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Circadian timing of metabolism in animal models and humans

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Abstract. Dibner C, Schibler U (University Hospital of Geneva; and University of Geneva, Geneva, Switzerland). Circadian timing of metabolism in animal models and humans (Review). *J Intern Med* 2015; **277**: 513–527.

Most living beings, including humans, must adapt to rhythmically occurring daily changes in their environment that are generated by the Earth's rotation. In the course of evolution, these organisms have acquired an internal circadian timing system that can anticipate environmental oscillations and thereby govern their rhythmic physiology in a proactive manner. In mammals, the circadian timing system coordinates virtually all physiological processes encompassing vigilance states, metabolism, endocrine functions and cardiovascular activity. Research performed during the past two decades has established that almost every cell in the body possesses its own circadian timekeeper. The resulting clock network is organized in a hierarchical manner. A master pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is synchronized every day to the photoperiod. In turn, the SCN determines the phase of the cellular clocks in peripheral organs through a wide variety of signalling pathways dependent on feeding cycles, body temperature rhythms, oscillating bloodborne signals and, in some organs, inputs of the peripheral nervous system. A major purpose of circadian clocks in peripheral tissues is the temporal orchestration of key metabolic processes, including food processing (metabolism and xenobiotic detoxification). Here, we review some recent findings regarding the molecular and cellular composition of the circadian timing system and discuss its implications for the temporal coordination of metabolism in health and disease. We focus primarily on metabolic disorders such as obesity and type 2 diabetes, although circadian misalignments (shiftwork or 'social jet lag') have also been associated with the aetiology of human malignancies.

Keywords: circadian oscillator, glucose homeostasis, human peripheral clocks, metabolic diseases.

Introduction: Molecular composition of circadian oscillators

Organisms belonging to phyla of all three domains of life, Archaea, Bacteria and Eukaryota, harbour endogenous timekeepers known as circadian clocks. These time-measuring devices enable the organisms to adapt their behaviour and physiology in a proactive manner to daily cycles of light intensity and temperature generated by the Earth's rotation around its own axis. During the past two decades, impressive progress has been made in the molecular and cellular understanding of circadian clocks in several biological systems, including cyanobacteria, fungi, green plants, insects and mammals. Genetic and biochemical approaches have revealed essential clock genes in all of these systems, and the number of these genes is currently increasing. The physicist and Nobel Laureate Richard Feynman stated: 'What I cannot create, I do not understand'. According to this notion, we are close to understanding the clockwork circuitry in only one system, cyanobacteria. By simply mixing the three clock proteins KaiA, KaiB and KaiC with ATP, Takao Kondo and *et al.* succeeded in reassembling a molecular oscillator generating 24-h rhythms of protein phosphorylation/dephosphorylation during extended time periods in the test tube [1]. However, none of the three Kai proteins of cyanobacteria bears any amino acid sequence similarity to the clock proteins of the other above-mentioned biological systems, and it is therefore unlikely that a similar reductionist approach will be successful in the next few years. In fact, even the circadian clocks of different eukaryotic systems (fungi, plants and metazoans) share little if any sequence homology and may thus have been generated independently by convergent evolution. The only commonality between the

known circadian oscillators of fungi, plants and metazoans is the involvement of negative feedback loops in gene expression. Those operative in mammalian cells are shown schematically in Fig. 1.

However, according to an alternative hypothesis, there may be a common basic oscillator relying on metabolic feedback loops. Evidence for such an oscillator driving daily redox cycles of antioxidant peroxiredoxin proteins has recently been provided. Circadian peroxiredoxin oxidation/reduction cycles that do not rely on canonical clock genes have been observed in cyanobacteria, archaea, algae, fungi, plants, roundworms, insects and mammals [2]. Remarkably, these circadian cycles even operate in human and mouse red blood cells, which are devoid of nuclei and ribosomes and thus incapable of transcription and translation (Fig. 2) [3, 4]. It remains to be investigated whether or to what extent the known circadian oscillators are coupled to the metabolic clock, the molecular 'cogwheels' of which have not yet been identified (Fig. 1).

As mentioned above, the canonical molecular oscillator of mammals relies on feedback loops in clock gene expression. These engage a complex array of transcriptional and post-transcriptional mechanisms that have been extensively reviewed elsewhere [5–7]. Therefore, here we will describe these mechanisms only to the degree required in order to understand the following sections. First, it is worth noting that the major purpose of a molecular clock is to generate oscillations in the expression of clock-controlled output genes, which eventually drive overt rhythms in physiology and behaviour. This has been accomplished by metazoan evolution through the selection of complex molecular interactions modulating high-amplitude fluctuations in the activity of some key transcription factors. The heterodimeric transcription factors CLOCK and BMAL1, which bind E-box motifs of hundreds of clock-controlled genes in a highly rhythmic fashion, are the central drivers of the molecular oscillator [8, 9]. These transcriptional activator proteins regulate their cyclic activity and balanced accumulation through two interconnected feedback loops (Fig. 1). In the primary feedback loop, they stimulate the transcription of the genes encoding the four corepressor proteins period 1 (PER1), period 2 (PER2), cryptochrome 1 (CRY1) and cryptochrome 2 (CRY2) through the binding of E-box motifs and the recruitment of various coactivators, including CBP/p300 [10, 11], TRAP150 [12] and SRC-2 [13]. The mRNA and protein levels of CRY and PER increase, and these corepressors assemble into large heterotypic protein complexes that bind to the CLOCK–BMAL1 heterodimers. This results in the removal and inactivation of the CLOCK–BMAL1 activator complexes from the promoters and enhancers of the CRY (*Cry*) and PER (*Per*) genes and thereby in the repression of *Cry* and *Per* transcription. As a consequence, the concentrations of CRY1/2 and PER1/2 decrease until they can no longer inhibit their own expression, and a new daily accumulation cycle of CRY–PER complexes can ensue. In a secondary feedback loop, which involves the nuclear orphan receptors REV-ERBα, REV-ERBβ, RORα, RORβ (only expressed in neurons) and RORγ, BMAL1 and CLOCK control their own temporal expression. Thus, the heterodimer CLOCK–BMAL1 binds to E-box promoter and enhancer elements with the *Rev-erbα* and *Rev-erbβ* genes, and its cyclic activity leads to the circadian expression of REV-ERBα and REV-ERBβ. The REV-ERB repressor proteins compete with ROR-PGC-1α activator/coactivator complexes for the binding to RORE elements within the *Bmal1* and *Clock* genes and win this competition when they reach their highest circadian concentration. Once bound to RORE elements, they recruit the NCoR1–HDAC3 corepressor complex and repress transcription of the *Bmal1* and *Clock* genes [14]. The transcriptional mechanisms operative in the primary and secondary feedback loops are assisted by a large number of post-translational mechanisms, including protein phosphorylation/dephosphorylation, ubiquitination, sumoylation, acetylation/deacetylation and poly ADP-ribosylation. Some of these post-translational protein modifications provide a link between circadian and metabolic cycles. Again, the complex interlocked gene expression circuitries outlined above have evolved for one major purpose, namely the circadian expression of genes leading to overt cycles in physiology and behaviour. As illustrated in Fig. 1, the transcription cycle depending on CLOCK–BMAL1/E-box interactions (i.e. the primary feedback loop) runs in antiphase with the transcription cycle driven by ROR/RORE interactions (i.e. the secondary feedback loop). Therefore, the transcription cycles of output genes controlled by CLOCK–BMAL1 and RORs display phase differences of ~180° (~12 h). Additional phases can be generated by clock output regulators, such as PAR bZIP transcription factors and E4BP4/NFIL3, whose rhythmic expression is governed by the primary and secondary

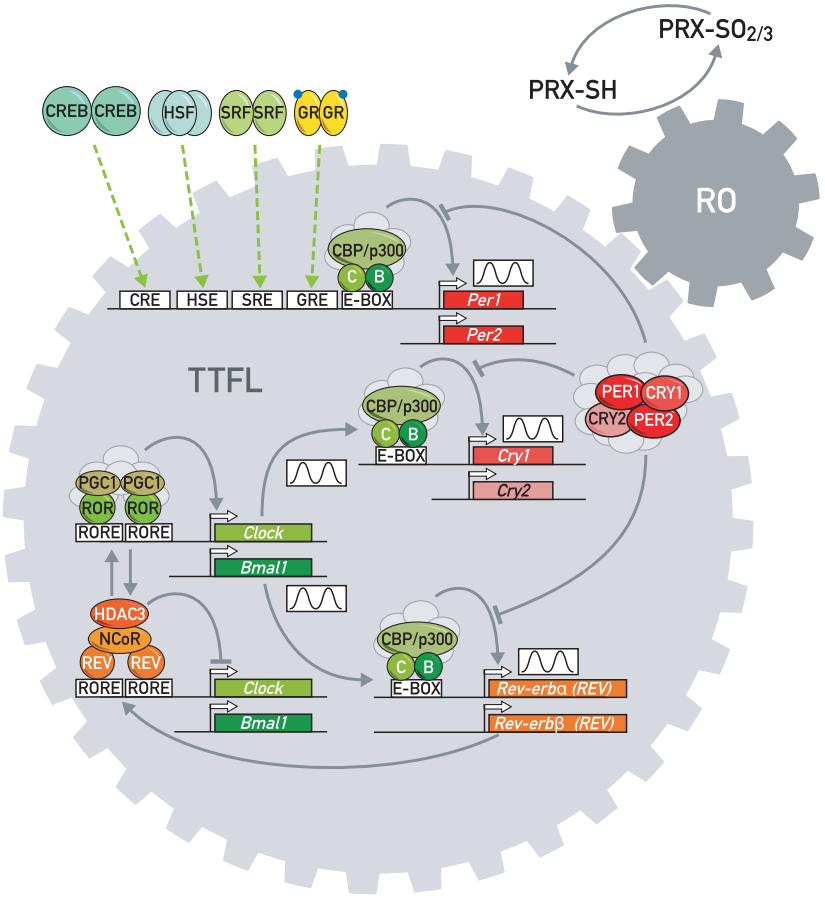

Fig. 1 *Mammalian core clock machinery. In mammals, the molecular oscillator circuitry is composed of two interlocked negative feedback loops of gene expression. This canonical oscillator is known as the transcriptional translational feedback loop (TTFL). The two transcription factors CLOCK (C) and BMAL1 (B) bind to E-box DNA motifs and recruit the coactivator proteins CBP/p300 and a few other polypeptides (shown in light grey). This leads to the activation of the genes* Per1, Per2, Cry1 *and* Cry2 *in the primary feedback loop, and* Rev-erbα *and* Rev-erbβ *in the secondary feedback loop. PER1, PER2, CRY1 and CRY2 assemble into large repressor complexes containing many additional polypeptides. These repressor complexes attenuate the transactivation potential of CLOCK and BMAL1, after having reached a threshold level of activity. As a consequence, the level of PER and CRY repressor complexes diminishes until they no longer inhibit the activity of CLOCK and BMAL1, and a new 24-h PER and CRY production cycle can be initiated. In a second feedback loop, CLOCK and BMAL1 activate and PER/CRY complexes repress the transcription of the genes encoding the two negatively acting nuclear orphan receptors REV-ERBα and REV-ERBβ (REV). Circadian REV expression then results in the cyclic repression of the* Clock *and* Bmal1 *genes. When REV concentrations are low, the positively acting nuclear orphan ROR receptors bind to RORE elements within promoter and enhancer regions of these two genes and activate transcription by PGC1 coactivator complexes. When REV concentrations are high, REVs compete with RORs for the binding to RORE elements and recruit NCoR/HDAC3 corepressor complexes.* Per1 *and* Per2 *also serve as immediate early genes in the synchronization of the molecular clockwork circuitry. Thus, their transcription can be stimulated by immediate early transcription factors whose activity is controlled by systemic cues, such as hormones, second messengers, temperature and neurotransmitters. cAMP responsive element (CRE)-binding protein (CREB), HSF1 binding to heat shock elements (HSEs), serum response factor (SRF) binding to serum response elements (SREs) and glucocorticoid receptor (GR) binding to glucocorticoid responsive elements (GREs) are amongst these immediate early transcription factors. The activation of* Per1 *and* Per2 *expression by these systemically regulated transcription factors plays an important role in the phase resetting of circadian clocks. A redox oscillator of unknown molecular composition drives circadian cycles of peroxiredoxin (PRX) oxidations. The schematic diagram suggests that this redox oscillator is coupled to the TTFL, but this remains to be determined, adapted with changes from [103] (Swiss Med Wkly. 2014 Jul 24;144:w13984. www.smw.ch.). Arrows and bars at the end of the connecting solid and dashed lines indicate activation and repression of transcription, respectively.*

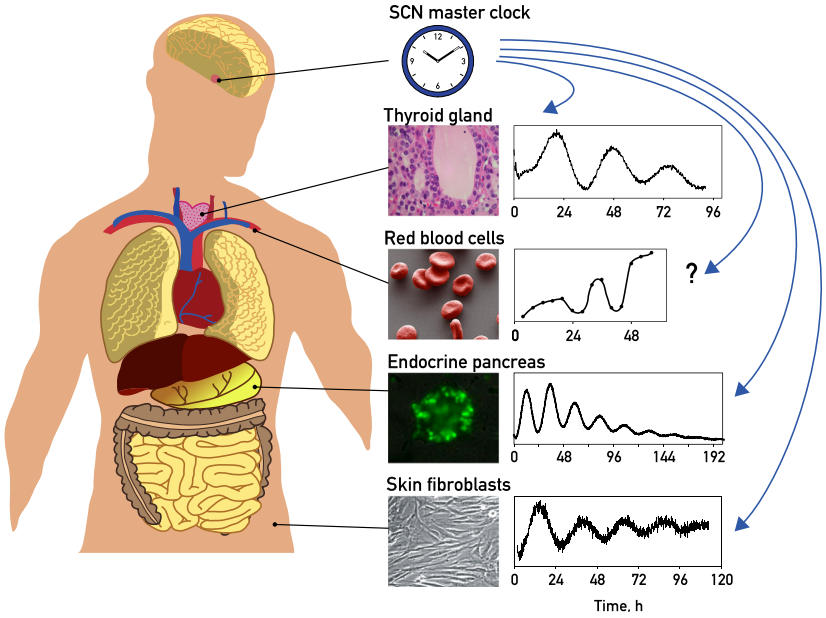

Fig. 2 *Peripheral clocks and redox oscillators in humans. In humans, as in other mammals, virtually all cells harbour cell-autonomous and self-sustained molecular oscillators (Fig. 1). The central clock in the suprachiasmatic nucleus (SCN) synchronizes these peripheral oscillators by a plethora of neural and humoral signals (for details, see ref. [6] and references therein). Circadian gene expression, in the form of luminescence cycles produced by luciferase reporter genes, has been monitored in several human tissues, including the thyroid gland [150], pancreatic islets [20] and skin fibroblasts [145]. Moreover, circadian peroxiredoxin redox oscillations have been described in human erythrocytes, which are devoid of nuclei and ribosomes and thus incapable of transcription and translation [4]. It is not yet clear whether these redox oscillations are also synchronized by the SCN master pacemaker.*

feedback loops, respectively, or by combinations of transcription factors with different phases in activity and/or accumulation [15, 16]. The generation of different phases in circadian gene expression is essential, as different metabolic reactions and other biological processes must be guided to distinct time windows in order to optimize cellular and organismic physiology. Indeed, one of the functions of biological clocks is to temporally isolate essential but chemically incompatible reactions. For example, it makes sense to separate glycogen synthesis and glycogen utilization (phosphorolysis) in time, and the circadian clock helps to coordinate these antagonistic processes (ref. [17] and references therein).

Architecture of the circadian timing system: central and peripheral oscillators

As is clear from the above discussion, the molecular clockwork circuitry functions in a cell-autonomous manner. In mammals, virtually every cell harbours such a self-sustained and cell-autonomous oscillator. Using circadian reporter genes encoding fluorescent proteins or firefly luciferase in conjunction with highly sensitive fluorescence or luminescence microscopy techniques, respectively, rhythmic gene expression can now be easily recorded in real time and in individual cells in tissue culture [18–21]. It has been determined using these approaches that the period lengths for individual cells can vary by several hours. With such large variations, the phase coherence between cells and organs would be expected to degenerate rapidly in the body if the network of cellular circadian oscillators was not periodically synchronized. These phase adjustments are accomplished by rhythmic signals controlled by the suprachiasmatic nucleus (SCN) located in the ventral hypothalamus (Fig. 2). The SCN, also known as the circadian master pacemaker, consists of two tiny clusters of cells, comprising about 100 000 neurons in humans and about 10 000 cells in mice [22]. It is commonly subdivided into ventrolateral and dorsomedian areas, known as the core and the shell, respectively. The SCN is

composed of various cell types that can be characterized by their expression of different neuropeptides, including vasopressin, vasoactive intestinal peptide, gastrin-releasing peptide and neuromedin S [23]. The molecular oscillators of individual SCN neurons, like those of individual fibroblasts, progressively desynchronize when kept in tissue culture. However, in organotypic brain slice cultures, in which the SCN tissue organization is conserved, the cellular oscillators remain synchronized for weeks, due to strong intercellular coupling through synaptic and paracrine mechanisms [23]. This explains why animals housed in the absence of external timing cues (constant darkness and temperature) display strongly rhythmic behaviour throughout their lifespan. Under normal conditions, the SCN is synchronized daily to geophysical time by the photoperiod (24-h light–dark cycle), and also by social cues in humans and some other species [24]. Although how the SCN is affected by social cues is poorly understood, some of the pathways involved in light-dependent phase entrainment of the SCN have been elucidated. Of note, a very small percentage of ganglion cells of the inner retinal layer, known as intrinsically photosensitive ganglion cells (ipRGCs), are both necessary and sufficient for photic phase entrainment of the SCN. These ganglion cells are directly connected to SCN neurons via the retinohypothalamic tract. ipRGCs convey photic signals that are perceived by two completely different but functionally redundant mechanisms to SCN neurons. First, ipRGCs are themselves photosensitive, due to the expression of the nonvisual photoreceptor melanopsin. Secondly, they serve as a relay station between a fraction of classical rod and cone photoreceptors in the outer retinal layer and the SCN. Due to this functional redundancy, melanopsin knockout mice with normal vision or blind mice lacking all classical rod and cone photoreceptors can synchronize the circadian clock to 24-h light–dark cycles. However, following genetic ablation of ipRGCs by the expression of the diphtheria toxin A subunit from the melanopsin gene locus, mice are no longer capable of synchronizing the SCN to daily light–dark cycles. These animals possess normal vision but free-run with an intrinsic period length of 23.8 h, irrespective of whether they are housed in constant darkness or under 24-h light–dark cycles [25]. The photic signals transmitted from ipRGCs to the SCN via the retinohypothalamic tract elicit an increase in Ca^{2+} in postsynaptic neurons. This in turn triggers the activation of various kinases and, as a consequence, of the immediate early transcription factor CREB. The latter binds to promoter and enhancer sequences of *Per* genes and thereby elicits the rapid accumulation of PER1 and PER2. These corepressor proteins attenuate the transactivation potential of CLOCK–BMAL1 and thus delay or advance the phase of circadian gene expression, depending on whether the light has been provided during the first or second half of the night, respectively. During subjective daytime, that is during the resting phase of (nocturnal) laboratory rodents in constant darkness, the phase of the SCN pacemaker is not susceptible to light [26, 27].

The SCN synchronizes the countless cellular clocks in peripheral organs by employing multiple signal transduction pathways depending on feeding cycles, oscillating hormones (e.g. glucocorticoids) and body temperature rhythms (Fig. 2). Feeding rhythms, which are driven by rest–activity cycles, are clearly the dominant *zeitgebers* (time givers) for most peripheral organs [28, 29]. If nocturnal mice or rats are offered food exclusively during the day (i.e. during their resting phase), the phase of circadian gene expression in peripheral tissues progressively changes until it is completely inverted after 1 or 2 weeks. Because the SCN master pacemaker is fairly resilient to feeding rhythms and maintains its original phase, the peripheral clocks become uncoupled from the SCN by inverted feeding rhythms. If food is again provided *ad libitum*, the peripheral oscillators resume their original phase within 2 or 3 days. A possible explanation for the slow phase inversion following daytime feeding and the rapid phase reinversion after food is again freely available is as follows. Under normal conditions (i.e. if food is provided *ad libitum* or exclusively during the nocturnal activity phase), the SCN synchronizes peripheral clocks indirectly via behaviour (i.e. feeding rhythms depending on rest–activity cycles) and more directly via hormones, the cyclic secretion of which remains roughly in phase with the SCN. If conflicting feeding cycles are imposed (i.e. daytime feeding for nocturnal animals), the SCN uses the more direct signals to counteract the indirect but dominant signals that depend on metabolic cycles. As soon as the conflicting feeding rhythms are terminated, the SCN immediately resumes its hierarchical role as a master pacemaker and adjusts the phase of the peripheral oscillators to normal within 2 or 3 days. Because of a recently developed *in vivo* luminescence recording device, the RT-Biolumicorder, circadian gene

expression in peripheral organs can now be monitored in real time in freely moving mice. By determining the phase-shifting kinetics of circadian liver gene expression in intact and SCN-lesioned mice, the synchronization of liver clocks by indirect feeding-dependent signals and more direct SCN-dependent signals could be experimentally confirmed [30]. Glucocorticoids, the daily plasma levels of which oscillate at least 10-fold in humans and laboratory rodents [31, 32], are amongst the SCN-dependent signals involved in the synchronization of peripheral clocks [33, 34]. By contrast, nicotinamide adenine dinucleotide (NAD+)-sensing enzymes such as the poly (ADP-ribose) polymerase 1 (PARP-1) [35] and the protein deacetylase sirtuin (SIRT)1 [36, 37] may serve as regulators for adjusting the phase of peripheral clocks (i.e. hepatocyte clocks) to metabolic cycles. Body temperature rhythms are perhaps the most unexpected *zeitgebers* for peripheral clocks [38–40]. The amplitude of body temperature fluctuations is <1% in mice (minimum 308 °K to maximum 311 °K) and humans (minimum 309 °K to maximum 310 °K). However, simulated murine body temperature cycles imposed on cultured fibroblasts efficiently synchronize their circadian clocks, and even human body temperature cycles phase-entrain the clocks of about 20% of the cultured cells. Heat shock transcription factor 1 (HSF1) [39, 40] and, possibly, the cold-inducible RNA-binding protein CIRP have a role in the synchronization of circadian gene expression by body temperature cycles. Nonetheless, few molecular details are known about the signalling pathways relevant for the synchronization of peripheral clocks *in vivo* (see ref. [41]).

Circadian coordination of metabolism

Interactions between circadian and metabolic cycles

The results of genomewide transcriptome profiling studies and the observation that feeding–fasting cycles are dominant *zeitgebers* for the synchronization of peripheral oscillators indicate that a major purpose of circadian clocks in peripheral tissues is the temporal orchestration of food processing (metabolism and xenobiotic detoxification). This conjecture is further supported by extensive metabolomic and lipidomic studies in laboratory rodents and humans, in which the levels of large numbers of metabolites were found to oscillate in tissues, plasma and saliva [42–50]. Several recent studies have demonstrated a strong relationship between metabolites and the activities of circadian core clock components (for reviews see [51–54]). For example, NADs, indicators of energy metabolism, can affect the activity of clock transcription factors directly or via NAD-dependent enzymes. Rutter *et al.* [55] were the first to provide compelling evidence for an NAD–clock connection by studying the impact of NAD cofactors on the binding of CLOCK/NPAS2–BMAL1 heterodimers to their cognate E-box DNA motifs. In these biochemical experiments, the DNA binding of these transcription factors strongly depended on the ratio of reduced to oxidized NAD [NAD(P)H/NAD(P)+]. The activation of PGC-1α by SIRT1-mediated deacetylation boosts *Bmal1* and *Clock* transcription in the SCN, and this enhances the amplitude of circadian gene transcription in SCN neurons [56] and, perhaps, in peripheral cell types [36]. As SIRT1 levels decrease with ageing, the amplitude of circadian gene expression is also reduced [56]. This may offer a mechanistic explanation for the deterioration of circadian behaviour and physiology in old animals and humans. PARP-1, an NAD-dependent enzyme that adds poly (ADP-ribose) residues onto its substrate proteins, displays robust diurnal activity cycles, and CLOCK was identified as a PARP-1 target protein. The results of genetic loss-of-function studies suggest that PARP-1 participates in the synchronization of liver clocks by feeding cycles [35]. Hepatic NAD+ levels indeed oscillate during the day, and at least in part, this rhythm is driven by the circadian expression of nicotinamide phosphoribosyltransferase (NAMPT) [57]. In fact, NAMPT expression and intracellular NAD+ concentrations follow a circadian rhythm even in cultured fibroblasts maintained under constant conditions, suggesting that the circadian clock drives these oscillations [58]. In liver, NAD+ fluctuations, through rhythmically activating SIRT3, also govern daily rhythms of oxidative phosphorylation in mitochondria [59].

Mutations in the essential clock genes *Bmal1* and *Clock* [60, 61] were found to cause various metabolic disorders. Conversely, perturbations of metabolic pathways in mice fed a high-fat diet dampen the amplitude of circadian oscillations and lengthen their period [62]. Moreover, the expression of circadian output genes dramatically changes in such animals, partly due to inhibition of CLOCK–BMAL1 recruitment to chromatin and activation of PPARγ [63].

The results of genomewide transcriptome profiling studies performed in various tissues suggest that

the clock orchestrates a large diversity of metabolic processes in the body. In rodent models, the presence of peripheral circadian oscillators and their impact on gene expression and organ function have been demonstrated in liver (e.g. ref. [64, 65]), pancreatic islets [61, 66–68], skeletal muscle [69–71] and adipose tissue ([72–74]. A large number of key metabolic functions are subject to daily oscillations. These include carbohydrate and lipid metabolism, and xenobiotic detoxification by the liver, kidney and small intestine [70, 75–77]. Of note, functional liver clocks contribute to glucose homeostasis by driving a daily rhythm of hepatic glucose export [78]. Further evidence for the interconnection between circadian oscillators and metabolism comes from several recent studies of the nuclear receptor and core clock gene *Rev-erbα*, which was found to be crucial for proper lipid and carbohydrate metabolism [76, 79–82]. Of interest, *Rev-erbα* has recently been shown to represent an important link between circadian clock and thermogenic networks, through the regulation of brown adipose tissue function. Low levels (during the early morning hours) or deletion of *Rev-erbα* rendered mice cold resistant, due to *Rev-erbα*-induced repression of uncoupling protein 1 in brown adipose tissue in a cell-autonomous manner [83]. Moreover, nuclear receptors play a critical role in controlling major metabolic networks in the liver, skeletal muscle and adipose tissue, allowing fine-tuning of the metabolic processes to feast and famine cycles [79, 81, 84]. Of note, FGF family proteins represent an important regulatory path through which nuclear receptors exert control on nutritional homeostasis [85–87]. Although most of these findings emphasize the role of peripheral clocks in the temporal orchestration of metabolism, other studies have shown that cyclic systemic signals, directly or indirectly depending on the SCN master clock, also contribute to daily oscillations of metabolic activities [88, 89].

During the past decade, research on circadian gene expression in mammals has largely focused on transcriptome analysis. However, recent proteomic studies have revealed that a large fraction of oscillating liver proteins is encoded by mRNAs, the accumulation of which does not vary throughout the day [90–92]. Moreover, the cyclic expression of numerous proteins persisted in genetically arrhythmic mice maintained on a daily feeding rhythm [90]. These results, in combination with mathematical modelling, highlight the importance of translational and/or post-translational mechanisms in the control of clock output pathways and emphasize the critical role of feeding cycles in these processes. Quantitative mass spectrometric analysis of the liver circadian acetylome revealed the possible roles of lysine acetylation in tuning metabolism in the cytosol and mitochondria [93]. Thus, enzymes involved in gluconeogenesis, glycolysis, citric acid cycle, amino acid metabolism and fatty acid metabolism exhibited strong circadian accumulation patterns according to this study. Aforementioned metabolomic studies conducted during the past few years in tissues directly verified the pervasiveness of diurnal metabolism.

Circadian clockwork perturbations and metabolic defects

Under homeostatic conditions, the clock acts as a driver of metabolic physiology. Perhaps not surprisingly, perturbations of either the circadian or metabolic system (e.g. behaviour misalignment, shiftwork or high-fat diet) can result in the disruption of metabolic pathways and circadian clock function (such as dampening of the amplitude and increasing the period length under free-running conditions [62, 94]). Mice homozygous for dominant negative *Clock* alleles develop hyperphagia, obesity and hyperglycaemia [62]. Similarly, mice with an adipocyte-specific *Bmal1* knockout become obese [95], and those with an islet-specific *Bmal1* ablation develop type 2 diabetes (T2D) [61].

There is robust evidence for a strong reciprocal association between a number of metabolic disorders, including obesity and diabetes, and the circadian clockwork [51, 60, 94, 96, 97]. In an elegant study, Joseph Bass and colleagues demonstrated a direct connection between clockwork function in endocrine pancreatic beta cells and T2D [61]. To investigate the effect of the endocrine pancreatic clock on islet function, the authors assessed circadian expression of the functional islet-specific genes, as well as insulin secretion and glucose-stimulated insulin response at different phases of the circadian cycle. In addition to mRNAs specified by core clock genes, including mRNAs encoding proteins involved in glucose metabolism, insulin signalling and cellular proliferation, islet transcripts were found to exhibit circadian oscillations. The oscillations of these transcripts were attenuated or abolished in the islets isolated from *ClockΔ19* mutant mice, consistent with the attenuation of PER2::luciferase bioluminescence cycles recorded from these islets. *ClockΔ19* mutant mice exhibited hyperglycaemia, significantly elevated

fasting glucose levels and impaired glucose tolerance and glucose-stimulated insulin secretion. Moreover, these conditions deteriorated with age. The decrease in insulin secretion in these mice was most probably due to a defect in insulin exocytosis. In keeping with the transcriptome profiling results, *ClockΔ19* mice had smaller islets with reduced proliferation rates. A similar phenotype was observed in *Bmal1* knockout mice, which also do not possess a functional CLOCK–BMAL1 heterodimer [61]. In line with these findings, a different pancreas-specific *Bmal1* knockout mouse model showed impaired insulin secretion and glucose homeostasis [68]. Of note, the adverse effects of islet-specific *Bmal1* deficiency on glucose levels, impaired glucose tolerance and insulin secretion were more pronounced than in either *ClockΔ19* mutant or global *Bmal1* knockout mice. As suggested by Charles Weitz and colleagues [78] on the basis of observations in mice with a hepatocyte-specific *Bmal1* knockout, the rest–activity and associated fasting–feeding rhythms governed by the SCN must be counterbalanced by metabolic rhythms controlled by clocks in peripheral organs, such as the liver and the endocrine pancreas.

An additional link between circadian clock components and glucose homeostasis has been provided by Zhang *et al.* [98]. They revealed that the core clock proteins CRY1 and CRY2 modulate fasting glucose levels through the inhibition of glucagon-induced gluconeogenesis. This is accomplished by dampening cAMP accumulation and thus CREB activity. Moreover, hepatic overexpression of *Cry1* lowered blood glucose levels and improved insulin sensitivity in the setting of insulin resistance (*db/db* mice), emphasizing the critical role of the clock proteins for glucose homeostasis [98]. Collectively, these findings indicate that obesity and high-fat feeding reciprocally affect the circadian system in rodents, highlighting the tight connection between metabolic and circadian rhythms.

Circadian clock and human metabolic diseases

Synchrony between the internal clock and the environment: circadian misalignment studies

A modern lifestyle, with exposure to artificial light, irregular meal times and short sleep duration, provokes chronic desynchrony between internal clocks and environmental cues; this is known as 'social jet lag'. Numerous epidemiological studies have suggested that such circadian misalignment, in particular prolonged periods of rotating shiftwork, might engender serious health problems including cardiovascular disease, metabolic disorders (see ref. [54] and references therein) and cancer [99]. The connection between malignant transformations and perturbed circadian clock function may be reciprocal. Thus, in a vicious feed-forward loop, circadian rhythms can be disrupted in patients with cancer [100] and tumour growth may be accelerated by circadian disruption [101, 102]. However, because of a number of possible confounding effects, the association between circadian disruption and health deterioration must be interpreted with caution. Indeed, it may be difficult to discriminate between the effects caused by the circadian misalignment *per see* and those elicited by changes in lifestyle generally associated with rotating shiftwork (for review, see ref. [103]).

Circadian clock and the aetiology of obesity and T2D

Insulin is released from pancreatic islets in a strongly circadian manner, and its levels in the blood exhibit a circadian pattern with a nocturnal dip in humans [104]. Of note, the sensitivity of skeletal muscle to insulin is also controlled by the clock [105]. Thus, glucose homeostasis is tightly controlled by the circadian system not only in laboratory rodents but also in humans [106]. Genetic linkage analyses in human subjects have indicated that *hPER2* and *hCRY2* polymorphisms might be associated with blood glucose levels [107, 108]. Polymorphisms in *hCLOCK* were linked to predisposition to obesity whereas two *hBMAL1* haplotypes were associated with T2D and hypertension (for review, see ref. [109]). Although the rs2287161 polymorphism of *hCRY1 per se* does not show a significant impact on glucose homeostasis, in combination with a high dietary carbohydrate intake, it is strongly associated with the development of elevated insulin resistance and diabetes in homozygous subjects [110].

Melatonin is a circulating neurohormone, which is predominantly secreted by the pineal gland during the night. It regulates circadian rhythms by assisting the translation of photoperiodic information in the brain. Melatonin signalling is mainly mediated by two receptors, MT1 and MT2, encoded by the *MTNR1A* and *MTNR1B* genes, respectively. The strongest MT2 expression levels are detected in the retina and in the SCN (for review, see ref. [104, 111, 112]). MT2-mediated melatonin signalling may indirectly regulate glucose level and insulin

secretion through the SCN [113]. As mentioned above, in healthy individuals, insulin secretion follows a circadian rhythm governed by the islets themselves, and the phase of this rhythm might be modulated by melatonin [114]. *MTNR1A* is in general more highly expressed than *MTNR1B* in pancreatic islets. However, the presence of MT2 has been confirmed both in islets and beta cells analysed by fluorescence-activated cell sorting, suggesting a possible role of this less-abundant melatonin receptor isoform in the regulation of insulin secretion [115]. Genomewide association studies in humans have demonstrated a connection between *MTNR1B* variants and hyperglycaemia, impaired early phase insulin secretion and beta cell function [115–117]. Moreover, it has been shown [115–117] that *MTNR1B* haplotypes predict T2D development. Thus, MT2, expressed in human pancreatic beta cells, might represent an important link between the circadian clock and glucose homeostasis and provide new opportunities for T2D diagnostics and possible treatments [118].

Patients with circadian misalignments show profound perturbations in plasma glucose and insulin levels (for review, see ref. [119]). There is accumulating evidence that insufficient sleep, which is widespread in modern society, represents a major risk factor for a number of conditions, in particular metabolic diseases [75, 109, 120]. Due to technical and ethical limitations associated with studies in human subjects, molecular investigations of the influence of circadian clocks on human metabolism have been initiated recently but are still relatively limited. As mentioned above, it has been demonstrated that obesity is tightly and reciprocally linked to circadian oscillator function in rodents [60, 62]. In humans, however, the situation is somewhat more complicated. In a study using biopsies of subcutaneous white adipose tissue harvested at 6-h intervals from lean and obese subjects and patients with T2D (n = 8–11 subjects per group), no major changes in circadian clock gene expression were detected between the groups [121]. The same group later reported a highly significant reduction in the amplitude of circadian melatonin production in a different cohort of patients with T2D [122]. In a study focused on obesity, Markwald *et al.* [123] found that sleep time restriction resulted in an increase in calorie consumption and weight gain, and a phase shift in circadian melatonin production. Yet, more calories are burned during waking hours than during sleep. Functional cell-autonomous clocks were characterized in human pancreatic islets (Fig. 2) and in beta cells synchronized in culture by a dexamethasone pulse and by cyclic changes in temperature [20]. These clocks drive rhythmic islet gene expression [20] and impact on the basal and induced insulin secretion in cultured human islet cells (C. Saini and C. Dibner, unpublished results). The analysis of clock gene expression in human islets suggested that the accumulation of the mRNAs encoding PER2, PER3 and CRY2 is downregulated in patients with T2D in comparison with their healthy counterparts [124].

When interpreting the impact of core clock transcription factors on insulin secretion and diabetes (e.g. [ref. 61]) or blood glucose levels and insulin sensitivity (e.g. ref. [98]), it should be acknowledged that these transcriptional regulatory proteins may implement functions not related to circadian rhythmicity. In other words, when studying organisms with mutations in core clock genes, we must discriminate between clock gene and clock phenotypes. For example, the susceptibility of different clock mutant mice to oncogenic transformations, metabolic disorders or bone defects [125, 126] may have no causal relationship with circadian rhythmicity. *Bmal1* knockout mice exhibit an early ageing phenotype and a number of age-related pathologies [127]. Yet, these phenotypes are not observed in *Clock*Δ*19* mutant mice or *Per1/Per2* and *Cry1/Cry2* double-knockout mice. Furthermore, BMAL1 acts as a negative regulator of the mammalian target of rapamycin complex 1 (mTORC1) pathway [128]. As a consequence, mTORC1 activity is increased in *Bmal1* knockout mice, and this may account for the accelerated ageing observed in these animals. Similarly, it has been reported that REV-ERBα modulates inflammatory responses by macrophages [129] by attenuating the production of inflammatory cytokines such as interleukin 6 [130] and Ccl2 [131, 132]. In this case, the high-amplitude expression of REV-ERBα may account for the strongly circadian gating of inflammatory symptoms, although what the value of this gating might be remains unclear.

Recent studies in rodent, as well as prospective human studies, have provided increasing evidence of the link between sleep disturbances, circadian system disruption and metabolic diseases [133]. Of note, obstructive sleep apnoea

(OSA), which combines sleep fragmentation and hypoxaemia, represents a major risk factor for the development of obesity, insulin resistance and possibly diabetes [133]. The association between OSA and the development of obesity and insulin resistance was observed in adult and paediatric populations [134]. A plausible explanation for the link between OSA, the circadian system and metabolic disturbances could involve changes in circadian patterns of metabolic hormones caused by sleep apnoea. However, in a recent study, no changes in circadian variations of ghrelin, leptin, resistin and adiponectin were revealed in patients with OSA compared to control subjects [135]. OSA is also strongly associated with cardiovascular diseases, including stroke and acute coronary syndromes. For example, plasminogen activator inhibitor-1 (PAI-1), a key circulating prothrombotic factor that inhibits fibrinolysis and exhibits a robust circadian rhythm in human subjects, is elevated in both cardiovascular diseases and OSA and might thus represent an important link between the two conditions. The sharp early morning peak in PAI-1 expression is caused by the circadian system and might explain the morning peak in adverse cardiovascular events [136]. The presence of OSA has been shown to adversely affect circadian fibrinolytic balance, with higher mean PAI-1 activity and lower tissue-type plasminogen activator activity in OSA patients compared with control subjects. This perturbation might account for the increased rate of cardiovascular events in patients with OSA, with alterations of circadian clock function these patients being responsible [137]. These findings suggest an important relation between OSA, the circadian system and metabolic disease aetiology. However, whether glycaemic control in patients with T2D can be improved by treating OSA remains controversial [133].

Circadian rhythm in synchronized primary cells and tissue explants of human subjects

The characterization of circadian rhythms in humans is an extremely challenging and costly endeavour, as it requires prolonged subject observation under controlled laboratory conditions. The 'bunker experiments' conducted by Aschoff [138] were the first and perhaps best-known examples of studies in which the free-running clocks of human subjects were investigated. In these studies, volunteers (mostly university students as well as the author) were housed for several weeks under conditions in which they did not receive any external timing cues. The period lengths of body temperature cycles and urine production rhythms were considerably longer than 24 h in 85% of the examined individuals (average: ~25 h).

Subsequent studies on human circadian rhythms have employed additional daytime-dependent parameters, such as saliva and blood levels of melatonin [122], plasma cortisol levels [32] and thoracic skin surface temperature [100, 139]. Moreover, several relatively noninvasive sampling procedures, including repetitive oral mucosal biopsy [140] and hair follicle analysis [141], have been developed. However, none of these methods provides reliable assessments of free-running circadian rhythms.

In view of these limitations, substantial efforts have been directed towards establishing techniques for examining human circadian oscillators in *in vitro* synchronized human primary explants/cells from tissue biopsies. Indeed, the results of a number of studies in cultured fibroblasts suggested that these cellular clocks represent an excellent experimental system to study mammalian circadian oscillator functions [142–144]. Moreover, fibroblasts expressing the luciferase reporter from various circadian promoters constitute an ideal model system for the molecular dissection of basic oscillator properties in living cells with high temporal resolution and precision [6, 19]. Brown and colleagues have demonstrated that cultured primary human skin fibroblasts represent an excellent experimental system for the dissection of human clock properties (Fig. 2); perhaps not surprisingly, circadian clock parameters measured by the continuous recording of circadian bioluminescence cycles produced by human skin fibroblasts varied widely amongst the cells harvested from different donors [145]. Remarkably, circadian oscillator characteristics measured in cultured skin fibroblasts correlate relatively well with rhythmic human behaviour, as demonstrated in human subjects in whom circadian physiology was examined under laboratory conditions [146] or individuals who completed the Munich chronotype questionnaire [147, 148]. The findings of these studies suggested that long and short periods of fibroblast clock gene expression were frequently associated with late ('owl-like') and early ('lark-like') chronotypes, respectively [149]. Accordingly, studies of peripheral organs

are promising for increasing understanding of the entire circadian timing system in humans. In the meantime, robust oscillations in gene expression have also been investigated in human pancreatic islets maintained in organotypic cultures [20], in human primary thyrocytes (Fig. 2, ref. [150]) and in *in vitro* differentiated skeletal myotubes (Laurent Perrin and C. Dibner, unpublished results). An exciting observation regarding the impact of human bloodborne factors on the period length of circadian gene expression in cultured cells was recently reported. Surprisingly, cultured fibroblasts exposed to serum collected from elderly subjects had shorter periods than fibroblasts exposed to serum harvested from young individuals [151]. This suggests that circadian molecular oscillators are more plastic than hitherto anticipated in that they can change their properties according to their environment. The recording of circadian gene expression in cultured cells may even be useful for diagnostic purposes. It will thus be exciting to examine circadian clock properties in cells treated with serum obtained from human subjects with various disorders, such as metabolic diseases, chronic inflammatory conditions and cancer [150].

Conclusions

Based on several observations, the temporal orchestration of metabolism seems to be one of the primary functions of the mammalian circadian timing system. Indeed, most if not all metabolic processes are subject to diurnal oscillations, and conversely, daily feeding–fasting rhythms are the dominant timing cues in the synchronization of circadian clocks in peripheral organs. The disruption of circadian physiology imposed by social constraints on many individuals (chronic 'social jet lag') can have severe consequences on their health. In addition to increasing sleep deficit and irregular meal times, there is a concomitant worldwide rise in the incidence of metabolic disorders and T2D. Both biological and epidemiological studies suggest a direct link between lifestyle and these metabolic disorders. However, the genetic and biochemical associations between human circadian clocks and metabolic disorders are still poorly understood. It is therefore of utmost scientific and clinical importance to further investigate the emerging connection between circadian oscillator function and the aetiology of obesity and T2D. If we succeed in establishing a molecular link between the circadian clock and these metabolic disorders, modulation of the oscillator could provide a potential novel therapeutic option. Here, we have summarized some recent studies performed in laboratory rodents and human subjects that have made significant contributions towards understanding the molecular link between circadian gene expression and metabolic dysfunctions, such as obesity, the metabolic syndrome and T2D. These studies highlight the importance of circadian clock function – or at least circadian 'clock gene' function – in energy homeostasis and metabolism. However, many other clinically relevant physiological processes are driven by the circadian timing system and are therefore daytime dependent. Particularly well-known examples are bronchial asthma with around-the-clock changes in dyspnoea frequency, symptoms of rheumatoid arthritis, oscillations in blood pressure (i.e. a morning rise preceded by night time dipping) (for review, see ref. [152]) and xenobiotic detoxification (for review, see ref. [102]). The enzymes involved in the latter process also metabolize therapeutic drugs and thus are relevant in determining pharmacokinetic parameters. Hence, the tolerability and efficacy of cancer chemotherapeutic agents can vary significantly as a function of delivery time [101, 153, 154]. In the clinic, a significant improvement in tolerability has been shown in randomized trials in which patients with cancer received the same sinusoidal chronotherapy schedule over 24 h, compared to constant-rate infusion or chronotherapy in which the timing was not optimized [100, 155]. Moreover, the quality of life of patients with regard to fatigue and weight loss was significantly improved in those receiving a daytime-adapted drug-delivery regimen [139]. Even though chronotherapeutics is still in its infancy, it has great potential for improving the efficacy of treatments and reducing the burden of unwanted side effects. In the modern era of personalized medicine, knowledge of individual chronotypes may be critical for treatment and may have to be included as an important component in diagnostic procedures. We hope that the number of clinicians willing to take into account this rhythmic physiology when treating patients will dramatically increase in the near future.

Conflict of interest statement

No conflicts of interest to declared.

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