Toward overcoming neurodegenerative disease by the circadian molecular clock study
— My 30 year history in a national institute —

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The mammalian clock gene, *Period2*, was discovered by my research group studying clock genes in 1998. I summarize the progress of understanding the circadian clock molecular mechanism after this discovery. Our group has demonstrated the importance of glycogen synthase kinase 3-dependent phosphorylation of *Period2* and its nuclear transfer and E4BP4 (vril) negative transcriptional regulation, as well as *Clock/Bmal, Period/Cry* E-box dependent negative feedback loop. A role of myo-inositol for elongation of the circadian clock was uncovered through collaboration on ice plant projects with Tsujiko Co., Ltd, Shiga prefecture. When we started the molecular study of the circadian clock, we only considered the daily rhythm. Fortuitously, our research on the peripheral clock mechanism (PPARα) revealed a new mechanism of seasonal clocks, which can count photoperiods to adapt to winter (torpor). Our generation of researchers entered Japanese national institutes during a period called “the basic research shift era.” But, basic research grants were cut significantly during the 24 to 25 year period after we joined the institutes, and our research mission was abruptly changed to applied science. After several years of frustration and contemplation, we gave up studies using mice and concentrated on using *Drosophila* to reduce costs and save time. Consequently, we found a causative role of sleep abnormality around a young age in two neurodegenerative (Gaucher’s and Parkinson’s) diseases by using fly models. I summarize an application for the molecular mechanism of neurodegenerative disease. I am greatly thankful that I was able to spend more than 30 years on the study of molecular circadian clocks with the people who have been involved, from when I started as a researcher in 1986 at the Fermentation Research Institute of the Agency of Industrial Science and Technology to the present day at AIST.

**Keywords**: Circadian clock, clock gene, transcriptional factor, sleep, neurodegenerative disease, Gaucher’s disease, Parkinson’s disease

1 Beginning of circadian clock gene research

The author joined the Fermentation Research Institute, the Agency of Industrial Science and Technology in 1986, after strong insistence and recommendation by Group leader Youji Mitsui of the Animal Cell Research Laboratory. At his lab, they were looking for a person with skills for animal gene cloning since they wanted to investigate the vascular endothelial cell growth factor. I had a couple of offers from national universities at that time, but I decided on this lab because it seemed they would allow me to do whatever I wanted to do. Immediately after I joined, I worked like a worker ant from early morning to midnight for isolating and sequencing various clones, but I could not pick out the target gene. Unable to just watch our hard work, the Group leader recruited a student, Shinichiro Nishimatsu (currently, Kawasaki Medical School), from Dr. Kazuo Murakami’s lab at the University of Tsukuba. However, in less than three months, we received reports of the successful FGF cloning from two places, one in Japan and the other in USA, and my first project was terminated. While I was filled with a sense of defeat, there was a paper published in *Nature* by Mike Young’s group that a specific RNA sequence similar to a *Drosophila* clock gene, *Period*, was detected in birds, mice, and humans. This paper got me excited. That is because the research of genes involved in behavior was the reason I jumped into this field. Furthermore, it was my dream to study behavior from genes ever since I became interested in psychology and behavior science in high school. At that time, for circadian clock genes, the *Period* gene of *Drosophila* was cloned for the first time in 1984, but it was a total mystery why such a gene could affect complex behavior. Therefore, I immediately shifted the target to clone circadian clock genes in mammals, and embarked on the labor of isolating and sequencing candidate clones every day. However, the sequences I obtained were all an RNA repetitive sequence (ACNGGC) of a specific RNA region partially similar to the *Period* gene, and I was unable to obtain the targeted homologous gene composed of 1,100 or more amino acids from mice. Looking back, this seemed to be the noncoding RNA that is the current megatrend in molecular biology. Excellent colleagues who joined the Biological Clock Group during this time included the following people: Miwa Matsu (in situ hybridization); Masae Kurama (diurnal expression of endothelin); Kaname Saida (cloning of endothelin family); Koichiro Kako (establishment of EMSA method and a role

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Commentary: Toward overcoming neurodegenerative disease by the circadian molecular clock study (N. Ishida)

2 Background of the biological clock research

When we started circadian rhythm research, most of the study was done in the field of physiology and anatomy, and the study at molecular and gene levels was very much behind. The master clock that governs the 24-hour rhythm in mammals is located in the brain region called the suprachiasmatic nucleus (SCN) in the hypothalamus. This nomenclature was given because it is situated directly above the optic chiasm. It is located right above the region where the left and right optical nerves cross each other in the brain. The SCN tissue consisting of about 10⁴ cells receives input from the optical nerves. (This is why light can entrain the phase of the clock to an environmental phase.) The output of the SCN tissue consisting of about 10⁴ cells receives input from the optical nerves. (This is why light can entrain the phase of the clock to an environmental phase.) The output of the SCN includes the pineal body (main producer of melatonin), the satiety center, the feeding center, the thermoregulatory center, and the autonomous nervous system. The firing frequency of individual nerve cells in the SCN is high during the day and low at night. It has been gradually discovered that there is a 24-hour rhythm in the hormone secretion from the SCN, but it was totally unknown which molecule (gene product) is required for the generation of such a 24-hour rhythm in mammals.

As mentioned above, this mystery has been unraveled through the advances in Drosophila molecular biology. It has now been discovered that in Drosophila, various gene products are involved in the generation of the 24-hour rhythm, as shown in Table 1, including the Period gene, using the forward genetic approach where genes of mutations of Drosophila that show rhythm abnormality are analyzed.

The clock gene is defined as the gene that affects the behavioral circadian rhythm (phenotype includes arrhythmic, long period, short period, or all of the above) when there is a mutation in one gene. Please refer to the original paper for the description of phenotypes of individual clock genes (Table 1). One of the important characteristics of such clock genes is 24-hour rhythm expression of the gene products (mRNA or proteins) in our body. I present as an example the rat per2 gene that we isolated for the first time in the world. Surprisingly, we found that the circadian clock gene, rat per2 gene, expressed not only in the central pacemaker clock (SCN) in the brain, but also in other tissue such as stomach, liver, kidneys, as well as blood cells, hair cells, and nail cells. (We called these present in places other than the brain peripheral clocks.) It is obvious that such peripheral clocks are controlled by the SCN, because the rhythmic expression of peripheral clock genes is abolished after the lesion of SCN and the fact that when the organs are cultured, the peripheral organs such as liver, kidneys, and heart lose their rhythmic gene expression in a few days while the SCN cells possess automaticity and the 24-hour rhythm is not lost for a long time. In our bodies, not only every cell, but also the expression of circadian clock genes showed a 24-hour rotation.

3 Circadian clock gene Period2 is a period determining factor for mammalian behavior

The molecular mechanism of the circadian clock in mammals is shown by the transcription-translation feedback loop of clock gene products including Period (Fig. 1b). The main idea of this molecular mechanism is based on the Drosophila model (Fig. 1a). The Drosophila clock mutant Period was isolated by Ronald Konopka and Seymour Benzer in 1971. Dr. Benzer unfortunately passed away in November 2007. He was the strongest candidate for the Nobel Prize in the field of circadian clocks. He can be considered the true founder of this field, as he moved from the field of physics and created the basic notion of the first generation of molecular biology along with Francis Crick and Sydney Brenner. That is because at that time when the behavioral trait was thought to

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be determined by multiple factors, he proposed the one-gene-one behavior theory which continues to shine. Such a unique bold hypothesis had emerged from his background in physics as a first generation of molecular biologist applying phase molecular genetics. In the early days, Dr. Benzer discovered the linearity of genes and the qualitative difference (cistron) of point mutation using the T4 phage. Benzer was one of the first scientists to rise to prominence in the field of behavioral molecular biology. Later, the Period gene was cloned in 1984 by the J Hall group and the M Young group (2017 Nobel Prize), and it was found that one amino acid mutation in a different position produced phenotypes for short period, long period, and arrhythmic periods (stop codon).\(^5\)

After a long period of dark ages, the existence of period homologous genes in mammals came into light again in the latter half of the 1990s after the Genome Project of human and rodents was completed. During this time, we won a NEDO grant, and many post-docs worked in my Group [Tomoko Kuroiwa, Marek Banasik, Toshiyuki Hamada (currently, Associate Professor, Hokkaido University), Yutaka Sadakane (currently, Professor, Suzuka University of Medical Science), Zhi-Qiang Qu (currently, Professor, Qingdao University), Naoto Hayasaka, and Norihiko Yamazaki]. At last we identified the rat Period2 (rper2) gene with the Kazusa DNA Research Institute and discovered its 24-hour oscillatory expression in peripheral organs.\(^3\) The greatest contributors to that paper were Takahiro Nagase (Kazusa DNA Research Institute) and Katsuhiko Sakamoto (Kazusa DNA Research Institute) and Katsuhiko Sakamoto who was then employed as a member of staff scientists in my laboratory after working as a post-doc, and others prepared the other rper2 gene cloning for humans and rats, but for mice period gene, we were secondary to Dr. Yoshiyuki Sakaki of the University of Tokyo and Dr. CC Lee of the University of Texas.\(^9\) There were three homologs (per1, per2, and per3) of the Drosophila Period gene in mammals including humans, and it was later revealed that per2 was deeply involved in the behavioral clock function. Accidentally, we were very lucky enough to start on the analysis of per2. Mice lacking these genes were created, and it was found that individually, per1 was for short period rhythms, and per3 had hardly any phenotypes [later, it was found that per3 was involved in delayed sleep phase syndrome (DSPS)\(^6\) and in peripheral clocks of lungs and adrenal glands]. However, per2 gene knock out mice or overexpressed per2 transgenic mice showed a drastic phenotype of arrhythmic behavior in the conditions of constant dark or constant light, respectively. Moreover, while not observed in per1-destroyed mice, in per2 gene knock out mice, the oscillatory expression of other important clock genes Bmal1 was lost in the SCN. From this fact, per2 was considered to be an important factor that controls the oscillatory expression of other clock genes. Moreover, we discovered the bipolar nuclear localization sequence\(^3\) in rper2 amino acids sequence.\(^7\) Therefore, Koyomi Miyazaki, who was then employed as a member of staff scientists in our lab after working as a post-doc, and others prepared the rper2 gene that deletes this nuclear localization sequence (NLS). When the NLS deleted rper2 was overexpressed in COS1 cells,\(^3\) we found that the Cry\(^\mathrm{Term}\) and per2 nuclear localization was inhibited by trapping in the cytoplasm.\(^7\) Therefore, we attempted to create a transgenic (TG) mouse with normal rper2 overexpressors, and another with NLS deleted rper2 overexpressors.\(^8\) As a result, the NLS deleted rper2 overexpressor mouse showed long period, while normal rper2 overexpressor mouse showed short period in the circadian behavior rhythm. This work was the first example in my laboratory where observation in vitro was reproduced in vivo.

Fig. 1 Compared biological clock molecular model for Drosophila (a) and mammals (b)
In both species, a negative feedback model of clock gene transcription with extremely similar sequences occurs.
Immuo-staining the nuclear translocation suggests ion of per2 was delayed in the long period type. We believe that the period was extended due to the delay of nuclear localization of regulating proteins such as Period and Cry, as shown in Fig. 1b. Moreover, it was observed that the rper2-overexpressed mice might have high body temperature and high wakefulness in active phase which suggests that the brain region other than the SCN might be involved in these phenomenon. Later, Chisato Idaka (current name Chisato Kinoshita; Department of Pharmacology, Teikyo University School of Medicine), who joined our Group as a graduate student at the Tokyo Institute of Technology, discovered that glycogen synthase kinase-3β (GSK-3β) was a factor that promoted the nuclear localization of per2.[10] The GSK-3β directly binds to per2, causes phosphorylation, and promotes nuclear localization. This pathway explained well the action mechanism of LiCl that was effective against depression. That is, when LiCl causes the self-phosphorylation of GSK-3β, it becomes inactive and delays the nuclear localization of per2, and this extends the period of activity. This pathway is attracting attention of researchers developing anti-depressant drugs as a target for new drug discovery. Recently, Kazuki Sakata, a graduate student at the University of Tsukuba, et al. found that high concentration of inositol extended the period of circadian rhythm and locomotor rhythm in Drosophila.[80] Myoinositol, an ingredient derived from ice plants (Mesembryanthemum crystallinum) grown in a plant factory of Tsukijio Co. Ltd. of Shiga Prefecture, was discovered originally by us during the screening for a substance that affects Drosophila mating rhythm. Myoinositol is highly expected to be an antidepressant in the future.

The mystery of GSK-3β was found in Drosophila. ATP-mediated protein kinase B (AKT, also called PKB), which was known in the signal pathway of nutrition stimulation, inhibited the nuclear localization of clock gene product Timeless through the target of rapamycin (TOR) pathway.[11] In AKT-overexpressed flies, TOR-86 kinase in the central pacemaker of the fly brain was activated, then the glycogen synthase kinase-3β (SGG) was phosphorylated, and inhibited the nuclear localization of clock gene product Timeless. In summary, nutrients signal from food delayed the peripheral clock, then the peripheral clock affected the central pacemaker in the brain by a feedback mechanism. This feedback mechanism is also common in mammals.

4 Rhythm disorder and clock gene mutation

Sleep-wake rhythm syndrome in humans include the advanced sleep phase syndrome (ASPS), the delayed sleep phase disorder (DSPS), and the non-24-hour sleep-wake syndrome. These syndromes occur on a familial basis, but the causal gene was totally unknown. However, in 2001, linkage analysis was carried out of a family of ASPS in Utah, USA, and the responsible gene was mapped to Chromosome 4. Moreover, it was observed that the rper2-overexpressed mice might have high body temperature and high wakefulness in active phase which suggests that the brain region other than the SCN might be involved in these phenomenon. Later, Chisato Idaka (current name Chisato Kinoshita; Department of Pharmacology, Teikyo University School of Medicine), who joined our Group as a graduate student at the Tokyo Institute of Technology, discovered that glycogen synthase kinase-3β (GSK-3β) was a factor that promoted the nuclear localization of per2. The GSK-3β directly binds to per2, causes phosphorylation, and promotes nuclear localization. This pathway explained well the action mechanism of LiCl that was effective against depression. That is, when LiCl causes the self-phosphorylation of GSK-3β, it becomes inactive and delays the nuclear localization of per2, and this extends the period of activity. This pathway is attracting attention of researchers developing anti-depressant drugs as a target for new drug discovery. Recently, Kazuki Sakata, a graduate student at the University of Tsukuba, et al. found that high concentration of inositol extended the period of circadian rhythm and locomotor rhythm in Drosophila. Myoinositol, an ingredient derived from ice plants (Mesembryanthemum crystallinum) grown in a plant factory of Tsukijio Co. Ltd. of Shiga Prefecture, was discovered originally by us during the screening for a substance that affects Drosophila mating rhythm. Myoinositol is highly expected to be an antidepressant in the future.

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5 Factors affecting the Period2 rhythmic expression

As mentioned in this paper, the 24-hour rhythmic expression of per2 is very important in maintaining the biological rhythm in mammals, and the main regulatory point is transcription. Until now, CACGTG, a non-canonical E-box, was known as the cis sequence involved in the per2 mRNA rhythmic expression. Tomoya Ohno, who joined our Group as a graduate student at Life Science and Technology, Tokyo Institute of Technology(TIT) because I was a visiting professor at TIT, biochemically identified that the bZIP transcription factor E4BP4 (Drosophila vrilie holomog) had an inhibitory effect on per2 rhythmic expression (Fig. 1b). As a result of detailed analysis, he found there were two E4BP4 binding sites, the A-site and the B-site, near the mper2 promoter DNA region. When a mutation was made into these two sequence sites, the per2 rhythmic

Human Period1 LANKA E$VS$TSQC $FS$TIVHVG DKKPP
Human Period2 LPGKA E$VS$LSQC $YS$TIVHVG DKKPP
Human Period3 LSTAM L$LG$SQC GYS$TIVHP PPETA

Fig. 2 Mutated amino acids in hPERIOD protein in human abnormal rhythm syndrome

Serines (S) in the region are target amino acids of phosphorylation by casein kinase I, and this region is important for the phase regulation of circadian rhythm. The arrows point to amino acid mutation in advanced sleep phase syndrome (ASPD) and delayed sleep phase syndrome (DSPS).

expression was inhibited specific to the B-site. Moreover, circadian time-specific binding was confirmed at the B-site by both a gel retardation assay and the ChiP method. Finally, the per2 promoter rhythmic expression activity was compared with the mutation to the A-site, the B-site, and the E2-box by the luciferase activity using the real-time monitoring system. Interestingly, the 24-hour oscillation was not lost in the single mutation of E2, but it was proven at a cellular level that the 24-hour oscillation disappeared with the mutation to both the E2-box and the B-site. Conventionally, the importance of the A-site only was reported in vitro,[14] but in a complex system such as oscillatory expression, it is important to conduct analysis by introducing to a cellular or an in vivo level system. Since the oscillatory expression was maintained in the single mutation of E2-box, it became clear that the per2 oscillation could not be explained only by the negative feedback model of Clock/Bmal1 and per/Cry. Moreover, we found that the E4BP4 bound with per2 and Cry2 in the cell.[15] From these facts, we proposed a new model for a negative transcription-regulating factor complex for biological clocks (Fig. 1b). In this new model, these complexes go through nuclear localization in circadian time specific manner, and per2 and Cry2 complex target the Clock/Bmal, while E4BP4 complex targets the B-site (D-box) to negatively regulate the transcription. There are several reports that E4BP4 is important in the 24-hour oscillatory expression of phosphatidylcholine transporter Mdr2,[16] cytochrome P450A4 (CYP3A4) involved in drug catabolism, and cholesterol 7α-hydroxylase (Cyp7α) that is involved in bile acid synthesis. Therefore, it is thought that the transcription factor E4BP4 acting negatively on the 24-hour rhythm transcript is important in peripheral circadian clocks, particularly in liver rhythm formation.

6 Biological clock regulation by chromatin

This chromatin topic was tackled by Yoshiaki Onishi (currently, AIST Kansai), who specializes in chromatin biochemistry and who moved from a different group in AIST. The Bmal1 which formed the heterodimer with a circadian clock gene product CLOCK protein is an extremely important protein that causes the 24-hour oscillation of the biological rhythm, and consists of the negative feedback loop. However, why this gene is transcribed through 24-hour oscillation remained a mystery. First, Transcriptional rhythmic regulation of Bmal1 gene was accomplished by the binding of clock transcription factor REV-ERBa and RORα to upstream region of Bmal1 gene, using various deleted DNA constructs. Onishi further found that the intranuclear matrix protein SAF-A (also called hnRNPU) bind rhythmically at the downstream of the cis element RORE, to control the transcription.[17] That is, the state of chromatin around Bmal1 gene was widely open or closed. As SAF-A has been reported to involve in the regulation of the c-Mycc-Max complex, Bmal1 will become very important in controlling cancer cells in the future.

7 Different functions of peripheral clock (Haradokei) and central clock in Drosophila and mammals

Until recently, most of the relationships between the peripheral clock (Haradokei) and the central clock was studied in mammals, but an interesting study has been published by Sehgal et al. using Drosophila.[18] In Drosophila, the tissue that has both the functions of liver and fat tissues of humans is called the fat body (Fig. 3). Using genetic engineering methods to transfer the dominant negative type Clock gene so that the biological clock function in the fat body was lost, a fly that was very weak against fasting (i.e. quick to starve to death) was created because its glycogen storage was significantly decreased. Therefore, this fly would feed frequently during the night. Interestingly, such abnormality was not seen in the CLKβK fly that had Clock gene mutation throughout the body. Therefore, they hypothesized that there must be different effects of clock genes on the central clock and peripheral clock, and created a fly in which the Clock gene function was lost specifically in the central pacemaker. As a result, the glycogen storage increased in the fat body, and the fly became stronger against fasting compared to a normal fly. Through this experiment, it was shown that the functions of the peripheral clock (Haradokei) and the central pacemaker were different, as observed in mammals. Unfortunately, in this paper, the direct relationship between the clock gene and glycogen storage was not demonstrated, but this point was proven using mammals by Ryosuke Doi (currently, Astellas Tsukuba Research Center), who was a graduate student at the University of Tsukuba of which I was a professor. I shall describe the glycogen study.[19]

The most important organ for glycogen storage and for maintaining blood glucose levels is liver in mammals. It was known in the 1970s that there was a circadian rhythm in the glycogen synthase that was the rate-limiting enzyme in glycogen synthesis. In later molecular biology analysis, it was
clarified that the glycogen synthase 2 (Gys2) was expressed mainly in the liver, and its family glycogen synthase 1 was expressed in muscles, nerves, and fat. Doi et al. looked at the rhythmic expression regulating the mechanism of Gys2 in the liver. First, the rhythmic expression of genes involved in glucose metabolism was investigated in the normal mouse and the mouse with mutation in the Clock gene (C1/C1) using the real-time PCR method. As a result, abnormalities were seen in the Clock-mutant mouse for the rhythm expression of phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphate, glucose transporter 2, and others. The most interesting difference was the almost complete disappearance of the expression rhythm of Gys2 mRNA in C1/C1 mice. On the other hand, there was no significant difference in glycogen phosphorylase, which was the rate-limiting enzyme of glycogen breakdown, between the normal and the Clock-mutant mice. The data indicate that the glycogen storage rhythm in the liver was under the rate limitation of glycogen synthesis but not in the breakdown. Moreover, arrhythmicity was detected not only in Gys2 mRNA but also in the Gys2 protein level in Clock-mutant mice. Furthermore, the data suggests that this glycogen rhythm was not dependent on insulin or glucagon levels that changed depending on food. From these facts, it was considered that the Gys2 rhythm was an endogenous regulatory expression of mRNA. We hypothesized that it might be directly controlled by clock gene products, and searched the genome sequence for the cis element binding to clock gene products. Since we could not find typical rhythm cis elements in about 3 kb of the 5' upstream region, after trial-and-error, we found two E-box elements in tandem in the first intron of the Gys2 gene. By making mutations into these two E-box elements, it was shown that both elements contributed equally by using the in vitro reporter assay and the chromatin immune-precipitation method from the liver extract. It was thought that these elements might function in vivo. Therefore, we addressed this point by investigating the effect on rhythm oscillation using the real-time reporter assay method. Using the mper2-dLuc as a positive control, E1, E2, and both were mutated and compared. As a result, both of E1 and E2 mutations were most attenuated. From this experiment, we showed that glycogen synthesis rate-limiting enzyme Gys2 was regulated rhythmically at a transcription level through the two E-box elements recognized by Clock and Bmal. We think the Gys2 gene was one of the output from the peripheral clock. This is the first data in the world that showed that the glycogen metabolism was under the direct control of the molecular clock. Haruhisa Kawasaki (Institute for Chronobiology, Foundation for Advancement of International Science), who joined our group after returning from studying in the United States, advanced this study, and found that C/EBPα, an important transcription factor involved in glycogen storage in fetal liver, was also regulated by core feedback loop through the E-box element in the upstream region of C/EBPα gene.20

8 Three cis elements that comprise the circadian clock

As input of the circadian clock system, various elements other than light are being found. That is because the concept of peripheral clocks in all tissues has become established because of the discovery of the rhythmic clock gene expression in all tissues. The most recent peripheral clock model is shown in Fig. 4.21 The cholesterol in food and HEME (hemoglobin) supplied from blood are becoming very important, as they have been considered to be important factors that synchronize with the circadian clock in liver and other internal organs. Cholesterol positively controls clock proteins Clock and Bmal through the transcription factor ROR dependent RORE binding, while HEME negatively controls Clock and Bmal gene through the transcription factor REV-ERB (Figs. 1 and 4).1 A recent paper showed

Fig. 4 Circadian clock synchronization with various environmental factors such as light, food, and fat
ROR, Rev-erb, Clock, Bmal, Period, and Cryptochrome (Cry) are clock genes. DBP, TEF, HLF, E4BP4, PPAR, RAR, RXR, and SREBP are circadian-clock-controlled transcription factors. RORE, E, D, CRE, and PPRE are target DNA sequences (cis element) for the transcription factor binding.
that when a ligand-binding site of RORα is expressed in an insect cell, cholesterol coprecipitates with RORα. Moreover, from X-ray crystal analysis, the possibility of RORα ligand being cholesterol is indicated, because cholesterol sulfate and 25-hydroxycholesterol bind to this ligand-binding site of RORα. On the other hand, in chronobiology, what is in the upstream of clock proteins Clock and Bmal is the big issue, and this is being solved. These clock proteins Clock and Bmal, other than regulating Period and Cry in the core loop, regulate the transcription factors DBP, TEF, and HLF as output, bind with the nine-base sequence called the D-box, and create a large 24-hour rhythm in the peripheral cells (Fig. 4). Recently, fasting-induced hormone, FGF21, was reported as the factor that had this D-box in its regulatory sequence.

Katsutaka Oishi, who was employed as a research scientist from research technician of the Biological Clock Group, previously conducted genome-wide expression analysis using clock-mutant mice. He discovered the peroxisome proliferator-activated receptor α (PPARα) that was a nuclear receptor and fatty acid as ligand, among the genes that was rhythmic expression regulated by clock gene products in the liver, and also discovered that fatty acid breakdown (β-oxidation) occurred specifically during the night via this PPARα. Moreover, It was found that FGF21 was expressed with a large circadian rhythm, when fibrate, a ligand of peroxisome proliferator receptor α, was administered to the abdominal cavity of the mouse. We also reported that fasting by the fibrate (particularly the catabolization of fatty acid) advanced the circadian rhythm in the body and enhanced the fluctuation of the FGF21 rhythm.

Since this phenomenon is not observed in PPARαKO mice, it was considered to be dependent on the peroxisome proliferator response element (PPRE). However, when a ketone diet (a diet method of ingesting high fat and low carbohydrates) was carried out, FGF21 was increased without depending on PPARα. That suggests a pathway for inducing FGF21 without depending on PPRE. Recently, it was reported that not only the known PPRE but also new D-boxes and E-boxes were discovered through the detailed analysis of the mouse FGF21 promoter (Fig. 4). More interesting is the fact that the transcription factor E4BP4 suppressed both the Bmal/Clock-dependent transcription and the PPARα-dependent transcription at the same time. That the E4BP4 regulated the Bmal/Clock-dependent transcription was easily predictable, because we had already made a complex with E-box (cis element consisting of CACCTG) dependent negative regulators, Period2 and Cry with oE4BP4. However, it is very interesting that E4BP4 suppressed the PPRE-dependent transcription with PPARα at the same time. This indicates that the suppression of rhythmic transcription of E4BP4 is much more powerful than we initially thought, acts on E-boxes and PPRE as well as D-boxes, and plays the role of a major brake on the peripheral clock. It is known that E4BP4 negatively controls the transcription targeting genes of the D-box with an insulin dependent manner after a meal. As mentioned in Chapter 5, the mechanism in which transcription factor E4BP4 acts negatively on the formation of 24-hour rhythm transcription is very important particularly in liver function. There is a report that E4BP4 is activated on time-specific light stimuli and induced at transcription factor SREBP in bird pineal cell. The effects of light and food are very complex even in one gene expression. This is a field that needs further attention.

In humans, there was an interesting report that blood FGF21 was expressed in circadian rhythm following fatty acid rhythms. In the same paper, they showed that FGF21 was induced by unsaturated fatty acid like linoleic acid rather than saturated acid like palmitic acid, using human hepatocarcinoma-derived cell HepG2. More surprisingly, when the circadian rhythm of FGF21 was compared in normal and obese (having BMI 25 or over) humans, the amplitude of rhythm was very small in obese humans (Fig. 5). In the future, by studying the diurnal dynamics of such fasting-induced hormone FGF21, it may become possible to prevent obesity and aging by increasing the amplitude of rhythm. Recently, it was reported that FGF21 was induced from brown adipose cells when Swiss mice were kept in low temperature. FGF21 is studied as the factor that induces hibernation and diurnal torpor, and please refer to other reviews for details. In summary, nuclear receptor PPARα binds with PPRE in the liver during fasting, and transcription factor ATF2 binds to CRE in brown adipose cells during low temperature to induce FGF21. However, in the case of a ketone diet when FGF21 is induced without PPARα pathway, the pathways through D-boxes and CRE are important for this induction. (Fig. 4). By analyzing FGF21 induction comprehensively, the relationship between fasting and fat metabolism will become clearer in the future.

Fig. 5 Three peaks of circadian rhythm of the fasting-induced hormone, FGF21 in humans
Clear circadian rhythm is detected in lean humans compared to obese humans.
9 Basic model of biological clock learned from Drosophila

As mentioned above, we learned of almost all the actors (molecules) and the relationships (pathways) from Drosophila (Table 1). Though quite late in the game, I decided to study the Drosophila system. Earlier, every time I ran into Dr. Sumihare Noji (currently, President, Tokushima University), at the Molecular Biology Society of Japan, he recommended me to introduce Drosophila system studies. Coincidentally, Director Tatsuo Katsura of the National Institute of Bioscience and Human Technology (currently, AIST) recommended a co-professorship of the University of Tsukuba to me, and it became possible to take in more students at the lab. At that time, Tetsuya Okada joined our Ishida Group of Clock Gene, AIST with the recommendation of Dr. Michiko Ohtomi of Toho University. Dr. Ohtomi had been studying the biological clock for a long time, and was a student under Dr. Takeo Deguchi (Tokyo Metropolitan Institute of Gerontology), who studied diurnal rhythm of melatonin in the pineal body. Later, Okada moved and received tutelage of Dr. Osamu Hayaishi, who was the President of the Osaka Bioscience Institute famous for sleep and prostat glandin study, but Dr. Hayaishi passed away at 2015. Here, I was in trouble, as I had never done Drosophila genetics before. I looked for someone who was experienced in Drosophila genetics and found Takehide Murata in Riken Tsukuba. He had directly learned molecular genetics from Dr. Teiichi Tanimura (currently, School of Science, Kyushu University) who identified the Timeless (named Ritsu) gene in Drosophila in Japan. After Okada studied at Riken for a while, he discovered the E-box, as well as the vrille/E4BP4 gene product binding element responsible for the rhythmic expression in the upstream region of Drosophila clock gene Timeless in our laboratory.[28] Later, Takaomi Sakai (currently, School of Science and Engineering, Tokyo Metropolitan University) joined our group as a post-doc from Dr. Yuzuru Oguma’s lab at the College of Biological Sciences, University of Tsukuba, and the Drosophila clock gene research was rapidly accelerated in my laboratory. Sakai was interested in the female mating behavior because he worked on it during graduate school, and found that this female mating behavior showed circadian rhythm depending on clock genes.[29] More interestingly, he discovered a diversity of female mating rhythms in different fly species. Therefore, he set up a hypothesis that such time specific mating is effective in species differentiation driven by reproductive isolation. To test this hypothesis, Izumi Nishinokubi, who was employed as a technical support member from Dr. Kenji Tomioka’s lab at Okayama University, carried on the experiment. The clock gene Timeless was isolated from Drosophila ananassae, transferred to the Timeless null mutant fly of Drosophila melanogaster, and this ananassae timeless gene, was regulated by a heat shock promoter. The result was not so simple as to reproduce the mating circadian behavior of D. ananassae with a replacement of one clock gene (we got an intermediate of D. ananassae and D. melanogaster), but we obtained an unexpected by-product where the locomotor rhythm of diurnal fly could be changed to nocturnal by shifting the expression period of the Timeless protein by 12 hours.[30] This result supports a recent ecological experiment where mice could be changed from nocturnal to diurnal by environmental adaptation. It is my pleasant memory that I wrote a review, “Time, love and species,” where I discussed these findings.[31] As mentioned earlier, the discoverer of the Period gene, Dr. Benzer passed away, but his book “Time, Love, Memory” became a bestseller in the United States. During the age from Sakai to Nishinokubi, we mainly studied the time specificity of female mating receptivity in circadian behavior. Then, Yasutaka Hamasaka, who got his degree in Sweden, joined our Ishida Group of Clock Gene. When I talked with him, he was interested in the brain neural connection of close-proximity (CP) rhythm, in which the male pursued the female fly. An American group started a new CP rhythm system using a CCD camera, after seeing our first paper in the Proceedings of the National Academy of Science.[32] This system had much better throughput than the arranged marriage method used by Sakai, and we incorporated this method. Hamasaka created various Drosophila with destroyed biological clock region in the brain, by utilizing a molecular genetic method (such as causing cell death in certain nerves only). As a result, we identified the center for close-proximity rhythm in Drosophila, and clarified that males stopped pursuing females when the brain area called evening oscillator was deleted.[33] Since this center became active during summer, it is most likely related to seasonal rhythms. Later, we attempted to develop an automating machine for close-proximity rhythm, locomotor and sleep rhythm. Jointly with Taisei Co., Ltd., we introduced moving image analysis of IR camera, and Takahiro Suzuki, a post-doc at the graduate school of the University of Tsukuba, created a program in C language. The device was named Automated Circadian System (AutoCircaS) (Fig. 6). The prototype of AutoCircaS was assembled uniting many parts that lay in the corner of our lab as junk, and Taisei and Suzuki (who set up a venture company Shigray Inc.) brushed up AutoCircaS as a selling product. For this accomplishment, AutoCircaS was awarded one of the Chiba Prefecture Monozukuri big prize in 2016. One day, I received a call from Masami Shimoda (National Institute of Agrobiological Sciences), an old friend of mine, and he asked for our help to figure out why a Drosophila fly that lacked a gene for a certain mental disease was showing abnormal behavior. According to him, there was a Drosophila family gene (dfmr1) of fragile X syndrome that was accompanied by mental retardation in humans. Furthermore, there were three family genes in mammals, but only one homologous gene in flies. I thought this would be very easy to analyze, and conducted behavioral analysis, and found that the locomotor rhythm was strikingly arrhythmic. Shimoda rescued the normal dfmr1 genes of the mutant fly (Fig. 7), and found that the behavior of the fly became normal, the core clock...
gene oscillation was maintained, and the eclosion rhythm showed normal. This experiment indicated that the dfmr1 gene possessed output functions from central pacemaker locomotor behavior. Later, it was clarified that poor growth of nerve cells was a common cause of disease in humans and Drosophila. As mentioned here, the Drosophila disease model contributes to the research of neurodegenerative disease with unknown mechanisms.

Schizophrenia is a disease that is most difficult among the psychiatric disease. Recently, through genetic analysis of a family with frequent occurrences of schizophrenia in Scotland, the disrupted-in-schizophrenia1 (DISC1) gene was reported to show frequent mutual translocation of Chromosome 1 and Chromosome 11. For DISC1, we worked jointly with Masami Shimoda mentioned earlier, Katsuo Sawa of Johns Hopkins School of Medicine, to analyze behavior for expressing human DISC1 in Drosophila. As a result, the human DSIC1 transgenic Drosophila showed tendency to have longer sleep time.

10 Essence of dementia learned from Drosophila genetics

Parkinson’s disease (PD) is the second most common neurodegenerative disease after Alzheimer disease, accompanied by so-called dementia symptoms, and is a major social issue confronting the current longevity society. As the Drosophila model for familial Parkinson disease, transgenic fly expressing human mutant α-synuclein is famous and published in Nature and other journals. Using this Drosophila model for Parkinson disease, we found that sleep disorder preceded behavioral abnormality such as tremors. Recently, there was a report that patients with a hetero variant of Gaucher disease causal gene had 28 times more risk for Parkinson disease. That is, the Gaucher disease causative gene is the most risk factor for Parkinson disease. However, the molecular mechanism is currently unknown. If this mechanism can be solved from the clock and sleep gene pathway, we can develop new treatment methods from new perspectives.

Takahiro Suzuki (currently, Shigray Inc.), who was a graduate student (the University of Tsukuba) in Ishida Group of Clock Gene, AIST, succeeded in creating a model animal by transforming a variant human Gaucher disease gene in a Drosophila compound eye. Gaucher disease is a lysosome disease that is specified as one of diseases in the Research on Measures for Intractable Diseases Project of the Ministry of Health, Labour and Welfare. Due to a genetic mutation, the activity of enzymes called glucocerebrosidase is lost before birth. Therefore, glucocerebroside which is a substrate of this enzyme cannot be transferred into ceramides, and the substrate accumulates in the liver, spleen, bones, nerves, and others. The gene of glucocerebrosidase was firstly cloned in USA by Dr. Shoji Tsuji (currently, Faculty of Medicine, the University of Tokyo) et al. in 1986. Many patients with human Gaucher disease die in childhood, and a complete cure of this disease is extremely difficult even today. Suzuki et al. set out to create a model Drosophila for Gaucher disease, and expressed this human variant glucocerebrosidase gene in Drosophila using a compound-eye-specific driver, and found a formation abnormality in the compound eye. With further detailed molecular biological analysis, we clarified that this eye morphological abnormality was caused by endoplasmic reticulum (ER) stress. Therefore, ambroxol, a chaperone drug thought to reduce endoplasmic...
reticulum (ER) stress was administered, and the above eye abnormality and ER stress were reduced (Fig. 8). From these data, we concluded that human Gaucher disease involved not only the accumulation of enzyme substrates as explained conventionally, but was a molecular mechanism of ER stress caused by mutant proteins themselves.

To link such neurodegenerative molecular mechanism to Parkinson disease and to propose a treatment method, we recently succeeded in creating a Gaucher disease model Drosophila where human mutant glucocerebrosidase was expressed throughout the body, as well as in specific sites. We recently succeeded in creating a disease model Drosophila that had both Parkinson disease and Gaucher disease because Drosophila allowed creation of models faster and easier than with mice.

We recently developed the second generation of the Gaucher disease model Drosophila. This was a model in which a Minos-insertion mutation was introduced into a GBA homologous gene in Drosophila. This mutant showed accumulation of hydroxyl glucocerebroside, an enzyme substrate, as observed in the Gaucher patients. By analyzing the gene expression in this second generation of Garucher disease model Drosophila, the increased expression of several genes (factors) for autophagy related Parkinson disease was observed. It was shown that this Gaucher disease model Drosophila had a short lifespan, and showed motor dysfunction as well as sleep disorder. Interestingly enough, in the new Gaucher model Drosophila and the Parkinson disease model Drosophila, sleep disorder was found in the young age (corresponding to about the late 20s to 30s in humans). From this fact, in neurodegenerative disease accompanying dementia including Parkinson disease and Gaucher disease.

We are still continuing drug screening using the dementia model Drosophila mentioned above.

11 Conclusion

The clock gene research at AIST was started by using mice and cell strains, but in the mice system, two years were necessary to manipulate one gene by genetic engineering, and the maintenance of mice and cultivation of the cell line were expensive. In the latter phase at AIST, we ran out of budget, and therefore, changed our research system to flies and mammalian cell cultures only. The merit of the Drosophila system is that it has a short life-span of about 60 days, the stock facilities for normal and all genetic variants are available all over the world, and the researchers and organizations in fly society are very friendly and any mutants are supplied readily by an e-mail request. We have succeeded in creating useful mutant fly lines for the development of new treatments for neurodegenerative disease such as dementia at AIST. Using such new mutants, primary screening of dementia drugs is still going on and we are receiving small funding from companies. Looking back at the 30 years of scientist life at a national research institute(AIST), I am very grateful that I can continue my work with many collaborators, participants including young students. and with the cooperation of many company people. I was unable to mention the names of all the collaborators, however, I express my deepest thanks here for the 30 years.

Now, a big stream of the science funding in Japan tends toward application, and there is an economical demand that science be immediately useful. It is becoming difficult to study basic science in national research institutes and universities. However, real innovation in science and technology comes from pure basic research, particularly for antibody medicine in Japan. In the future, I hope national...
agencies and ministries nurture real organizers with good judgement (mekiki in Japanese) over cross-sectional basic research so that bioscience in Japan does not decline.

The author established the Institute for Chronobiology in the Foundation for Advancement of International Science (FAIS) (3-24-16 Kasuga, Tsukuba) in April 2016. Here, we are continuing our basic research on clock genes, sleep, and neurodegenerative disease. I hope you might drop in and talk to us at my new laboratory if you stop by at Tsukuba Science City. While proofreading this article, the 2017 Nobel Prize in Physiology or Medicine was awarded to “Discoveries of Molecular Mechanisms Controlling the Circadian Rhythm.” I would like to say congratulations to Drs. Jeffrey C. Hall, Michael Rosbash, Michael W. Young who are my good old friends and teachers in this field.

Terminologies

Term 1. Bipolar nuclear localization sequence: a special amino acid sequence region within a protein to transport a specific protein from the cytoplasm to the nucleus.

Term 2. COS cell: A cell line name, which comes from SV40 transformed renal fibroblast cells of African Green Monkey. Since it shows good expression efficiency of foreign genes, it is often used in transient expression of proteins.

Term 3. Endogenous: In general, a situation where the root or cause of a phenomenon exists within itself.

Term 4. Cry: Blue-light photoreceptor protein that was originally discovered in plants. Later, it was found in many animals including humans.

Term 5. Cis element: Also called cis-acting element. Existing in the vicinity of the gene, gene transcription (reading of RNA from DNA) is modulated when a regulating protein binds to this site. This region itself does not synthesize proteins, and it is solely for binding with other proteins (a transcription factor).

Term 6. TE2-box: When an E-box sequence is categorized, it can be divided into the canonical E-box CAGNTG sequence and the second E-box. CACGTT sequence was named E2-box. An E-box is a DNA sequence to which transcription factors such as Clock and Bmal can bind and activate transcription. In Fig. 1, it is referred to as E cis element.

Term 7. bZIP transcription factor: bZIP (basic zipper protein) is one of the motifs of a secondary protein structure, and has the ability to bind with DNA.

Term 8. mper2 promoter: Transcription factor binding region in the upstream of mouse Period 2 gene.


Term 10. B-site: DNA sequence for the binding with E4BP4 transcription factors on the downstream region of Period 2 gene.
References


Commentary: Toward overcoming neurodegenerative disease by the circadian molecular clock study (N. Ishida)

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