB, Friedman JR (2011) Circulating microRNA is a biomarker of pediatric Crohn disease. Journal of Pediatric Gastroenterology and Nutrition 53:26-33.

Zhang X, Zeng Y (2010) Regulation of mammalian microRNA expression. Journal of Cardiovascular Translational Research 3:197-203.

Zhang Y et al. (2010) Secreted monocytic miR-150 enhances targeted endothelial cell migration. Molecular Cell 39:133-144.

Zheng B, Albrecht U, Kaasik K, Sage M, Lu W, Vaishnav S, Li Q, Sun ZS, Eichele G, Bradley A, Lee CC (2001) Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105:683-694.

Zylka MJ, Shearman LP, Weaver DR, Reppert SM (1998) Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20:1103-1110.