Genomics Demystifies the Gears of Circadian Clocks

RNA sequencing is uncovering the genetic mechanisms driving circadian tissue rhythms and controlling body functions.

Introduction

Humans, plants, and even single-cell organisms have a master clock inside. It's certainly not as large as the Abraj Al Bait Towers clock in Mecca, Big Ben in London, or the Kremlin clock, but its mission is the same. It keeps time and ensures that each organism's life processes are synchronized.

The master internal clock in humans is guided by the daily light and dark cycles influenced by the sun. It coordinates internal metabolic processes by synchronizing the gene-controlled local clocks found in tissues and organs. Local clock genes are autonomous, encoding proteins whose levels rise and fall in rhythmic patterns during a 24-hour cycle. These oscillating biochemical signals control body functions, such as heart rate, blood pressure, and metabolism. Disruption in the master clock alters the biological rhythms it coordinates, impacting the body's health and well-being.

There is an active community of researchers seeking to understand the molecular mechanism of circadian rhythms and how they keep time in the brain and other tissues. Mouse and fruit fly (*Drosophila melanogaster*) studies have identified a number of clock genes, with appropriate names such as *clock*, *frequency*, *period*, and *timeless*.

Michael Hughes, Ph.D., Assistant Professor at the University of Missouri-St. Louis is studying how circadian clocks influence output processes such as blood pressure and sleep cycles. Performing targeted RNA profiling on the MiSeq® System with TruSeq® Targeted RNA Expression panels is enabling him to identify cycling transcripts of clock genes in *Drosophila* and mice. iCommunity spoke with Dr. Hughes to learn how RNA sequencing is transforming circadian rhythm research.

Q: Where is the circadian clock found in humans?

Michael Hughes (MH): It's located in a tiny area of the hypothalmus called the suprachiasmatic nuclei (SCN). It takes light cues from retinal ganglion cells and translates them into transcriptional rhythms that coordinate the biochemical signal oscillations in the body.

Michael Hughes, Ph.D. is an Assistant Professor at the University of Missouri-St. Louis

Under normal circumstances everything is well synchronized. Conditions such as jet lag offer a perfect example of what happens when the master and local clocks are out of sync. Jet lag is caused when you fly across several time zones, causing your master clock and all your peripheral clocks to reset to the new time. Internal clocks have different capacities for retraining than others. As a result, the peripheral clocks are out of sync with each other for several days as they adapt to the time change. That's why you feel so awful when you are jet lagged.

Q: What other ways can the master clock be disrupted?

MH: People suffering from certain forms of blindness have disruptions in their master clocks. There are two sets of retinal ganglion cells. One set is responsible for visual perception, receiving input from photo receptors and sending that information to the brain where it's processed into images. The other set sends data directly to the SCN. Damage to both sets of retinal ganglion cells renders a person blind, and disrupts their sleep/wake and circadian rhythm cycles.

Recently, it was found that many cataract patients experience sleeping disturbances after their cataracts are removed and new lenses implanted. It turns out that for decades surgeons have added a blue light filter between the lens and the retina, inadvertently blocking input to the retinal ganglion cells responsible for circadian input.

Q: Are clock genes involved in the pathogenesis of certain diseases?

MH: Studies show an increased risk of cancer, heart conditions, and diabetes in people who work the night shift or in environments that alter their circadian clocks, such as flight attendants and miners. Many tumor suppressor genes that regulate cell cycle and growth, such as Wee1, are also clock genes.

We also know that heart attack risk for patients with cardiovascular disease is highest at two times during the day—early in the morning and mid-morning. It's a consequence of an imbalance of blood pressure, heart rate, body temperature, and other organismal rhythms.

Q: How are clock gene studies advancing circadian rhythm research?

MH: A large part of the field is interested in the molecular mechanism behind the master circadian clock, how it keeps time in the body, and how circadian rhythms are generated in the brain and other tissues. The discovery of thousands of clock genes, such as bmal, clock, period, and timeless is a testament to that.

Q: What is your research focused on?

MH: My research focuses on understanding how the circadian clock influences cellular and organismal physiology. I'm interested in how the clock regulates output processes such as sleep/wake, blood pressure, and body temperature cycles. To do this, we need to look at transcriptional rhythms.

In many different tissues, the circadian clock is generating huge numbers of rhythmic transcripts. For example, the mouse liver has about 4,000 cycling genes, and the kidneys and the lungs have almost as many. Heart and skeletal muscle, white fat, brown fat, all have substantial numbers of cycling transcripts as well. We created a website, CircaDB[1] that includes gene expression data sets covering the known cycling transcripts.

> "We're studying how the circadian transcriptome changes in different situations. That's a question that is perfectly suited for using a targeted sequencing approach."

Q: What technologies did you use initially to identify rhythm transcripts?

MH: We started with qPCR and microarray technologies, and obtained a lot of great data from those studies. Now we're using next-generation sequencing (NGS). The data quality and the number of things that you can do with the data is so much greater. There's just a lot more power in sequencing approaches. Everything we've done has been on Illumina platforms, whether genotyping arrays or sequencing.

Q: What NGS system are you using to perform sequencing?

MH: I have a MiSeq System in my department and that's what I've used to perform most of my sequencing lately. We'll continue to use the MiSeq System for these small studies. When we scale up to large profiling studies, we'll perform them on a HiSeq® System. The work that I did as a post-doc and the work that appears on CircaDB was all performed on HiSeq Systems.

Q: Was there a reason why you chose the MiSeq System over other desktop sequencers?

MH: We talked to a number of core facilities that have run Illumina platforms and desktop sequencers from other companies. Every person we called had great things to say about the MiSeq System.

Q: What model organisms are you working with?

MH: A lot of the rhythm transcript research is being performed in the mouse. My colleagues at the University of Pennsylvania and several other places are doing mouse work, while I'm using fruit flies in my new lab at the University of Missouri-St. Louis.

Q: Why is Drosophila *a good model organism for studying rhythm transcripts?*

MH: Some of the earliest circadian research was performed in *Drosophila*, and historically, it's been a great model organism. The architecture of the clock in *Drosophila* is similar to that found in humans and mice.

I have a small lab and it's more cost-effective for me to perform these experiments in flies and mice. In particular, the fly transcriptome is smaller so I can get away with fewer reads. Flies also have fewer circadian cycling genes than mice, about 100–200 cycling genes per tissue in a fly versus 4,000 per tissue in a mouse. It's more manageable to find the function of a fly cycling gene, and we can move quickly in terms of manipulating genes and making transgenic animals.

Q: What experimental approaches are you using now to perform your research?

MH: We conduct behavioral analyses, looking at fly behavior over the course of many days to see how their activity and sleep rhythms change.

Of course, we perform a lot of RNA sequencing and RNA expression studies on Illumina platforms. I've been relying exclusively on Illumina library preps as well. I started using them as a post-doc and they've been working great for us.

Q: What library preparation kits are you using in your research?

MH: I'm collaborating with Dr. Karyn Esser at the University of Kentucky who is a distinguished expert on circadian rhythms in murine skeletal muscle. We're using a TruSeq Targeted RNA Expression Kit that consists of a custom targeted panel of skeletal muscle cycling genes to perform that research. We're studying how the circadian transcriptome changes in different situations. That's a question that is perfectly suited for using a targeted sequencing approach. We've already performed the whole-genome screens and identified the cycling genes. At this point, we're most interested in about 150–200 of the 4,000+ cycling genes. By drilling down into those specific targets, we can move faster and more efficiently.

Q: What are the advantages of the TruSeq Targeted RNA Expression Kit Panels you're using?

MH: The sample prep protocol is straightforward and manageable, which eliminates a bottleneck at the front end of the study. The multiplexing capability of the TruSeq Targeted RNA Expression Kit is also a strong point and is essential for targeted sequencing, where you want to analyze many samples in a single run. The TruSeq kits and the MiSeq System enable us to accomplish that. We're generating good data and able to move a lot faster.

In fact, multiplexing enables us to generate even better data, because as the cost per replicate decreases so does the cost in terms of labor, time, and money. It means we can build more biological replicates into our experiments, increasing the statistical power of the study, and giving us the confidence to make conclusions from the data.

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Q: What have you seen so far in your murine skeletal muscle research?

MH: We've found that circadian rhythms change in mice subjected to restricted feeding. Mice are nocturnal and eat at night when they are more active. Forcing them to eat during the day inverts their transcriptional rhythms, changing the phase completely. That's telling us that the rhythms in skeletal muscle are finely tuned to feeding and nutrient cues. Other researchers have seen this occur in the liver and other tissues. It validates that the profiling we're doing is on the right track.

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Q: Are you combining the TruSeq Targeted RNA Expression panel data with data from other sequencing studies?

MH: We would like to combine the RNA sequencing results with data from ChIP-Seq, RIP-Seq, and other studies to investigate DNA/protein and RNA/protein interactions. We just haven't had the opportunity yet.

Q: How will understanding circadian clock regulation impact disease research?

MH: We're looking for the key regulators of circadian output. Specifically, the key genes downstream of the circadian clock, how they're regulated, and what they influence. Long term, we hope to manipulate these genes to understand what's going on in the system and find out how they impact the initiation or progression of disease.

Moving forward, I'd like to investigate how circadian rhythms impact neurodegenerative diseases. We've found a number of genes involved in neurodegeneration that are regulated by the clock, such as TARDBP (TDP-43). The open question is whether sleep disorders caused by circadian clock disruption contribute to neurodegenerative disease or whether neurodegeneration alters the circadian clock, resulting in sleep disorders.

Q: What's the next step in establishing your library?

MH: I'm finalizing the lab setup and focused on obtaining a grant. There's also a major teaching component to my job. I'll be teaching a lab class for first- and second-year Ph.D. students, where I'll be walking them through one of my circadian rhythm experiments. They'll make TruSeq Targeted RNA Expression libraries and run them on the MiSeq System, which is ridiculously easy. I'll also have them perform some of the simpler aspects of the bioinformatics, including processing and aligning the data, and completing some differential expression analysis.

My goal is to demystify the whole process of next-generation sequencing. Graduate students at the beginning stages of their careers might not think about performing a sequencing or bioinformatics project. There's value in having them experience doing it themselves and realizing that it's not as hard as they expected.

When you start in a Ph.D. lab, you learn techniques from that lab and it's easy to get bogged down into one way of thinking. I'm hoping to decrease their activation energy to try new things. We'll see how it works in practice.

References

1. CircaDB Circadian Expression Profiles Data Base, bioinf.itmat.upenn.edu/circa (April 17, 2014).

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