1. INTRODUCTION

The circadian clock in suprachiasmatic nuclei (SCN) is one of the most indispensable biological functions in all living organisms. The SCN acts as a multifunction timer to control homeostatic systems, including sleep and activity, hormone levels, appetite, and other functions with a 24 h rhythmicity. Biological rhythms affect the pathophysiology of diseases as well as physiological functions. When the risk and/or intensity of symptoms of a disease change rhythmically over time, chronotherapy is especially relevant in the case of allergic rhinitis, arthritis, asthma, myocardial infarction, congestive heart failure, stroke, and peptic ulcer. The clock genes regulate circadian rhythms involved in biological, physiological, and behavioral processes under the control of the circadian clock. Chronopharmacological finding are influenced by pharmacodynamics (PD) as well as the pharmacokinetics (PK) of a drug. An information of the 24 h rhythmicity in the risk of disease and the evidence of 24 h rhythmic dependency of drug PK and PD play an important role in the rationale for pharmacotherapy.

The clock genes have been identified to ultimately regulate a vast array of circadian rhythms involved in biological, physiological, and behavioral processes such as homeostatic functions of steroid hormones and their receptors. Perturbation of these rhythms is closely related to sleep disorders, depression, diabetes, and cancer diseases. The circadian strategies of monitoring rhythm, overcoming rhythm disruption, and manipulating rhythm are useful to improve chronopharmacotherapy from the perspective of the molecular clock. Such an approach should be performed by overcoming the new challenges in drug delivery systems (DDS) that match the circadian rhythm (Chrono-DDS). Gene delivery and antibody delivery, targeting specific molecules for some diseases have been focused on recent studies on pharmacotherapy. One of important candidates should also be clock genes.

This review aims to introduce the role of the molecular clock in time-dependent dosing changes in the therapeutic effect and safety of a drug and the possibility of drug discovery and development based on the molecular clock.

Key words circadian rhythm; molecular clock; chronopharmacokinetics; chronopharmacology; chronotherapy

2. CHRONOBIOLOGY OF DISEASE

The clock genes regulate circadian rhythms involved in physiology and behavior. The circadian rhythm of Per mRNA expression is discovered in other tissues as well as in the SCN. Since the circadian rhythm of physiological functions and Pers mRNA expression was abolished in SCN-lesioned rats and Clock mutant mice, the circadian rhythm in the peripheral tissue is considered to be regulated by that in the SCN. Such a cascade of clock genes might control biological rhythms in the whole body. SCN controls a circadian rhythm of plasma glucocorticoid levels via the hypothalamic–pituitary–adrenal axis. Glucocorticoids control various physiological responses and developmental processes by binding to and modulating the transcriptional activity of their cognate receptors.
nuclear receptor. Transit induction of Per1 and albumin D-site binding protein (Dbp) mRNA levels was demonstrated after a single dose of dexamethasone. Glucocorticoid hormones are particularly attractive candidates, as they are endogenous substances and contribute to the entrainment of peripheral oscillators but not SCN. However, both nervous and humoral signals seem to be involved as the mechanisms employed by circadian output pathways. Moreover, the regulatory system of biological rhythms should be clarified on the basis of clock genes.

Three mammalian clock genes are rhythmically oscillated in the SCN. Although Per1 and Per2 are induced in response to light, the induction of Per1 plays an important role in an initial event in the light-induced reset and entrainment of the circadian clock. The transcriptional machinery of the core clock controls a clock-controlled rhythmicity in Fig. 1. Namely, CLOCK-BMAL1 heterodimers act via an E-box enhancer to activate the transcription of Per, vasopressin, and Dbp mRNA, contributing a specific output function. CLOCK mutation affects the expression of the non-rhythmic genes as well as the rhythmic genes. The clock gene controls sleep disorders, metabolic syndromes, and cancer disease and so on.

Myocardial infarction frequently occurs in the early morning. This might partly result from the circadian rhythm of...
fibrinolytic activity associated with plasminogen activator inhibitor-1 (PAI-1) rhythmic activity. Cycle-like factor (CLIF) forms a heterodimer with CLOCK and upregulates PAI-1 through E-box sites in endothelial cells. Furthermore, PER2 and CRY1 inhibit the activation of the PAI-1 promoter by the CLOCK:CLIF heterodimer. CLIF controls the circadian rhythm of PAI-1 and potentially provides a molecular basis for the morning onset of myocardial infarction.

Bmal1, a transcription factor controlling the circadian rhythm, contributes to the control of adipose differentiation and lipogenesis in mature adipocytes. BMAL1 controls adipogenesis and lipid metabolism in mature adipocytes.

In humans, sleep disorders are related to a genetic mutation that affects circadian clock function. Familial advanced sleep-phase syndrome has been demonstrated in three families. The condition of a disease show early evening sleepiness and early morning awakening. Patients with familial advanced sleep-phase syndrome have a circadian period of approximately 1 h shorter than normal. Genomic analysis using multiple sets of dense genomic markers to map the associated mutation for one of the familial advanced sleep-phase syndrome families concluded that the mutant gene is hPer2, the human homolog of mPer2.

3. CHRONOPHARMACOKINETICS

Chronergy, the time-dependent action of drugs on an organism, pertains to rhythmical changes in both effect and side effect of drugs. Chronergy is associated with both the chronopharmacokinetics of a given drug as well as the chronesthesia of the affected biological targets. The 24 h rhythms during each process such as absorption, distribution, metabolism, and elimination involved in chronopharmacokinetics are regulated by the rhythmicity of many physiological factors such as gastrointestinal, cardiovascular, hepatic, and renal changes. The lifestyle, such as active-rest cycle, posture, and eating schedule in addition to physicochemical properties of a drug (lipophilicity or hydrophilicity) influence chronopharmacokinetics.

A significant portion of the transcriptome under circadian clock control in mammals includes the PAR-domain basic leucine zipper (PAR-bZip) transcription factors D-site-binding protein (DBP), hepatic leukemia factor (HLF), and TEF. Triple mutant mice were born at the expected Mendelian ratios, but were epilepsy prone, aged at an accelerated rate, and died prematurely. A highly circadian manner of DBP, TEF, and HLF has been demonstrated in several peripheral tissues. The liver and kidney transcriptomes of PAR bZip triple knockout mice were compared with those of wild-type or heterozygous mutant mice to clarify the PAR bZip target genes. The gene expression patterns of several proteins involved in the drug metabolism and the liver and kidney responses to xenobiotic agents were altered by the disruption of three genes in mice. The PAR bZip proteins control the expression of several enzymes and regulators involved in the detoxification and metabolism of drugs, such as Cyp enzymes and so on. PAR bZip directly might controls some genes encoding detoxification enzymes, such as CYP2A5, CYP2C50, and CES3. The constitutive androstane receptor (CAR), whose circadian transcription is governed by PAR bZip proteins, controls the expression of other detoxification enzymes, such as CYP2B10. However, both CAR and PAR bZip proteins control other enzymes in the xenobiotic defense, such as aminolevulinic acid synthase and P450-oxidoreductase. The various stage at which PAR bZip transcription factors might compose in the coordination of xenobiotic detoxification have been demonstrated (Fig. 2).

In the mouse liver, CYP2E1 enzymatic activity, clinically and toxicologically important, exhibits circadian oscillations. The expression of CYP2E1 mRNA increases from the late light phase. CYP2E1 promoter activity is increased by the hepatic nuclear factor-lalpha (HNF-1alpha) and is repressed by CRY1 based on the results of the luciferase reporter gene analysis. The repressor activity of CRY1 is demonstrated on the HNF-1alpha binding site of the CYP2E1 promoter region with a mutated E-box. To study the molecular mechanism of circadian clock, serum-shocked HepG2 cells are used as an in vitro model in the human liver. A 24 h rhythm in the expression of clock genes is demonstrated after the brief exposure of HepG2 cells to 50% serum. A 24 h rhythm in the mRNA levels of CYP2E1 is observed after treatment of 50% serum with HepG2 cells. The transfection of small interfering RNA against HNF-1alpha and CRY1 into serum-treated HepG2 cells represses the rhythm of CYP2E1 mRNA expression. CRY1 periodically interacts with HNF-1alpha transcriptional complexes, including coactivator p300 on the HNF-1alpha binding site in the CYP2E1 promoter, as observed from the results of the chromatin immunoprecipitation reimmunoprecipitation analysis.

Since CYP3A4 plays an important role in the metabolism of many drugs, it is clinically important. Although CYP3A4 metabolism varies according to the time of dosing, the mechanism of this variation remains unclear. Rhythmic fluctuations with a period of about 24 h are demonstrated for both metabolic activity and mRNA levels of CYP3A4 in serum-shocked HepG2 cells. DBP, a circadian transcriptional factor, activates the transcription of the CYP3A4 by binding to the DNA sequence upstream of the transcriptional initiation site. The transactivation of the CYP3A4 by DBP is suppressed by E4 promoter-binding protein-4 (E4BP4), a negative component of the circadian clock.

CYP2D6 is one of the major drug-metabolizing enzymes in humans and shows a circadian rhythm. Differentiated embryo chondrocyte-2 (DEC2) participates in the circadian rhythm of hepatic metabolism by controlling the expression of CYP2D6. DEC2, also known as bHLHE41 or Sharp1, is a pleiotropic transcription repressor. DEC2 controls the expression of genes involved in cellular differentiation process, hypoxia response, apoptosis process, and circadian rhythm regulation. DEC2 interacts with CCAAT/enhancer-binding protein (C/EBPa) associated with the formation of a complex with histone deacetylase-1, which represses the transcriptional activity of C/EBPa and increases the expression of CYP2D6. The rhythm in DEC2 expression, which is nearly opposite to that in the CYP2D6 mRNA expression, was observed in 50% serum-treated HepG2 cells. The amplitude of CYP2D6 mRNA rhythm decreases after the transfection of cells with small interfering RNA against DEC2.

The expression of genes involved in xenobiotic detoxification is controlled by the circadian clock. The aryl hydrocarbon receptor (AhR) is a transcription factor contributing to the activation of detoxification enzymes in response to xenobiotic toxins, and AhR expression is controlled by molecular circadian clock. The expression of AhR and its DNA-binding abil-
ity in the lungs of mice exhibit rhythmic oscillations. Clock mutant mice fail to exhibit rhythmic oscillations in the expression of AhR. The mRNA levels of AhR in the lungs of Clock mutant mice are lower as compared with those in wild-type mice. CLOCK protein affects the toxin-induced expression of detoxification enzymes by modulating AhR activity.

Xanthine oxidase (XOD) is a xanthine dehydrogenase, a rate-limiting enzyme in purine nucleotide degradation, which produces uric acid. A circadian rhythm of uric acid concentrations in the blood and liver has been demonstrated in both humans and rodents. Circadian oscillations in the expression of XOD and its enzymatic activity are observed in the mouse liver. XOD expression is transcriptionally activated by the orphan nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) and suppressed by bile acids. The synthesis of bile acids is regulated by the circadian clock. PPARα-mediated transcription by bile acids causes a rhythm in the hepatic expression of XOD, which leads to circadian rhythm in uric acid production.

P-glycoprotein (P-gp) is an ATP-binding cassette transporter encoded by ABCB1 in humans. Abcb1a and Abcb1b, two members of P-gp, in rodents have the same functional role as P-gp in humans. P-gp is a xenobiotic transporter that contributes to the intestinal barrier. Circadian rhythm under the molecular components of the circadian clock are demonstrated for the expression of Abch1a and efflux pump function of P-gp. The HLF and E4BP4 play an important role in regulating the transcription of Abch1a by competing with each other for the same DNA-binding site using a luciferase reporter assay and gel mobility shift assay. The transactivation of mdr1a by HLF is periodically repressed by E4BP4. Although the circadian rhythm in intestinal accumulation of digoxin as a result of the efflux pump function of P-gp is observed in wild-type mice, the efflux pump function of P-gp is decreased in Clock mutant mice.

The intestinal absorption of digested proteins, small peptides composed of two or three amino acids, is mediated only by the proton-coupled peptide transporter-1 (PepT1) encoded by SLC15A1. In mice, the intestinal expression of PepT1/Slc15a1 oscillates during the daily eating cycle. The increase of bile acids in intestinal epithelial cells after food intake interferes with the recruitment of the co-transcriptional activator cAMP response element binding protein (CREB)/p300 on the promoter region of Slc15a1, thereby repressing the transactivation of Slc15a1 induced by PPARα. The circadian repression of PPARα-mediated transactivation by bile acids in the intestine causes circadian changes in PepT1/Slc15a1 and small peptide absorption during the daily eating cycle.

Slc22a4 encoding organic cation transporter novel type-1 (OCTN1) is identified as a PPARα-regulated gene using microarray analysis. The circadian repression of PPARα-mediated transactivation by bile acids in the intestine induces circadian changes in OCTN1/Slc22a4 expression during the
daily eating cycle. The circadian expression of OCTN1 induces dosing-time-dependence in the absorption of gabapentin and pregabalin from intestine, and their analgesic effects on neuropathic pain hypersensitivity.

Several diverse cell-surface proteins are anchored to the cytoskeleton via scaffold proteins, which are implicated in the regulation of membrane expression of various cell-surface proteins. Na+/H+ exchanger regulatory factor-1 (NHERF1) is a scaffold protein encoded by Slc9a3r1. PER2 modulates the transcription of mouse Slc9a3r1 and the circadian accumulation of NHERF1 in the mouse liver. Namely, PER2 binds to the p65 protein and prevents transcriptional activation of Slc9a3r1 by p65/p50. Circadian accumulation of NHERF1 protein stabilizes the plasmalemmal localization of fatty acid transport protein-5 (FATP5), thereby enhancing hepatic fatty acid uptake at certain times of the day. Since NHERF1 interacts with many diverse cell-surface proteins, it may also contribute to the circadian control of plasmalemmal localization of other plasma membrane proteins and their functions in hepatic cells.

The biological function of skin exhibits a circadian rhythm from the viewpoint of skin permeability related to the transport of water and glycerol. Aquaporin 3 (AQP3), located in the basal layer of the epidermis, controls the biological functions of the skin including optimum water content and trans-epidermal water loss. The expression of mAQP3 also shows circadian rhythm in HaCaT cells after synchronization with 50% serum shock. Oscillation is controlled by the CLOCK/BMAL1 heterodimer. Although the expression of mouse Aqp3 shows circadian rhythm in the epidermis, the rhythm is dampened in Clock-mutated mice. The hydration of the stratum corneum is decreased in Clock-mutated mice. The transcription of mAqp3 is activated by DBP. Although the rhythmic expression between mAqp3 and hAQP3 shows different molecular mechanisms, the circadian rhythm of skin hydration is controlled by clock genes.

4. CHRONOPHARMACODYNAMICS

Chronesthesia describes rhythmic change in the susceptibility or sensitivity of a biological target including receptors, membrane permeability, cells and organ systems. Biological rhythms can cause dosing-time-dependent differences in the PD of medications that are unrelated to their PK. This phenomenon, termed chronesthesia, is explained by circadian oscillations in receptor number, secondary messengers, and metabolic pathways, and so on.

The dopamine D3 receptor (DRD3) in the ventral striatum, associated with motivation and motor functions, is regulated by the circadian molecular clock (Fig. 3). The transcription of DRD3 by retinoic acid-related orphan receptor α (RORα) is repressed by the orphan receptor REV-ERBa. The expression of DRD3 oscillation in serum- or dexamethasone-treated cultured cells is abrogated by the downregulation or overexpression of REV-ERBa. Locomotor hypoactivity by an agonist of DRD3, 7-hydroxy-N,N-dipropyl-2-aminotetralin (7-OH-DPAT), is increased when DRD3 is enhanced. The molecular interaction between the circadian clock and the functional activity of DRD3 modulates the pharmacological actions of DRD3 agonists and antagonists.

5-HTT, a serotonin transporter, plays an important role in the regulation of 5-HT neuronal activity via the reuptake of extracellular 5-HT from the synaptic cleft. Circadian rhythm is demonstrated by the mRNA levels of 5-HTT and its uptake in the mouse midbrain. The activating transcription factor-4 (ATF4), a member of the ATF/cAMP response element (CRE)-binding protein family, contributes to circadian oscillations of CRE-mediated gene expression under the regulation of the molecular clock. ATF4 activates 5-HTT transcription by rhythmically binding to the CRE site in the 5-HTT promoter. The time-dependent binding of ATF4 to the CRE site in the 5-HTT promoter is disrupted by a mutation in the Clock gene. The anti-immobility effect of fluvoxamine in the forced swimming test depends on the rhythmic changes in serotonin transporter
mRNA expression and uptake activity in the midbrain.

Neuropathic pain, hypersensitivity to normally innocuous stimuli, is a chronic condition that often occurs after peripheral nerve injury. Neuropathic pain hypersensitivity in sciatic nerve-injured mice shows a circadian rhythm associated with the rhythmic secretion of glucocorticoids from the adrenal glands. Since the adrenal secretion of glucocorticoids is controlled by clock gene products, the circadian system also influences pain hypersensitivity induced by peripheral nerve injury. Rhythmic exacerbation of pain hypersensitivity is mediated by glucocorticoid-induced increase of the extracellular release of ATP in the spinal cord, which stimulates purinergic receptors on microglia in the dorsal horn. Serum- and glucocorticoid-inducible kinase-1 (SGK-1) plays an important role on the glucocorticoid-enhanced release of ATP from astrocytes. SGK-1 expression levels in spinal astrocytes are enhanced depending on glucocorticoid stimuli and increase ATP release by opening pannexin-1 hemichannels.

The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and serves as a regulator of cell growth and survival. The circadian expression of mTOR is demonstrated in renal adenocarcinoma cells implanted in mice. Consequently, the amount of phosphorylated mTOR also follows a circadian rhythm. Fbxw7 acts as an E3 ubiquitin ligase that targets mTOR. The expression of Fbxw7 at mRNA and protein levels in renal adenocarcinoma cells are under the control of the molecular circadian clock. The circadian rhythm of Fbxw7 affects the stability of mTOR through its degradation. The survival of tumor-bearing mice is improved by the injection of everolimus, mTOR inhibitor, during the day when mTOR increases.

Since angiogenesis plays a key role in tumor growth and metastasis, hypoxia-induced expression of vascular endothelial growth factor (VEGF) is essential for tumor-induced angiogenesis. The expression of VEGF mRNA in tumor cells implanted in mice increases substantially in response to hypoxia, showing a circadian rhythm. The activity of VEGF promoter induced by hypoxia is suppressed by PER2 and CRY1 in the luciferase reporter gene analysis. The administration of antiangiogenic agents at the increased time of VEGF production enhances the antitumor effect of antiangiogenic agents.

Methionine aminopeptidase 2 (MetAP2) contributes to the growth of endothelial cells during tumor angiogenesis. MetAPs show a rhythmic oscillation in implanted tumor cells. The transactivation of the MetAP2 promoter induced by the CLOCK:BMAL1 heterodimer is suppressed by PER2 or CRY1. The transcription of clock genes within clock feedback loops triggers the circadian rhythm of MetAP2 activity. The antitumor efficacy of the MetAP2 inhibitor is increased by dosing the drugs at the time when MetAP2 activity increases.

The sensitivity of cancer cells to antitumor drugs changes depending on the rhythmic oscillation of the p53 expression in tumor cells. The circadian accumulation of p53 is regulated by ATF4. ATF4 induces the rhythmic oscillation of p19ARF (Cdkn2a) in murine fibroblast tumor cells. Oscillation of
p19ARF expression coincides with MDM2 (a specific ubiquitin ligase of p53) expression in a time-dependent manner, resulting in rhythmic prevention of its degradation by MDM2. The circadian accumulation of p53 depends on the time of the p19ARF–MDM2 interaction. The ability of nutlin-3, p53 degradation inhibitor, to kill murine fibroblast tumor cells is increased by the time-dependent accumulation of p53. This circadian accumulation of p53 under the control of ATF4 contributes to the time-dependent difference in the sensitivity of cancer cells to antitumor agents.

5. CHRONO-THERAPEUTIC DRUG MONITORING (TDM)

Circadian-stage-dependent differences in the PK of drugs have been demonstrated for several drugs. In the case of antiepileptic drugs, valproic acid shows a circadian-stage-dependent PK as a function of the time of administration. The influence of dosing time on the predictive accuracy of valproic acid concentrations in plasma by using the Bayesian approach has been investigated for an oral tablet form of valproic acid using a dose of twice daily. The predictive accuracy varies depending on the time of the day at which valproic acid is administered. In the case of antiasthmatic drugs, the accuracy of predicting theophylline concentration in the serum by using the Bayesian approach has been investigated for an oral slow-release form of theophylline using a dose of twice daily. The predictive accuracy varies depending on the time of the day at which theophylline is administered. For both drugs, the predictive accuracy of drug concentration is improved when time-of-day matching PK parameters are used. Intraindividual variability as well as interindividual variability should be considered in dose adjustment.

6. CHRONO-DDS

The principal objective of chronopharmaceutics is to introduce the drug at high concentrations at the time of the highest demand for maximum therapeutic efficiency and at low concentrations when the demand is lower to minimize side effects. Chronopharmaceutics presents contemporary challenges to DDS. The traditional goal of pharmaceuticals aiming at controlling over-thecounter (COER-verapamil), Cardizem LA (diltiazem), and InnoPran XL (propranolol), Zocor (simvastatin) and Pepcid (famotidine). Most data have been compiled from the U.S. Food and Drug Administration (FDA) electronic orange book, specific product package inserts, United States patents, and specific pharmaceutical company websites. There are several technologies in chronopharmaceutics such as physico-chemical modifications of the active pharmaceutical ingredient, chronomodulating infusions pumps, three-dimensional printing, controlled-release erodible polymer, and controlled-release microchip strategies. Future challenge in chronopharmaceutics may be achieved at the interface of other emerging disciplines including nanomedicine and systems biology. The safer and more efficient disease therapies in the future might be performed by novel and more biological approaches to drug delivery.

The rate of cell proliferation correlates with the abundance of cell-surface expression of transferrin receptor 1 (TTR1) controlling the uptake of iron-bound transferrin. Since the expression of TTR1 is higher in cancer cells than in normal cells, the expression of TTR1 offers a target for cancer therapy. Transferrin N-glutaryl-dioleoyl-phosphatidylethanolamine (Tf-NGPE) liposomes are designed as intracellular targeting carriers of chemotherapeutic drugs into tumors. The intratumoral delivery of a Pt-based anticancer drug, oxaliplatin, using Tf-NGPE liposomes changes according to the oscillation of transferrin receptors on tumor cells in colon 26 tumor-bearing mice. Furthermore, the time-dependent dosing of Pt incorporation into tumor DNA is parallel to that of the antitumor effect after the intravenous injection of Tf-NGPE liposomes encapsulating oxaliplatin. The intratumoral delivery of antitumor drugs using Tf-NGPE liposomes is increased by injection of the drug when the tumor expression of TTR1 is high.

Iron is an important biological catalyst that is critical for DNA synthesis during cell proliferation. The circadian expression of iron regulatory protein 2 (IRP2) is demonstrated in colon-26 tumors implanted in mice. IRP2 controls the expression of Tfr1 mRNA at the post-transcriptional level by binding to RNA stem-loop structures as iron-response elements. BMAL1 and CLOCK heterodimers regulate Irp2 mRNA transcription. These pieces of evidence show that the molecular clock plays an important role in tumor cell proliferation by regulating iron metabolism.

7. CHRONOTHERAPEUTIC STRATEGY

The effectiveness and toxicity of several drugs change associated with the time of dosing depending on the 24 h rhythm of biochemical, physiological, and behavioral processes controlled by molecular clock. A scheme of chronotherapeutic strategies are shown from the viewpoint of monitor rhythm, overcome rhythm disruption, manipulate rhythm, develop chrono-drug delivery systems, and discover chrono-drugs (Fig. 5). To identify a rhythmic marker for selecting the time of administration will lead to further progression and spread of chronopharmacotherapy. To choose the most appropriate time of the day for the administration of drugs by monitoring rhythmic markers such as clock genes might lead to increase their therapeutic effects and/or reduce their side effects. The 24 h rhythm of biochemical, physiological, and behavioral processes are disrupted by several drugs. The disruption of rhythm may sometimes lead to disrupted homeostatic regulation and then illness. Changes in the biological rhythm should be closely monitored and considered an adverse effect when they lead to the disrupted control of the circadian system. Furthermore, the manipulation of rhythmic drug administration and feeding schedule appears to produce new rhythmicity and then lead to a new concept of chronopharmacotherapy.
Chrono-drug discovery and chrono-DDS can help provide insights into chronotherapy as a way to optimize current therapies and develop new therapeutic strategies for several diseases.

8. RHYTHM MONITORING

Clock genes play an important role in the molecular clock of the SCN and peripheral tissues including the liver, kidney, and intestines so on. Both nervous and humoral signals contribute to the communication between the SCN and peripheral tissues. Since body clock information can be exploited to maximize potency and minimize toxicity during drug administration, the evaluation of internal body clock by a few-time-point assay has been a challenge in medicine. A convenient, reliable, and less invasive method was developed for the detection of clock gene expression using biopsy samples of hair follicle cells in human. The circadian phase of individual behavioral rhythms accurately reflects clock gene expression in hair follicle cells. A method to detect individual body clock under various conditions is developed by measuring the body clock of the day by profiling the expression patterns of substances in the molecular timetable based on circadian blood metabolomics. Furthermore, a molecular timetable based on circadian-oscillating substances in multiple mouse organs or blood is designed to estimate the body clock from samples collected at only a few time points. This molecular timetable is applied to evaluate and estimate body clock in humans. The metabolite-timetable method accurately determines the internal body clock and will lead to the development of chronotherapy and personalized medicine.

9. DISRUPTION AND MAINTENANCE OF BIOLOGICAL RHYTHMS

The disruption of circadian rhythms cause the alteration of sleep-wake cycle, hormonal secretions and then various physiological and psychiatric disorders. It can also be a side effect of several drugs. To evaluate the alteration of the circadian time structure, evidenced by delayed, advanced, or disrupted sleep-wake circadian rhythm, plays an important role in detecting a new side effect of medications that might manifest only as a result of a particular administration schedule.

The relationships between rest-activity circadian rhythm parameters, health-related QOL, and survival have been investigated in chemotherapy-naive patients with metastatic colorectal cancer. Several health-related QOL parameters are correlated with the main rest-activity circadian rhythm parameters. The QOL and survival of patients with cancer are improved by the interventions that normalize circadian timing system dysfunction.

Although interferons (IFNs) have been well known as antiviral and antitumor agents, they induce adverse neuropsychiatric effects including depression, neurosis, and suicide. The administration of IFNs during the early active phase in diurnally active humans alters circadian rhythmicity of the cortisol level and lymphocyte count. IFN-α disrupts the rhythm of Per mRNA expression in the SCN. It is supported by the suppressive effect of IFN-α on the mRNA expression of Clock and Bmal1. The circadian rhythmicity of locomotor activity and body temperature are also severely blunted by the repetitive dose of IFN-α. IFN-α acts on the SCN, as evidenced by Interferon-stimulated gene factor (ISGF) expression and the SCN-controlled rhythm in the peripheral tissue. The photic induction of Per1 in SCN, a functional disorder in the resetting and entrainment of SCN, is also completely blunted by the repetitive dose of IFN-α in the early active phase.

Interestingly, a suppressive effect on the mRNA expression of each clock gene in the SCN has been demonstrated by the repetitive dose of IFN-α during the early active phase, but not the early rest phase. Similar time-dependent suppression of Per1 mRNA expression has been observed during the repetitive dose of IFN-γ, which can be caused by IFN-α or IFN-β in

Fig. 5. Schematic Diagram of Chronotherapeutic Strategies Based on the Monitoring of Rhythm, Overcoming Rhythm Disruption, Manipulation of Rhythm, Chrono-Drug Delivery System and Chrono-Drug Discovery from the Viewpoints of Molecular Clock.

The chrono-DDS and chrono-drug discovery can help develop new therapeutic strategies for several diseases and provide insights into chronotherapy as a way to optimize current therapies.
combination with other cytokines. IFN-γ receptor in SCN shows a circadian expression with a peak at the early active phase. This might be why IFN-α dose during the early rest phase can decrease its side effects. The observations of the disrupted expression of clock gene caused by IFN-α dose during the early active phase in nocturnally active rodents correspond well to those of the alteration of circadian rhythm caused by IFN-α dose during the early active phase in diurnally active humans.

10. ADJUSTMENT AND MANIPULATION OF BIOLOGICAL RHYTHMS

The 24h rhythm of physiology and behavior is influenced by various environmental factors, such as eating schedules, genetic factors, and social interactions, as well as several triggering conditions and several drugs. The manipulation of the eating schedule can modify the rhythm of the molecular clock, the chronopharmacological action, and chronopharmacokinetics of a drug.

Circadian synchronization of cell proliferation is demonstrated in malignant solid tumors as well as in normal healthy tissues. However, DNA synthesis of normal bone marrow cells show a circadian rhythm that is different from that of tumor cells implanted in mice. The inhibition of platelet-derived growth factor signaling alters the circadian rhythm of tumor cell proliferation. Continuous administration of platelet-derived growth factor receptor tyrosine kinase inhibitor substantially represses DNA synthesis in implanted tumor cells, but not in healthy bone marrow cells. The rhythm of DNA synthesis in tumor cells is synchronized with that in bone marrow cells during the administration of platelet-derived growth factor receptor tyrosine kinase inhibitors. The manipulation of the conditions of organs using rhythmic administration of altered eating schedules or several drugs lead to produce new rhythmicity and one of the new concepts in chronopharmacotherapy.

11. CHRONO-DRUG DISCOVERY AND DEVELOPMENT

Recent trends have led to the development of biopharmaceuticals and genomic drug discovery. Antibodies and nucleic acids have been used in clinical practice. Antibodies have expanded the biopharmaceutical market, and nucleic acid therapy is urgently needed. The genome has recently become the mainstream source of drug discovery and development. Gene analysis was used for exploratory research into the causes of disease. Drug discovery is performed by clarifying targets based on disease-specific molecule information obtained from patients. Clock genes are expressed in other brain regions, peripheral tissues as well as SCN. Such an organization of clock genes may play an important role to the systematic circadian integration of biological rhythms in the whole body. An example of molecular targets under circadian transcriptional factors has been shown for metabolic enzymes and transporters of the drugs described above. The rhythm of molecular targets is demonstrated not only for transcription, but also for the post-transcriptional modifications, protein degradation, and genome editing, which play an important role to the rhythm of molecular targets. Other examples of chrono-drug discovery are shown below.

The circadian rhythm in the physiological functions affecting drug disposition underlies the circadian stage-dependence of drug PK in laboratory rodents. However, it is difficult to predict circadian changes in drug PK in diurnal humans by the use of sampling data from nocturnal rodents. Cynomolgus monkeys, diurnal active animals, are evaluated for the relevance of the intestinal P-glycoprotein (P-gp) expression to the PK of the time-dependent administration of its substrates. The circadian expression of clock genes in the SCN of cynomolgus monkeys are similar to those reported in nocturnal rodents. In contrast, the rhythmic pattern of clock genes in the intestinal epithelial cells of monkeys oscillates at opposite phases to that in rodents. A circadian time dependency is observed for the intestinal expression of P-gp in the small intestine of monkeys. Furthermore, the intestinal absorption of quinidine and etoposide (P-gp substrates) is substantially repressed by dosing the drugs at the time-of-day when P-gp expression increases. On the other hand, no significant time-dependence is observed in the absorption of acetaminophen (non-P-gp substrates). The chronopharmacokinetics of P-gp substrates seem to be influenced by the rhythmic expression of P-gp in intestine. To identify the circadian factors affecting drug disposition in laboratory monkeys is very helpful to improve the probability of success in the predictive accuracy of chronopharmacokinetics in humans.

Although malignant phenotypes of triple-negative breast cancer show a circadian rhythm, the role of cancer stem cells has not been understood in defining this change. High aldehyde dehydrogenase (ALDH) activity, a characteristic of cancer stem cells, is related to the malignancy of cancer cells and is used in order to identify and isolate cancer stem cells. Circadian alterations are observed in the population of ALDH-positive cells in a mouse 4T1 breast tumor model. The circadian stage-dependent increase and decrease in the expression of Aldh3a1 are generated by changes in the number of ALDH-positive cells. Interestingly, clock genes are rhythmically oscillated in ALDH-negative cells, but not in ALDH-positive cells. The circadian stage-dependent release of Wingless-type mmtv integration site family 10a (WNT10a) from ALDH-negative cells induces the rhythmicity of Aldh3a1 in ALDH-positive cells. Furthermore, the antitumor and antimetastatic effects of the ALDH inhibitor N,N-diethylaminobenzaldehyde are increased by dosing it at the time-of-day when ALDH activity is high in 4T1 tumor cells. These results show a new role of molecular clock within the tumor microenvironment in controlling the circadian dynamics of cancer stem cells and may also lead to the development of novel therapeutic strategies for the treatment of triple-negative breast cancer with ALDH inhibitors.

Chronic kidney disease (CKD) is a global health problem, and novel therapies to treat CKD are urgently needed. CKD is often aggravated with an increased retinol in serum; however, the reason for this has not been clarified yet. The dysfunctions of the circadian system are closely related to the development of various disease, since molecular clocks are time-organizing systems that induce rhythmic changes in various physiological and biochemical processes. The liver is the major organ responsible for retinol metabolism; the expressions of Cyp3a11 and Cyp26a1, encoding key enzymes of retinol metabolism, are decreased in 5/6-nephrectomized (5/6Nx) mice. This decrease is caused by the reduced expression of DBP.
In addition, a raise in the expression of plasma transforming growth factor-β1 (TGF-β1) in 5/6Nx mice leads to the reduced expression of Dbp. As a result, the damage in retinol metabolism and renal malfunction in 5/6Nx mice are improved by the dosing of anti-TGF-β1 antibodies. The raise of serum retinol causes the apoptosis of renal cells in 5/6Nx mice fed a normal diet, whereas renal malfunction is decreased in mice fed a retinol-free diet. Thus, the constitutive decrease of DBP expression causes the alteration of retinol metabolism in hepatic cells under CKD conditions. Therefore, the exacerbation of
Renal rhythmic expression of chemokine (C–C motif) ligand 2 (CCL2) is increased in response to p65 activation in the kidneys of 5/6Nx mice. G0s2 plays an important role for p65-induced transactivation of mCcl2 in 5/6Nx mice. These pathologies in the kidney of 5/6Nx mice are ameliorated by down-regulation of G0s2. The evidence for the feasibility and value of this approach is being accumulated for both scientific and therapeutic purposes. (Color figure can be accessed in the online version.)

The application of high throughput screening approach is performed to search for small molecules capable of pharmacological modulation of the molecular clock. The preparation of compounds, pull-down assay and LC/MS/MS analysis are performed for identification of inflammation-related molecules. The functional analysis leads to the discovery of new functions of inflammation-related molecules such as nuclear acetyl-CoA production and epigenetic control. (Color figure can be accessed in the online version.)
renal malfunction in patients with CKD may be improved by the recovery of the hepatic function, potentially through therapies targeting DBP and retinol. Chrono-drug discovery should focus not only on the kidney-liver axis but also on the kidney-other organs axis since CKD causes many complications. 79)

Renal expression of chemokine (C–C motif) ligand 2 (CCL2) is raised in response to p65 activation in the kidneys of 5/6Nx mice and G0s2 contributes to p65-induced transactivation of mCCL2 in these mice 77) (Fig. 8). These pathologies in the kidneys of 5/6Nx mice are improved by the downregulation of G0s2. Furthermore, a novel small-molecule inhibitor of G0s2 expression is identified via high-throughput chemical screening, and the inhibitor represses renal inflammation in 5/6Nx mice. The high-throughput screening strategy is used to search for small molecules capable of pharmacological modulation of clock genes 77) (Fig. 9). Evidence for the feasibility and value of this strategy is being accumulated from viewpoints of scientific and therapeutic purposes.

12. CONCLUSION

An information of the 24 h rhythm of disease and the evidence of 24 h rhythm of drug pharmacokinetics and pharmacodynamics contributes to the rational pharmacotherapy. Figure 10 shows the chronotherapeutic strategy focusing on the molecular clock system of chronopharmacokinetics, chronopharmacodynamics, and cancer chronopathological factors in the xenobiotic detoxification and transporter, 47–50) the receptor and transporter, 47–50) cell growth, DNA synthesis, apoptosis, angiogenesis, autophagy, and cancer stemness. 47–50) The elucidation of chronopharmacokinetic and chronopharmacodynamic mechanisms from the viewpoint of the clock genes plays an important role to improve the progression and spread of chronotherapy. Chrono-TDM and chrono-DDS may provide insights into chronotherapy as a way to optimize current therapies and aid in the development of new therapeutic approach for several diseases. Clock genes should also be an important candidate for chrono-drug discovery.

The clock genes ultimately regulate a vast array of circadian system. They are closely related to sleep disorders, metabolic syndromes, and cancer so on. The chronotherapeutic strategies based on rhythm monitoring, overcoming rhythm disruption, rhythm manipulation, chrono-drug delivery system, and chrono-drug discovery from the viewpoint of the clock genes contribute to improve the progress and diffusion of chronopharmacotherapy. The chrono-drug discovery can provide insights into chronotherapy as a way to optimize current therapies and help develop new therapeutic strategies for several diseases. Clock genes can also be one of important candidates. To stabilize circadian phase and enhance circadian amplitude, thereby consolidating and coordinating circadian organization, which in turn is likely to help prevent and control several diseases in humans, several academic laboratories are screening for small molecules targeting the circadian rhythm monitoring, overcoming rhythm disruption, manipulation of rhythm, chrono-therapeutic drug monitoring, chrono-drug delivery system and chrono-drug discovery. The screening for small molecules targeting the clock genes is now in progress to stabilize circadian phase and enhance circadian amplitude, thereby consolidating and coordinating circadian organization. The academic research along with a combination of chemical and biological information is essential to promote the research and development of new modality drug discovery such as clock genes. (Color figure can be accessed in the online version.)
clock. Further elucidation of the connections between clock genes, and chronopharmacokinetics and chronopharmacodynamics could provide insights into chronotherapy as an approach to optimize current therapies and benefit the development of new therapeutic strategies for several diseases.

Pharmaceutical companies focus on individual research topics reported by researchers, increase their value to match global needs, and contribute to both the creation of pharmaceuticals originated from academia and the acceleration of social implementation. The development of resources to lead drug discovery through activities, as well as support the strengthening of intellectual property strategies, have been the focus. To establish the chrono-drug discovery and development, the essential criteria for new drug discovery and development are to increase the probability of success in clinical trials for proof of concept, which involves the development of a promising strategy for drug target identification and validation to decrease the gap between non-clinical and clinical trials. To achieve this purpose, collaboration among the academia, the basic research institutes, the hospital institutions, the government, and the industry such as pharmaceutical companies, is essential. In addition, to promote the methodology, research, and development of new modality drug discovery represented by antibodies, nucleic acids, and clock genes is important for academic research, along with a combination of chemical and biological information is essential. Overall, clock genes are important candidates for therapy, as demonstrated by the accumulated data.

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Conflict of Interest The author declares no conflict of interest.

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