Table 1 Characteristics of participants in study 1

Characteristic	Non-diabetic individuals	Patients with type 2 diabetes
N	6	8
Men (*n*, %)	5 (83)	5 (63)
Age (years)	28±7	60±10**
BMI (kg/m^2)	21.4±2.5	26.3±8.9
Current smoker (*n*, %)	1 (17)	2 (25)
Current drinker (*n*, %)	1 (17)	3 (38)
Diabetes treatment (*n*, %)		
Diet alone	–	5 (63)
Oral agents	–	3 (38)
Insulin	–	0
Fasting glucose (mmol/l)	4.7±0.7	7.8±1.9**
Fasting insulin (pmol/l)	49±14	46±28
HOMA-IR	1.5±0.6	2.3±1.5
HbA_{1c} (%)	4.9±0.4	8.1±1.8**
Aspartate aminotransferase (U/l)	16±2	21±9
Alanine aminotransferase (U/l)	16±6	21±10
Total cholesterol (mmol/l)	4.6±1.1	5.0±1.0
Triacylglycerol (mmol/l)	0.9±0.5	1.1±0.7
HDL-cholesterol (mmol/l)	1.5±0.5	1.2±0.2

Values are *n* or means±SD
**$p<0.01$ vs non-diabetic individuals

Table 2 Characteristics of participants in study 2

Characteristic	Healthy individuals	Patients with type 2 diabetes
N	14	12
Age (years)	59±6	58±6
BMI (kg/m^2)	23.1±2.6	24.8±2.4
Current smoker (*n*, %)	8 (57)	3 (25)
Current drinker (*n*, %)	12 (86)	11 (92)
Diabetes treatment (*n*, %)		
Diet alone	–	5 (42)
Oral agents	–	6 (50)
Oral agents + insulin	–	1 (8)
Fasting glucose (mmol/l)	5.6±0.6	9.6±2.2**
Fasting insulin (pmol/l)	45±27	55±27
HOMA-IR	1.7±1.1	3.4±1.9**
HbA_{1c} (%)	5.0±0.3	7.3±1.0**
Aspartate aminotransferase (U/l)	22±7	25±6
Alanine aminotransferase (U/l)	23±9	31±13
Total cholesterol (mmol/l)	5.4±0.6	5.7±0.7
Triacylglycerol (mmol/l)	1.4±0.8	1.5±0.8
HDL-cholesterol (mmol/l)	1.5±0.3	1.5±0.3

Values are *n* or means±SD
**$p<0.01$ vs healthy individuals

Study 2 The next study was carried out from November 2007 to January 2008. Twenty-six men with ages in their 50s to 60s were recruited from LTA PS Clinic (Fukuoka). Twelve individuals were outpatients with type 2 diabetes, whereas 14 individuals were healthy volunteers. All of the participants kept regular hours for at least 2 weeks until the study day. Most of them usually awoke between 05:00 and 07:00 hours and went to bed between 22:00 and 24:00 hours. One healthy participant consumed four meals a day, another healthy individual did not eat breakfast, and the other participants usually ate three meals a day. As shown in Table 2, seven patients were treated for type 2 diabetes with medications (glimepiride + metformin, $n=4$; glimepiride + an α-glucosidase inhibitor, $n=2$; glibenclamide + human insulin, $n=1$). In addition, five and three patients were on medication to treat hypertension (an angiotensin II receptor antagonist, $n=4$; amlodipine + olmesartan, $n=1$) and hyperlipidaemia (pravastatin, $n=2$; bezafibrate, $n=1$), respectively. Venous blood samples for RNA isolation and blood chemistry were collected between 08:30 and 09:30 hours at the LTA PS Clinic after an overnight fast.

Isolation of leucocytes and purification of RNA Immediately after blood sampling, leucocytes were isolated and stabilised using the LeukoLOCK Fractionation and Stabilization Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 10 ml EDTA-anticoagulated blood was passed through a LeukoLOCK filter that captured only leucocytes, and the filter was flushed with PBS to remove residual erythrocytes. The filter was then filled with RNAlater to stabilise leucocyte RNA. The stabilised cells were stored on the filter at −20°C until RNA extraction. The isolation of total RNA was achieved using the LeukoLOCK Total RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions.

cDNA synthesis and real-time quantitative PCR cDNA was synthesised from 1 μg total RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Gene expression was analysed by real-time quantitative PCR using the Applied Biosystems 7900HT real-time PCR system, as previously described [16, 22]. All specific sets of primers and TaqMan probes (TaqMan gene expression assays) were obtained from Applied Biosystems. To control for variation in the amount of cDNA available for PCR in the different samples, gene expression levels of the target sequences were normalised to the expression of an endogenous control gene (*GAPDH*). The GenBank accession numbers, assay ID, and the target exons were NM_004898.2, Hs00231857_m1, 18-19 for *CLOCK*; NM_001178.4, Hs00154147_m1, 9-10 for *BMAL1*; NM_002616.1, Hs00242988_m1, 22-23 for *PER1*; NM_022817.1, Hs00256143_m1, 8-9 for *PER2*; NM_016831.1, Hs00213466_m1, 15-16 for *PER3*; NM_004075.2, Hs00172734_m1, 2-3 for *CRY1*; NM_001001928.2, Hs00947538_m1, 6-7 for *PPARA*; NM_138711.3, Hs01115512_m1, 4-5 for *PPARG*; NM_006238.3, Hs00602622_m1, 3-4 for *PPARD*; NM_002046.3, Hs99999905_m1, 3-3 for *GAPDH*; and NM_001002.3,

Hs99999902_m1, 3-3 for ribosomal protein, large, P0 (*RPLP0*), respectively. Data were analysed using the comparative threshold cycle method.

Blood chemistry Samples obtained after an overnight fast were assayed for plasma glucose, serum insulin, total cholesterol, HDL-cholesterol, triacylglycerols, aspartate aminotransferase, alanine aminotransferase and HbA_{1c}. Each variable was measured using a commercial kit. Insulin sensitivity was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR) [23].

Statistical analysis Differences in the variables and mRNA levels between patients with diabetes and control individuals were evaluated using the Mann–Whitney test. The rhythmicity of each gene was assessed using the Friedman test. The values are presented as means±SD, and $p<0.05$ was considered significant. All calculations were performed using SPSS version 11 for Windows (SPSS Japan, Tokyo, Japan).

Results

Study 1 Because biological clock function in leucocytes is controversial [24], we first analysed the daily variation in mRNA expression of the clock genes in all 14 individuals. As shown in Fig. 1b–e, the mRNA expression of *BMAL1*, *PER1*, *PER2* and *PER3* exhibited slight but significant 24 h rhythmicity ($\chi^2=12.9$, $p<0.01$ for *BMAL1*; $\chi^2=22.9$, $p<0.001$ for *PER1*; $\chi^2=22.0$, $p<0.001$ for *PER2*; $\chi^2=25.0$, $p<0.001$ for *PER3*; Friedman test to evaluate rhythmicity). Similarly to previous reports [6–8, 21], the levels of *PER1*, *PER2* and *PER3* peaked in the early morning and dropped to a trough level in the evening. On the other hand, the mRNA levels of *CLOCK*, *CRY1* (Fig. 1a,f) and another endogenous control gene (*RPLP0*) remained constant throughout the day ($\chi^2=6.8$, $p=0.08$ for *CLOCK*; $\chi^2=4.5$, $p=0.21$ for *CRY1*; $\chi^2=3.3$, $p=0.34$ for *RPLP0*; Friedman test). When the mRNA levels of clock genes were normalised to the expression of *RPLP0*, *CRY1* ($\chi^2=11.4$, $p<0.01$) as well as *BMAL1*, *PER1*, *PER2* and *PER3* ($\chi^2=13.0$–25.1, each $p<0.01$) showed significant 24 h rhythms with a peak in the morning. These results support the idea that the circadian clock functions in leucocytes.

Next, we compared the mRNA expression rhythms of the clock genes in patients with diabetes with those in non-diabetic individuals (Fig. 1a–f). Interestingly, *BMAL1* mRNA peaked in the evening in non-diabetic individuals ($\chi^2=8.0$, $p<0.05$; Friedman test to evaluate rhythmicity), whereas that peaked in the morning in patients with diabetes ($\chi^2=11.9$, $p<0.01$). In addition, the transcript levels of *BMAL1*, *PER1*, *PER2* and *PER3* were significantly lower in patients with diabetes than in non-diabetic individuals at

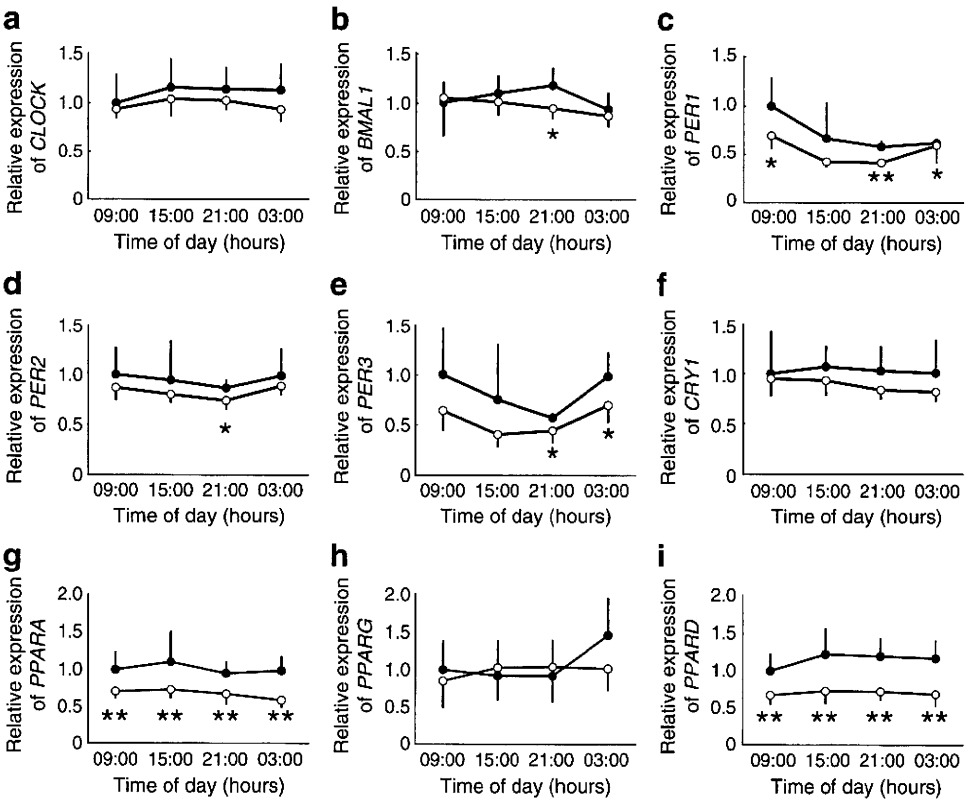

Fig. 1 Daily mRNA expression profiles of clock genes in the peripheral leucocytes of patients with diabetes and non-diabetic individuals. **a** *CLOCK*; **b** *BMAL1*; **c** *PER1*; **d** *PER2*; **e** *PER3*; **f** *CRY1*; **g** *PPARA*; **h** *PPARG*; **i** *PPARD*. Peripheral leucocytes were obtained from six non-diabetic individuals (black circles) and eight patients with type 2 diabetes (white circles) at 09:00, 15:00, 21:00 and 03:00 hours. Transcript levels of the clock genes were determined by real-time quantitative PCR. The mean value of non-diabetic individuals at 09:00 hours was set to 1 for each gene. Means±SD. *$p<0.05$, **$p<0.01$ vs non-diabetic individuals

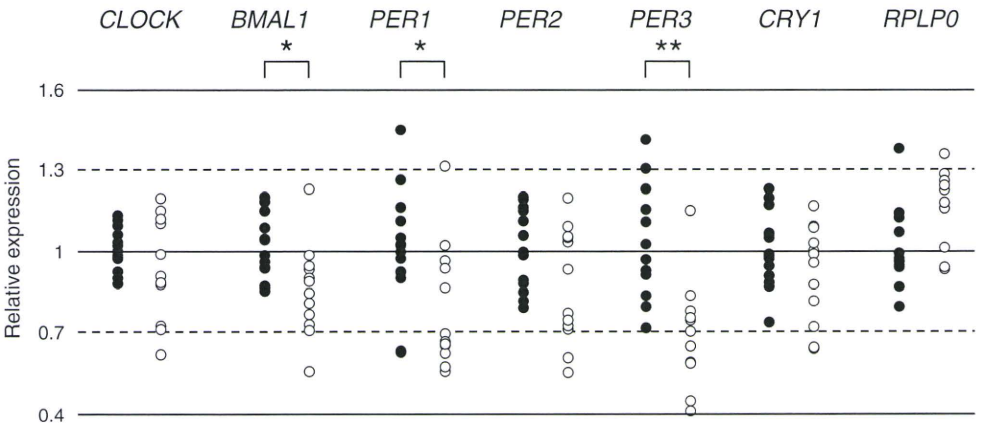

Fig. 2 Transcript levels of the clock genes and *RPLP0* in peripheral leucocytes of patients with diabetes and control individuals. Peripheral leucocytes were obtained from 14 healthy individuals (black circles) and 12 patients with type 2 diabetes (white circles) at 09:00 hours. Transcript levels of the target genes were determined by real-time quantitative PCR. The mean value of healthy individuals was set to 1 for each gene. *$p<0.05$, **$p<0.01$

one or more observation points. A multiple regression analysis identified that both *PER1* and *PER3* mRNA levels at 21:00 hours were correlated with the presence or absence of type 2 diabetes, but not with age (data not shown). Furthermore, the amplitudes of *PER1* and *PER3* were diminished in patients with diabetes (56% and 38% of those in non-diabetic patients; $p=0.06$ and $p<0.01$, respectively). The amplitude of *PER3* determined using *RPLP0* as an internal control also tended to be lower in patients with diabetes (72% of that in non-diabetic patients; $p=0.09$). These results suggest that the function of the circadian clock was impaired in peripheral leucocytes of the patients with diabetes examined in this study.

Because peroxisome proliferator-activated receptors are known to exhibit circadian expression in liver and adipose tissues [14], their daily mRNA expression profiles in the leucocytes were determined as an indicator of the circadian clock function. The mRNA expression levels of *PPARA* and *PPARD* were maintained constant throughout the day (Fig. 1g–i; $\chi^2=5.5$, $p=0.14$ for *PPARA*; $\chi^2=4.7$, $p=0.19$ for *PPARD*), although those of patients with diabetes were significantly lower than those of non-diabetic individuals. On the other hand, *PPARG* mRNA showed a significant 24 h rhythm ($\chi^2=9.6$, $p<0.05$) with a peak in the small hours. This rhythmicity disappeared in patients with diabetes ($\chi^2=5.0$, $p=0.18$) as was expected. This result supports the view that the clock function was diminished in patients with diabetes.

Study 2 The patients with diabetes were older than the non-diabetic individuals in study 1. Because senescence might impair the circadian clock [25], the possibility exists that the age differences in part affected the results. Therefore, we next compared the transcript levels of the clock genes in patients with type 2 diabetes with those from age-matched healthy individuals. Moreover, we recruited only men for this study to exclude a sex effect suggested previously [16, 26]. As shown in Table 2, fasting glucose, HOMA-IR and HbA_{1c} levels were significantly higher in patients with diabetes, but the other variables were similar between the two groups.

Consistent with the findings of study 1, the mRNA levels of *PER1* at 09:00 hours were significantly lower in patients with diabetes than in control individuals (Fig. 2). Moreover, *BMAL1* and *PER3* mRNA levels were also lower in the patients. Conversely, no differences were observed between the two groups in the transcript levels of *CLOCK*, *PER2*, *CRY1* or the endogenous control gene *RPLP0*. Note that *BMAL1*, *PER1* and *PER3* mRNA levels were inversely correlated with HbA_{1c} levels (Spearman's rank correlation coefficient $\rho=-0.47$, $p<0.05$ for *BMAL1*; $\rho=-0.52$, $p<0.01$ for *PER1*; $\rho=-0.55$, $p<0.01$ for *PER3*; Fig. 3). Thus, this study provides evidence that mRNA expression of a subset of clock genes is diminished in patients with type 2 diabetes, especially those with poorly controlled blood glucose.

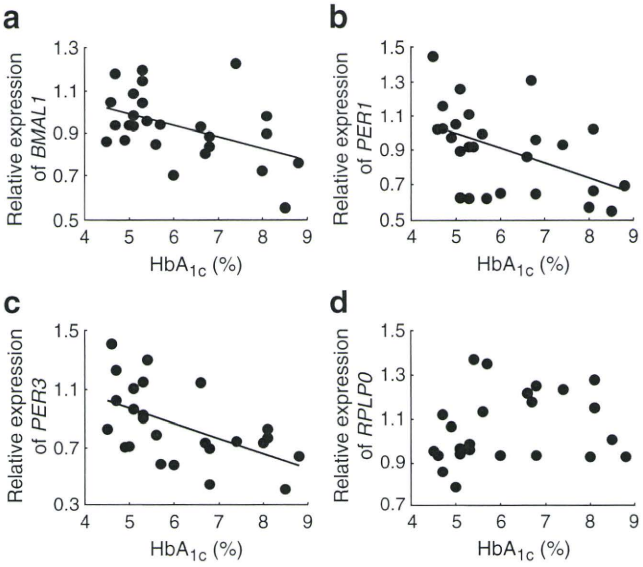

Fig. 3 Relationships between HbA_{1c} levels and mRNA levels of *BMAL1* (**a**), *PER1* (**b**), *PER3* (**c**) and *RPLP0* (**d**)

Discussion

Recent studies have correlated metabolic diseases such as metabolic syndrome and type 2 diabetes with the circadian clock. Our previous study [16] revealed that the rhythmic expression of clock genes was slightly diminished in the peripheral tissues of mildly diabetic KK mice and was greatly blunted in severely diabetic KK-A^y mice. In this study, we demonstrated for the first time that the circadian clock of peripheral leucocytes is diminished in patients with type 2 diabetes, particularly in those with poorly controlled blood glucose.

High glucose has been shown to downregulate *Per1* and *Per2* mRNA expression in cultured fibroblasts [27]. In addition, Kohsaka et al. [26] reported that a high-fat diet affected the rhythmic mRNA expression of *Clock*, *Bmal1* and *Per2* in the liver and adipose tissues of mice. Considering these findings, alterations in glucose, lipid and energy metabolism and/or changes in the concentrations of humoral factors such as plasma glucose appear to influence the peripheral clock.

As shown in Fig. 1, human leucocytes exhibited very weak clock gene oscillations compared with the clocks of the liver and adipose tissues in mice [16]. The peripheral clocks are thought to be synchronised by the SCN through neural and humoral signals [2]. Because peripheral blood leucocytes, unlike the other peripheral tissues, are not controlled directly by neural signals, their oscillators might be easily desynchronised and greatly affected by humoral signals. The observation that the mRNA expression rhythms of *PPARA* and *PPARD* disappeared in leucocytes (Fig. 1g,i) supports this hypothesis. However, *PPARG*, as well as the clock genes, exhibited weak, but significant 24 h rhythmicity, suggesting that the each circadian clock in leucocytes works the same way as those in liver and adipose tissues. Whether each intracellular clock of individual leucocytes is impaired in patients with diabetes remains to be determined; however, attenuation of overall rhythmicity in peripheral leucocytes may cause leucocyte dysfunction. Leucocyte function is known to be depressed in patients with diabetes, which may contribute to their increased susceptibility to infection [28].

That the mutation and genotypes of core clock genes are associated with metabolic diseases [15, 17–19] leads us to speculate that the circadian clock contributes to the development of diabetes. Oishi et al. [29] demonstrated that clock function is preserved to a great extent in the livers, hearts and kidneys of mice with streptozotocin-induced insulinopenic diabetes. We could not confirm impairment of the circadian clock in the liver and adipose tissues of mice fed a high-fat diet, which was reported by Kohsaka et al. [26], even though the mice developed metabolic syndrome characterised by obesity, hyperlipidaemia and hyperglycaemia [30]. Thus, impairment of the circadian clock by type 2 diabetes or metabolic syndrome remains controversial. Further studies are needed to determine whether impaired clock function can be improved by glycaemic control in patients with type 2 diabetes.

Sedentary lifestyles and high dietary fat intake are thought to be instigators of metabolic diseases such as type 2 diabetes and metabolic syndrome. Additionally, it is not uncommon for modern people to keep irregular hours, live rather nocturnal lives or eat late-night snacks. Because light and dietary intake strongly entrain the master and peripheral clocks, respectively [2], these lifestyle features could cause malfunction of peripheral oscillators. Almost all of the individuals enrolled in this study kept regular hours and ordinary lifestyles. Nevertheless, their lives were rather nocturnal compared with the lives of ancient peoples. Therefore, the possibility exists that life in modern society could affect the biological clock, especially in highly susceptible individuals. Although the genotypes of the clock genes were not determined in this study, patients with type 2 diabetes might have defective or fragile circadian clocks.

In summary, rhythmic mRNA expression of clock genes was dampened in peripheral leucocytes of patients with type 2 diabetes. The impairment of the circadian clock appears to be closely associated with the pathophysiology of type 2 diabetes in humans.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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