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RESEARCH PAPER

Timed high-fat diet in the evening affects the hepatic circadian clock and PPARα-mediated lipogenic gene expressions in mice

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Abstract A long-term high-fat diet may result in a fatty liver. However, whether or not high-fat diets affect the hepatic circadian clock is controversial. The objective of this study is to investigate the effects of timed high-fat diet on the hepatic circadian clock and clock-controlled peroxisome proliferator-activated receptor (PPAR) α-mediated lipogenic gene expressions. Mice were orally administered high-fat milk in the evening for 4 weeks. The results showed that some hepatic clock genes, such as Clock, brain-muscle-Arnt-like 1 (Bmal1), Period 2 (Per2), and Cryptochrome 2 (Cry2) exhibited obvious changes in rhythms and/or amplitudes. Alterations in the expression of clock genes, in turn, further altered the circadian rhythm of PPARα expression. Among the PPARα target genes, cholesterol 7α-hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase, low-density lipoprotein receptor, lipoprotein lipase, and diacylglycerol acyltransferase (DGAT) showed marked changes in rhythms and/or amplitudes. In particular, significant changes in the expressions of DGAT and CYP7A1 were observed. The effects of a high-fat diet on the expression of lipogenic genes in the liver were accompanied by increased hepatic cholesterol and triglyceride levels. These results suggest that timed high-fat diets at night could change the hepatic circadian expressions of clock genes Clock, Bmal1, Per2, and Cry2 and subsequently alter the circadian expression of PPARα-mediated lipogenic genes, resulting in hepatic lipid accumulation.

Keywords High-fat diet · Clock genes · Peroxisome proliferator-activated receptor α · Lipid metabolism · Mice

Introduction

The circadian clock is an endogenous oscillator with a period of approximately 24 h, and rhythmicity refers to the ability of organisms to adapt to environmental changes (Panda et al. 2002). While the master circadian clock is located in the hypothalamic suprachiasmatic nucleus similar clock oscillators may be found in some peripheral tissues, such as liver, intestines, and adipose tissues (Froy and Chapnik 2007; Zvonic et al. 2007; Ando et al. 2005). The hepatic clock oscillator consists of a pair of transcriptional activators, Clock and brain-muscle-Arnt-like 1 (Bmal1), and two classes of repressors, Period (Per1, Per2, Per3) and Cryptochrome (Cry1, Cry2) gene families (Schmutz et al. 2012). In general, each clock gene regulates some physiological function by regulating clock-controlled genes in peripheral tissues. For example, nearly half of all known nuclear receptors, including peroxisome proliferator-activated receptor (PPAR) α, exhibit circadian expressions in the liver and adipose tissues (Yang et al. 2006). Thus, disruptions in circadian clock either by mutation in mice or by shiftwork in humans are associated with increased risks for the development of multiple organ diseases (Ha and Park 2005; Kennaway et al. 2007).

Previous studies have indicated that high-fat diets may result in fatty liver formation. However, whether or not high-fat diets affect the hepatic circadian clock is controversial. Kohsaka et al. observed that a constant high-fat diet

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across 24-h light/dark cycles in mice may lead to alterations in the rhythmic expressions of circadian clock genes Clock, Bmal1, and Per2 in the liver (Kohsaka et al. 2007). Hsieh et al. also reported that obesity induced by high-fat diets may alter the circadian clock system and clock-controlled genes (Hsieh et al. 2010). By contrast, Ando et al. demonstrated that the clock function is preserved in the liver of mice with non-alcoholic steatohepatitis induced by a constant high-fat diet across 24-h light/dark cycles (Ando et al. 2009). Recent studies have also reported that high-fat feeding during daylight hours has limited effects on hepatic clock gene expressions (Reznick et al. 2012). However, the dietary habits of animals are not consistent with those of humans. In many countries, dinner is usually arranged in the evening. Few studies have investigated the impact of timed high-fat diet patterns on the hepatic circadian clock and expressions of clock-controlled genes. Some studies have revealed that feeding time may affect the gut molecular clock (Nisembaum et al. 2012; Yamajuku et al. 2009). In this study, we observed the effects of a timed high-fat diet in the evening on the hepatic circadian clock and expressions of the clock-controlled gene PPARα as well as several other PPARα-mediated lipogenic genes including those of cholesterol 7α-hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), low-density lipoprotein receptor (LDLR), lipoprotein lipase (LPL), diacylglycerol acyltransferase (DGAT), and fatty acid synthase (FAS), in ICR mice.

Materials and methods

Reagents

The assay kits for total cholesterol (TC) and triglycerides (TG) were purchased from Beijing Beihua Kangtai Clinical Reagent Company (Beijing, China). The assay kit for free fatty acids (FFA) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Trizol was a product of Invitrogen (Carlsbad, CA, USA). Taq DNA polymerase and reverse transcriptase were products of Thermo Scientific (USA). The primers (Table 1) used for amplification by reverse transcription polymerase chain reaction (RT-PCR) were synthesized by Shanghai Sangon Gene Company (Shanghai, China). All other reagents used in this study were of analytical grade.

Animals and treatments

ICR male mice (22 ± 2 g) were supplied by the Animal Breeding Center of Soochow University, housed in regular cages in a room with controlled humidity and temperature with a 12–h light (8:30–20:30)/12-h dark (20:30–8:30) cycle, and allowed free access to food and water. The animals were allowed to acclimatize to the laboratory environment for 3 days prior to the study. The animal study was approved by the University Ethics Committee and conducted according to the regulations for the Use and Care of Experimental Animals at Soochow University.

Forty-eight ICR mice were studied in this experiment. These mice were randomized to either a high-fat diet group ($n = 24$) or a match control group ($n = 24$). The former was orally administered a high-fat milk containing 10 % cholesterol, 20 % lard, 1 % propylthiouracil, 2 % bile salt, 20 % propylene glycol, and 20 % Tween-80 at 0.2 ml/10 g (body weight per day) by gavage in the evening (19:00–20:00) for 4 weeks, whereas the latter was given an equal volume of distilled water in the same manner. Finally, all of the mice were killed by stunning and cervical dislocation at the following time points: 6:00, 12:00, 18:00 and 24:00, and 6 animals in each group were randomly killed at each time point. The liver was collected for parameter measurement, and partial hepatic tissues were quickly taken and frozen in liquid nitrogen and stored at −80 °C for RT-PCR.

Histopathological observations

Liver specimens of mice were fixed in 10 % formaldehyde solution and embedded in paraffin for HE staining and then examined under a light microscope. The degree of lipid accumulation was graded by estimating the proportion of hepatocytes containing fat droplets and expressed as "−, +, ++, +++." A grade of "−" means no fat present, "+" indicates the presence of fat in less than 1/3 of the hepatic lobule, "++" indicates the presence of fat in 1/3–2/3 of the hepatic lobule, and "+++" indicates the presence of fat in more than 2/3 of the hepatic lobule.

Measurements of liver TC, TG, and FFA

Hepatic tissues were taken and homogenized (10 %, wt/vol) in cold normal saline, after which the tissue homogenate was mixed with a solution of chloroform/methanol (2:1) according to a ratio of 1:1. The prepared sample was then placed in a 4 °C refrigerator for 24 h and centrifuged at 3,000×*g* for 10 min. The substratum obtained was used to measure TC, TG, and FFA contents using colorimetric methods following the manufacturer's instructions.

RT-PCR

Clock, Bmal1, Per1, Per2, Cry1, Cry2, PPARα, CYP7A1, HMGCR, LDLR, LPL, DGAT, FAS, and GAPDH mRNA expressions in the hepatic tissues were determined by RT-PCR. The total RNA was extracted according to the manufacturer's instructions (CA, Sinopharm Chemical

Table 1 The specific primers used for the PCR amplification

Gene	Forward and reverse primers	bp	Annealing temperature (°C)
Clock	5′-TTCGACAGGACTGGAAACCC-3′	201	56
	5′-GTTCTCCCTCTGGGATAAAA-3′		
Bmal1	5′-GCAGTGCCACTGACTACCAGTT-3′	207	55
	5′-TGTGCTGAACAGCCATCCTTAG-3′		
Per1	5′-GGGAGCTCAAACTTCGACTG-3′	437	60
	5′-GAGACCTGAACCTGCAGAGG-3′		
Per2	5′-GTGAAGCAGGTGAAGGCTAAT-3′	312	58
	5′-AAGCTTGTAAGGGGTGGTGTAG-3′		
Cry1	5′-TTCGGGGACAGCCAGCTGAT-3′	421	60
	5′-TCTCCTCCTGGCCACACTGCAGA-3′		
Cry2	5′-GGCAGAAACCACCCCTTA-3′	159	53
	5′-CCAGGGAAGGCACGCCAT-3′		
PPARα	5′-CCTGGAAAGTCCCTTATCT-3′	412	58
	5′-GCATTTGTTCCGGTTCTTC-3′		
CYP7A1	5′-CCTTGGGACGTTTTCCTGCT-3′	515	56
	5′-GCGCTCTTTGATTTAGGAAG-3′		
HMGCR	5′-GAATGCCTTGTGATTGGAGTTG-3′	125	54
	5′-ACACAGGCCGGGAAGAATG-3′		
LDLR	5′-GTGTGAACCTGGAAGGCAGCTA-3′	375	52
	5′-TCTGTCCAGTAGATGTTGCGGT-3′		
LPL	5′-TCAGAGCCAAGAGAAGCAGCAA-3′	118	58
	5′-TTGTGTTGCTTGCCATCCTCA-3′		
DGAT	5′-GTGCACAAGTGGTGCATCA-3′	180	58
	5′-CAGTGGGACCTGAGCCATC-3′		
FAS	5′-AGGGGTCGACCTGGTCCTCA-3′	132	60
	5′-GCCATGCCCAGAGGGTGGTT-3′		
GAPDH	5′-GTATGACGTGGAGTCTACTG-3′	728	56
	5′-TACTCCTTGGAGGCCATGTA-3′		

Reagent Co., Ltd). The concentration and purity of the RNA were determined spectrophotometrically by the ratio of the absorbance at 260 nm to that at 280 nm. Then total RNA (5 μg) was used for the RT reaction performed in 40 μl of the final volume at 42 °C 60 min. The PCR was carried out under the following conditions: 1 cycle of 94 °C for 3 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing (the temperature was seen in Table 1) for 60 s, and extension at 72 °C for 30 s. A final extension was 72 °C for 10 min. The PCR products were separated on 1.5 % agarose gel and quantitated by densitometry using an Image Master VDS system and the associated software (Pharmacia, USA). GAPDH was used as an internal control. Data were expressed as the ratio of the signals of interest to that of the GAPDH band.

Statistical analysis

Data are expressed as mean ± SD, and one-way ANOVA followed by a post hoc LSD test was used for comparisons between groups. Statistical analysis was conducted using SPSS 18.0 software. $P < 0.05$ was considered statistically significant.

Results

Effect of high-fat milk feeding in the evening on hepatic lipids

The results showed that 1/3–2/3 of the hepatocytes filled with empty lipid vacuoles after mice were fed high-fat milk in the evening for 4 weeks (Fig. 1b). No such changes observed in control mouse hepatocytes (Fig. 1a), suggesting that hepatic lipids were accumulated and fatty livers had developed in mice administered high-fat diets. In parallel, hepatic TC and TG contents were higher in high-fat diet-fed mice compared with matched control mice, especially at 6:00, 18:00, and 24:00 (Fig. 2a, b, $P < 0.05$ or $P < 0.01$). Both indices also exhibited similar profiles in

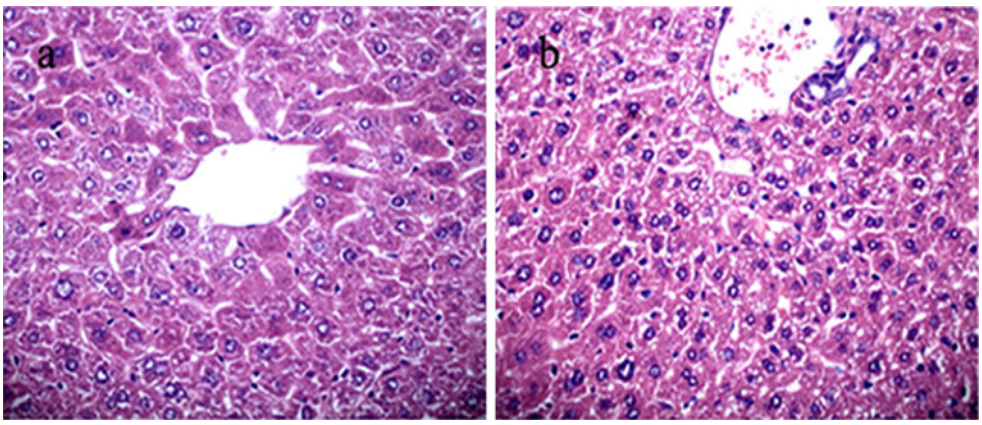

Fig. 1 Histopathological changes in mouse hepatic tissues (HE staining, ×40). No steatosis was observed in matched control mice (**a**) and 1/3 to 2/3 of hepatocytes filled with empty lipid vacuoles were seen in high-fat milk-fed mice (**b**)

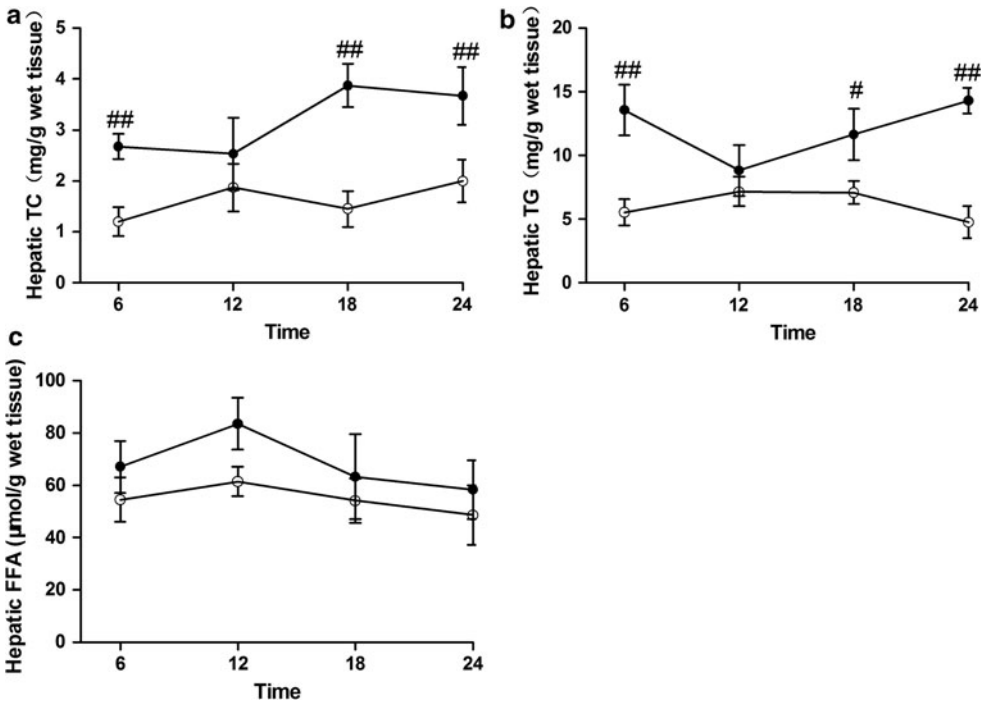

Fig. 2 Hepatic TC, TG, and FFA contents in mice fed with high-fat milk in the evening for 4 weeks. *White circles* represent the matched control group, and *black circles* represent the high-fat milk-fed group. Values indicate the mean ± SD of $n = 6$ mice per group for each time point. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the matched control group at the same time point

the high-fat diet-fed mice, but the peak was present in a 6-h delay for TC and a 12-h advance for TG relative to matched control mice (Fig. 2a, b). There were no significant variations in the content and circadian rhythm of hepatic FFA though with the increasing tendency across the entire four time points in mice fed high-fat milk (Fig. 2c).

Effect of high-fat milk feeding in the evening on hepatic clock gene expression

The present results revealed that all clock genes exhibited circadian oscillation (Fig. 3). Administration of a high-fat diet in the evening resulted in the amplitude attenuation of Clock and Bmal1 as well as amplitude increase in Cry2 and shifted the temporal expressions of Clock at 12:00 (Fig. 3b), Bmal1 at 24:00 (Fig. 3c), Per2 at 12:00 (Fig. 3e), and Cry2 at 6:00 (Fig. 3g) to the opposite phase relative to the matched control mice ($P < 0.05$), but did not affect the rhythms and amplitudes of the mRNA expressions of Per1 (Fig. 3d) and Cry1 (Fig. 3f) in the liver at any time point. These data indicate that the expressions of hepatic clock genes are partly altered in the high-fat diet-fed mice.

Effect of high-fat milk feeding in the evening on PPARα and lipogenic gene expressions

Compared with matched control mice, hepatic PPARα mRNA expression in the high-fat diet-fed mice decreased at 12:00, 18:00, and 24:00. This decrease was especially significant at 12:00, during which the temporal expression

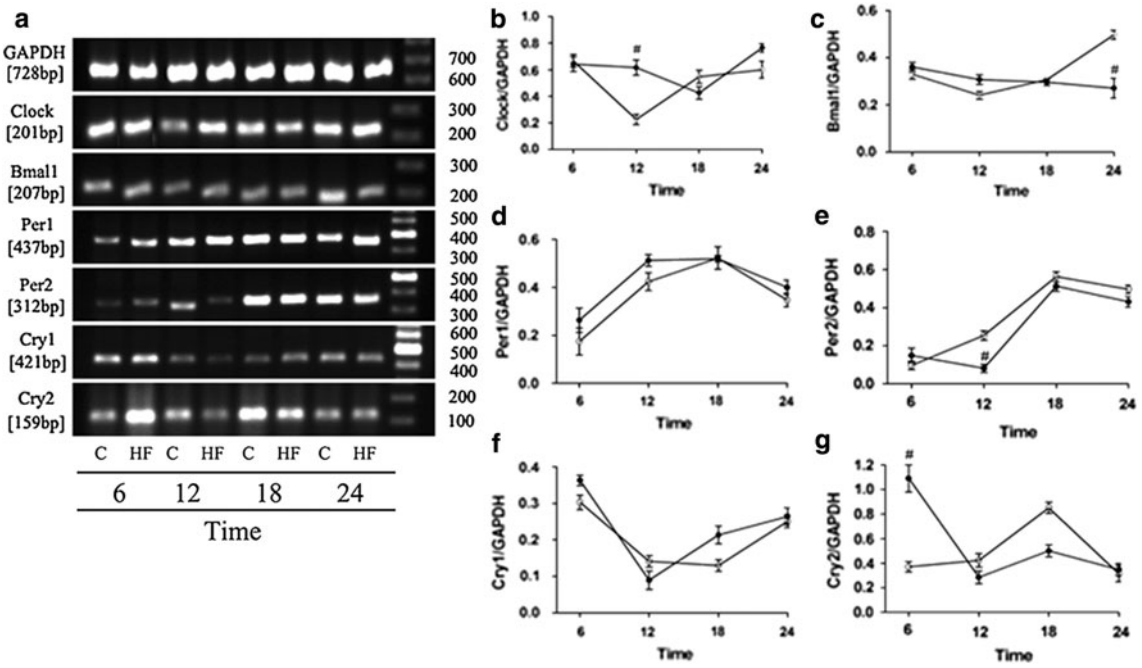

Fig. 3 Hepatic Clock, Bmal1, Per1, Per2, Cry1, and Cry2 mRNA expressions in mice fed with high-fat milk in the evening for 4 weeks. *White circles* represent the matched control group, and black circles represent the high-fat milk-fed group. C: the matched control group; HF: the high-fat milk group. Values indicate the mean ± SD of $n = 6$ mice per group for each time point. Gene bands were quantified relative to GAPDH. $^{\#}P < 0.05$ versus the matched control group at the same time point

showed the opposite phase relative to the matched control mice (Fig. 4b, $P < 0.05$). The amplitudes of expression also decreased under the high-fat diet. The mRNA expressions of CYP7A1 and HMGCR in the liver of high-fat diet-fed mice exhibited normal circadian variations in both groups, but their expression amplitudes significantly decreased at 18:00 (Fig. 4c, d, $P < 0.05$). In contrast to matched control mice, the rhythm of LDLR mRNA expression was altered, and the valley value was present in a 6-h delay and significantly decreased at 24:00 (Fig. 4e, $P < 0.05$). Interestingly, the oscillated amplitudes of the mRNA expressions of LPL and DGAT increased (Fig. 4f, g) but that of FAS decreased (Fig. 4h) in high-fat diet-fed mice. The diurnal rhythms of LPL and DGAT mRNA expressions were also disrupted in high-fat diet-fed mice and presented as a 6-h advance for LPL and dampened for DGAT compared with matched control mice. Thus, significant differences were observed between high-fat diet-fed and matched control mice at several time points (Fig. 4f, g, $P < 0.05$ or $P < 0.01$). The high-fat diet did not affect circadian variation in hepatic FAS mRNA expression though with an increasing tendency across all four time points (Fig. 4h).

Discussion

Previous studies have indicated that changes in clock gene expression participate in the development of some metabolic diseases (Kovac et al. 2009; Marcheva et al. 2009; Maury et al. 2010). In the present study, we compared hepatic clock gene expressions between control and high-fat diet-fed mice to determine whether or not the molecular clockwork is disrupted in fatty liver. As expected, some hepatic clock genes, such as Clock, Bmal1, Per2, and Cry2, exhibited obvious changes in rhythm and/or amplitude after administration of a timed high-fat diet in the evening, consistent with previous reports (Kohsaka et al. 2007; Hsieh et al. 2010). By contrast, no significant circadian variations in hepatic Per1 and Cry1 mRNA expressions were observed. Martino et al. reported that some clock gene rhythms in cardiac hypertrophic mice could remain unchanged and hypothesized that rhythmicity is an important mechanism that contributes to compensatory hypertrophy (Martino et al. 2007). As such, we believe that Per1 and Cry1 may play important protective roles in high-fat diet-induced fatty livers.

Under normal circumstances, the Clock/Bmal1 heterodimer is involved in lipid homeostasis by regulation of the clock-controlled gene PPARα (Yang et al. 2006; Inoue et al. 2005), which is a subtype of the PPAR receptors and predominantly expressed in the liver (Braissant et al. 1996). Bmal1 deficiency may induce dyslipidemia and ectopic fat formation (Shimba et al. 2011). As the rhythmic expression of clock genes is altered in fatty livers, PPARα loses its circadian expression rhythm with severely declining mRNA level, indicating that impaired clock gene

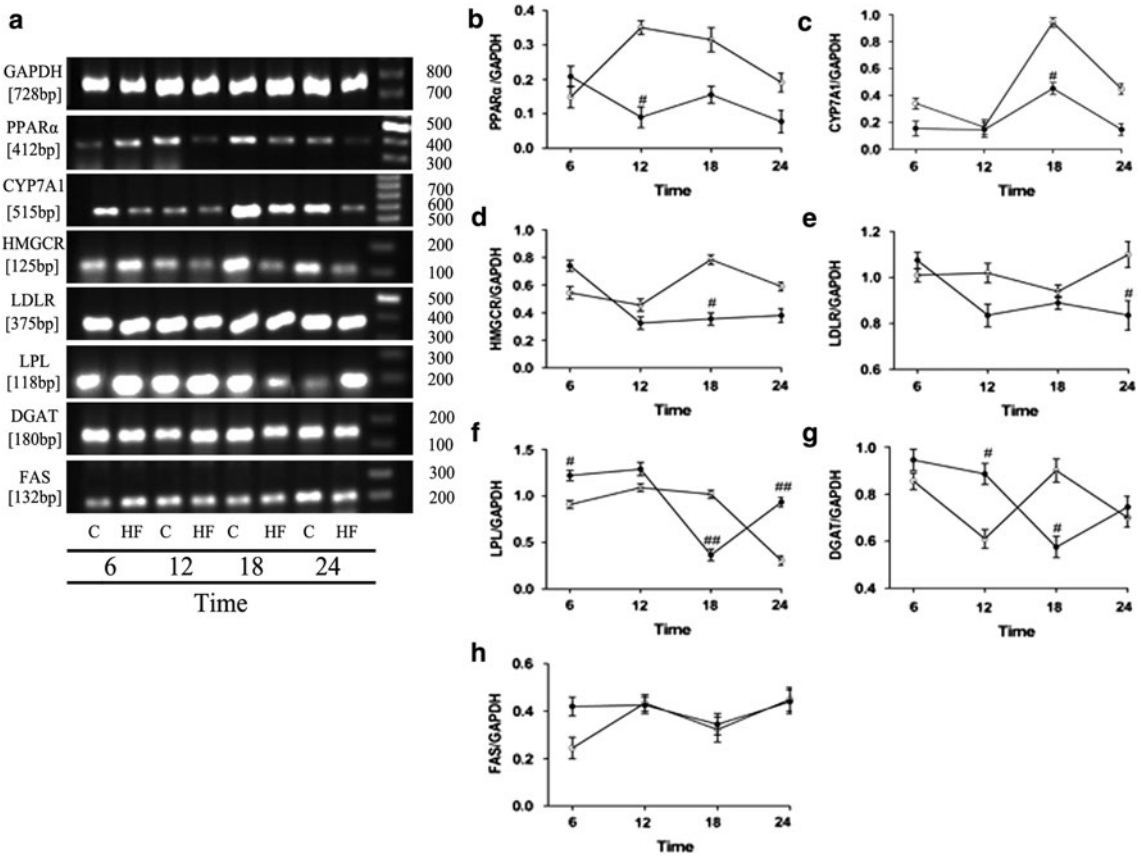

Fig. 4 Hepatic PPARα and lipogenic genes CYP7A1, HMGCR, LDLR, LPL, DGAT, and FAS mRNA expressions in mice fed with high-fat milk in the evening for 4 weeks. *White circles* represent the matched control group, and black circles represent the high-fat milk-fed group. C: the matched control group; HF: the high-fat milk group. Values indicate the mean ± SD of $n = 6$ mice per group for each time point. Gene bands were quantified relative to GAPDH. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the matched control group at the same time point

expression may result in disruption of the rhythm of PPARα expression.

PPARα is known to play a crucial role in regulating the expressions of CYP7A1, HMGCR, LDLR, LPL, DGAT, and FAS genes involved in lipid homeostasis (Desvergne and Wahli 1999). CYP7A1 is a liver-specific enzyme in the bile acid biosynthetic pathway that converts cholesterol into bile acids. HMGCR is a rate-limiting enzyme involved in cholesterol biosynthesis, and its daily variation in the liver has been recognized (Hamprecht et al. 1969). LDLR expression in hepatocytes may increase the circulatory uptake of LDL and lead to cholesterol accumulation in hepatocytes. After administration of a timed high-fat diet in the evening, the hepatic mRNA expressions of CYP7A1, HMGCR, and LDLR decreased with the reduction in PPARα. In particular, CYP7A1 mRNA expression significantly decreased in a manner that was almost consistent with the increase in hepatic TC level. This finding suggests that CYP7A1 may be a pivotal gene that controls cholesterol homeostasis under high-fat environmental conditions.

FAS is a key enzyme that controls the rate of fatty acid synthesis (Hammes 1985). DGAT is a microsomal enzyme expressed in the liver and a rate-limiting enzyme during TG synthesis (Farese et al. 2000). Conversely, LPL may catalyze the hydrolysis of TG and release FFA from TG. In the present study, we found that rhythmic changes in the three genes varied significantly. After administration of a high-fat diet in the evening for 4 weeks, FAS expression showed no rhythmic alteration. By contrast, the rhythm of the LPL expression shifted and that of the DGAT expression was substantially disrupted. The temporal expression of DGAT over light/dark cycles was notably similar to the alteration in hepatic TG level and contrasted the alteration in hepatic FFA level. These results reveal that DGAT may be a pivotal gene involved in hepatic TG accumulation under high-fat environmental conditions.

In conclusion, our results demonstrated that timed high-fat diets in the evening could change the hepatic circadian expressions of clock genes such as Clock, Bmal1, Per2, and Cry2 and subsequently alter the circadian expression of PPARα-mediated lipogenic genes, resulting in hepatic lipid

accumulation. However, some limitations are presented by the current study. The exact effects of variations in the protein expressions of PPARα and its related target genes must be investigated, and further study is necessary to elucidate how high-fat diets affect clock genes in greater detail.

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Conflict of interest The authors declare no conflicts of interest.

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