**LGR4 acts as a link between the peripheral circadian clock and lipid metabolism in liver**

Feng Wang1,2,*, Xianfeng Zhang2,*, Jiqiu Wang2,†, Maopei Chen2, Nengguang Fan2, Qinyun Ma2, Ruixin Liu2, Rui Wang2, Xiaoying Li2, Mingyao Liu3 and Guang Ning1,2

1Laboratory for Endocrine and Metabolic Diseases, Institute of Health Sciences, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) and Shanghai Jiao Tong University School of Medicine (SJTUSM), Shanghai, China
2Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Institute of Endocrinology and Metabolism, Shanghai Key Laboratory for Endocrine Tumors and E-Institute of Shanghai Universities, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin 2nd Road, Shanghai 200025, China
3Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas 77030, USA

*(F Wang and X Zhang contributed equally to this work)
†J Wang is now at Department of Endocrine and Metabolic Diseases, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

Email jiqiuwang@163.com

Abstract

The circadian clock plays an important role in the liver by regulating the major aspects of energy metabolism. Currently, it is assumed that the circadian clock regulates metabolism mostly by regulating the expression of liver enzymes at the transcriptional level, but the underlying mechanism is not well understood. In this study, we showed that Lgr4 homozygous mutant (Lgr4<sup>m/m</sup>) mice showed alteration in the rhythms of the respiratory exchange ratio. We further detected impaired plasma triglyceride rhythms in Lgr4<sup>m/m</sup> mice. Although no significant changes in plasma cholesterol rhythms were observed in the Lgr4<sup>m/m</sup> mice, their cholesterol levels were obviously lower. This phenotype was further confirmed in the context of ob<sup>/ob</sup> mice, in which lack of LGR4 dampened circadian rhythms of triglyceride. We next demonstrated that Lgr4 expression exhibited circadian rhythms in the liver tissue and primary hepatocytes in mice, but we did not detect changes in the expression levels or circadian rhythms of classic clock genes, such as Clock, Bmal1 (Arntl), Pers, Rev-erbs, and Crys, in Lgr4<sup>m/m</sup> mice compared with their littermates. Among the genes related to the lipid metabolism, we found that the diurnal expression pattern of the Mttp gene, which plays an important role in the regulation of plasma lipid levels, was impaired in Lgr4<sup>m/m</sup> mice and primary Lgr4<sup>m/m</sup> hepatocytes. Taken together, our results demonstrate that LGR4 plays an important role in the regulation of plasma lipid rhythms, partially through regulating the expression of microsomal triglyceride transfer protein. These data provide a possible link between the peripheral circadian clock and lipid metabolism.

Key Words
- circadian rhythm
- G protein-coupled receptor
- respiratory exchange ratio
- lipid metabolism

Correspondence should be addressed to J Wang
Email jiqiuwang@163.com

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Introduction

Circadian rhythms are the daily oscillations of a lot of physiological functions in almost all mammals. This circadian system is developed during the course of evolution to adapt to the daily changes in environmental conditions by anticipating sleep and activity periods (Eskin 1979, Takahashi 1995). These oscillations are driven by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Schwartz & Gainer 1977). Similar clocks, which are synchronized by central clocks, have been found in peripheral tissues, such as liver and adipose tissue (Welsh et al. 2004).

At the molecular level, central and peripheral oscillators share a common molecular circuitry, with a battery of transcriptional activators and repressors forming feedback loops and generating oscillations in circadian transcription factors (Ko & Takahashi 2006). These transcription factors then exert effects on broader physiological processes through the rhythmic expression of output genes, a remarkable number of which are metabolic enzymes. In recent decades, data on the circadian transcriptome and circadian modulation of SREBP signaling at the transcriptional level and is involved in cholesterol and lipid metabolism (Storch et al. 2002, Reddy et al. 2006, Eckel-Mahan et al. 2012, Vollmers et al. 2012).

As an important component of energy homeostasis, plasma lipid concentration is maintained within a narrow range and exhibits circadian rhythm in mammals (Maillot et al. 2005, Pan & Hussain 2007). Many molecules have been found to participate in the regulation of this process. For example, NR1D1 (REV-ERBA) participates in the circadian modulation of SREBP signaling at the transcriptional level and is involved in cholesterol and lipid metabolism (Le Martelot et al. 2009). A number of other genes, such as IRE1α, can influence metabolism at the posttranscriptional level (Cretenet et al. 2010). To gain further insights into the mechanisms that coordinate the control of the circadian clock and diverse metabolic pathways, additional output genes need to be found.

Leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4/GPR48) is a member of the G protein-coupled receptor (GPCR) superfamily. LGR4 has been reported to play a broad role in development, and Lgr4 gene mutant mice display early neonatal lethality (Mazerbourg et al. 2004). Our group and others have reported that LGR4 is involved in male infertility, electrolyte homeostasis, the development of the ocular anterior segment, and bone formation through different downstream targets (Weng et al. 2008, Luo et al. 2009, Li et al. 2010, Wang et al. 2012, Siwko et al. 2013).

Recently, a rare nonsense mutation within the Lgr4 gene was found strongly associated with low bone mineral density, electrolyte imbalance, late onset of menarche, and reduced testosterone levels in human (Styrkarsdottir et al. 2013). Several members of the leucine-rich repeat-containing GPCR family were found to participate in the regulation of the circadian rhythms of certain physiological activities. For example bursicon, the natural ligand of a Drosophila homolog to LGR4, DLGR2, was reported to act downstream of the neuropeptide CCAP, which controls the circadian timing of ecysis behavior in Drosophila (Park et al. 2003, Mendive et al. 2005). LGR5, which is closely related to LGR4, was reported to be regulated by clock genes in epidermal stem cells (Lin et al. 2009, Janich et al. 2011). However, whether LGR4 plays a role in circadian physiological behavior is not yet clear.

In this study, we report a new role for LGR4 in the connections between circadian rhythms in gene expression and circadian oscillations in metabolic activity. We found that Lgr4 homozygous mutant (Lgr4<sup>m/m</sup>) mice displayed a higher respiratory exchange ratio (RER) at night and an altered circadian rhythmic pattern of energy homeostasis, especially regarding lipid metabolism. We further demonstrated that LGR4 showed a rhythmic expression pattern in the liver, and a lack of LGR4 impaired the rhythmic expression of microsomal triglyceride transfer protein (MTTP). These findings demonstrate that LGR4 appears to serve as a molecular link between the circadian oscillator and energy metabolism in peripheral tissue.

Materials and methods

Animals

Lgr4<sup>m/m</sup> mice were generated as previously described (Weng et al. 2008, Wang et al. 2012). Three PCR primers were used for genotyping: the common upstream primer A: 5′-CCA GTC ACC ACT TTT ACA CAA TGG CTA AC-3′; downstream primer B: 5′-ATT CCC GTA GGA GAT AGC GTC CTA-3′; and downstream primer C: 5′-GGT CTT TGA GCA CCA GAG GAC-3′. Lgr4<sup>m/m</sup> mice and their WT littermates were age and gender matched throughout the experiments. Male mice at the age of 8–10 weeks were used in the experiments except the indirect calorimetry experiment in which both male and female mice were used. Lgr4 and Leptin double mutant mice were produced by intercrossing Lgr4 heterozygous mutant mice with Leptin heterozygous mutant mice. The mice were maintained on a 12 h light:
12 h darkness cycle and fed ad libitum. Dissected tissues were quickly frozen and stored at −80 °C. All procedures were approved by the Animal Care Committee of Shanghai Jiaotong University School of Medicine.

**RNA isolation and RT**

Total RNA was extracted from liver samples using the standard TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The absorbance ratio at 260/280 nm of all of the RNA samples was checked using a Nano-Drop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and all of the RNA samples were adjusted to the same concentration. The integrity of the RNA samples was also examined by agarose gel electrophoresis. RT of 1 μg of RNA was performed with the Reverse Transcription System (Promega, Madison, WI, USA).

**Table 1** The circadian parameters of the plasma lipid levels in WT mice and Lgr4 gene mutant mice (Lgr4^m/m^) calculated using JTK_CYCLE analysis

<table>
<thead>
<tr>
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<td>amplitude</td>
<td>phase</td>
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<tr>
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<td>No</td>
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<td>NA</td>
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<td>Yes</td>
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<td>No</td>
<td>0.1075</td>
<td>8</td>
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</table>

Figure 1

Lgr4^m/m^ mice displayed an impaired circadian rhythm of the RER. The RER of male (A) and female (B) mice were measured in metabolic chambers over 24 h (left, 24 h period; right, dark:light ratio of RER for WT and Lgr4^m/m^ mice). Lights on is indicated by a white bar, and lights off is indicated by a black bar (n = 4 for each genotype at each time point). ZT, Zeitgeber time; m/m, Lgr4^m/m^; *P < 0.05; **P < 0.01; error bars, S.E.M.
RT-PCR analysis

RT-PCR was performed using the LC480 system (Roche, Penzberg, Germany) with SYBR Green Supermix (Takara, Otsu, Shiga, Japan). The following RT-PCR conditions were applied over 50 cycles: 94°C (30 s), 94°C (5 s), and 60°C (30 s). The primers used in this study are presented in Supplementary Table S1, see section on supplementary data given at the end of this article.

Plasma lipid analysis

Blood was collected in EDTA-coated tubes. All samples were maintained on ice until being centrifuged at 960g for 10 min. Serum lipids were measured using commercial kits according to the manufacturer’s instructions. Specially, LabAssay NEFA kit (294-63601) and total triglyceride kit (290-63701) were obtained from Wako (Osaka, Japan). Total cholesterol, HDL-C, and LDL-C kit (KH-G-C-005) was obtained from Shanghai Kehua Bio-engineering Co., Ltd (Shanghai, China).

Isolation of mouse hepatocytes

Primary hepatocytes were isolated from mice by hepatic portal collagenase perfusion as described previously (Hengstler et al. 2000). Briefly, the whole liver was first perfused with Hank’s Balanced Saline buffer (HBSS) in situ and then with collagenase solution (1% BSA and 0.05 collagenase in HBSS) for 10 min in plate. Dispersed cells were resuspended and seeded onto a plate. The hepatocytes were grown in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS).

Serum shock

The cells were starved in high-glucose DMEM containing 0.5% (v/v) FBS for 12 h, followed by synchronization with DMEM containing 50% (v/v) horse serum (t=0) for 2 h, and the medium was then changed back to the starvation medium (Balsalobre et al. 1998).

Indirect calorimetry

The consumption of O2 and production of CO2 were determined using the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH, USA), according to the manufacturer’s instructions. Data were recorded every 10 min. The RER was calculated as the ratio of the volume of CO2 produced to the volume of O2 consumed.

Statistical analysis

Values are expressed as the mean±s.e.m. One-way ANOVA was applied, followed by a t-test. Circadian parameters were analyzed using a new version of JTK_CYCLE software as described previously (Hughes et al. 2010, Miyazaki et al. 2011).

Results

Loss of LGR4 changes the rhythm of the RER

To determine whether LGR4 plays a role in the circadian regulation of metabolism, the RER was measured in male (Fig. 1A) and female (Fig. 1B) Lgr4<sup>m/m</sup> mice and their WT

Figure 2

Diurnal variations in plasma lipids in WT and Lgr4<sup>m/m</sup> mice. Plasma was obtained from male mice fed ad libitum at the indicated times. The time reflects circadian hours, with the lights on at ZT0 and off at ZT12. Total triglyceride (A), total cholesterol (B), NEFA (C), LDL-C (D), and HDL-C (E) levels were measured as described. Each time point represents the mean±s.e.m.; n=3–4 for each genotype at each time point. TG, total triglycerides; TC, total cholesterol; ZT, Zeitgeber time; m/m, Lgr4<sup>m/m</sup>; *P<0.05.
littermates over 24 h using indirect calorimetry. Consistent with the findings of a previous report (Tu et al. 2005), the RER is higher in the dark phase than in the light phase in WT mice, suggesting the existence of a circadian rhythm in substrate utilization for energy source during the day, more glucose in the dark phase, and more lipid usage in the light phase. However, the RER of \textit{Lgr4} \textit{m/m} mice was higher than that of their WT littermates during the dark phase ($P<0.010$) and showed no difference during the light phase of the day, suggesting that the lack of LGR4 altered the circadian rhythm of lipid metabolism, and \textit{Lgr4} \textit{m/m} mice consumed lower amounts of lipids, but more sugar compared with WT mice (Hawley et al. 2012).

**Loss of LGR4 changes the lipid rhythm in mice**

To reveal further details regarding the diurnal variation of lipid metabolism, we next measured the plasma lipid levels in WT and \textit{Lgr4} \textit{m/m} mice at different time points. Table 1 provides the results of the statistical analysis with the circadian parameters of the plasma lipid levels. As shown in Fig. 2A, the \textit{Lgr4} \textit{m/m} mice exhibited higher plasma triglyceride levels at some time points and lost the rhythmic pattern compared with WT mice. \textit{Lgr4} \textit{m/m} mice also presented a changed phase in their plasma non-esterified fatty acid (NEFA) levels, reflected by lower plasma NEFA levels during the light phase and higher levels in the dark phase in \textit{Lgr4} \textit{m/m} mice in comparison with WT mice (Fig. 2B and Table 1). The cholesterol levels in \textit{Lgr4} \textit{m/m} mice, including those of total cholesterol (Fig. 2C), LDL-C (Fig. 2D), and HDL-C (Fig. 2E), did not show significant changes in their rhythmic patterns compared with WT mice, with the exception of some slight alterations at certain time points (Table 1). These results demonstrate that loss of LGR4 results in an impaired plasma triglyceride rhythmic pattern.

It is known that lipid levels and rhythmic patterns are greatly altered in obese mice (Hems et al. 1975). To determine whether LGR4 can exert functions on the circadian rhythms of plasma lipids in obese mice, we measured the plasma lipid levels in \textit{Leptin}\textit{-deficient} mice (\textit{ob}/\textit{ob}) and \textit{Leptin} and \textit{Lgr4} double-mutant (DKO) mice. Similar to the results obtained in \textit{Lgr4} \textit{m/m} mice and WT mice, the total plasma triglyceride levels loss circadian rhythms which were found in the WT mice (Fig. 3A and Table 2), while the total plasma cholesterol levels were lower and showed no significant change in their rhythmic pattern (Fig. 3B and Table 2).

**Table 2** The circadian parameters of the plasma lipid levels in \textit{ob}/\textit{ob} (OB) mice and \textit{Leptin} and \textit{Lgr4} double-mutant (DKO) mice calculated using JTK\_CYCLE analysis

<table>
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<tr>
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<th>JTK_CYCLE P value</th>
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<th>JTK_CYCLE phase</th>
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<tr>
<td>OB</td>
<td>TG</td>
<td>(3.15 \times 10^{-8})</td>
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<tr>
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<tr>
<td>OB</td>
<td>TC</td>
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<tr>
<td></td>
<td></td>
<td>8.35 \times 10^{-5}</td>
<td>2</td>
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Figure 4
Expression pattern of clock genes in WT and Lgr4\textsuperscript{m/m} mice. Male mice fed ad libitum were sacrificed at indicated time points. mRNA levels of the clock genes were measured by real-time PCR and normalized to Gapdh. The data represent the mean ± S.E.M.; n=3–4 for each genotype at each time point. ZT, Zeitgeber time; m/m, Lgr4\textsuperscript{m/m}. 

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Loss of LGR4 does not affect the central clock genes in the liver

The circadian rhythms of physiological activities are controlled by clock genes. To examine whether the phenotype found in Lgr4
m/m mice is a result of altered rhythms of components of the circadian clock, we next investigated the daily expression pattern of circadian genes in the liver tissue in WT and Lgr4
m/m mice. As shown in Fig. 4, the circadian rhythms of all of the circadian genes in WT mice were similar to those presented in previous reports (Kume et al. 1999, Preitner et al. 2002, Yang et al. 2006), but no significant changes were found between WT and Lgr4
m/m mice (Fig. 4). These results suggest that LGR4 does not directly regulate clock genes.

LGR4 expression shows circadian rhythms in vivo and in vitro

Previous studies show a lot of genes involved in circadian rhythms as downstream output genes of the circadian clock (Benito et al. 2010, Tong et al. 2010), so we examined whether the expression level of LGR4 shows a circadian rhythm in following study. We measured the Lgr4 mRNA levels in the liver tissue of WT mice and Lgr4
m/m mice during a 24 h period. As shown in Fig. 5A, Lgr4 expression of WT mice was higher overall during the light phase than the dark phase, presenting a peak at ZT4 and a nadir at ZT16, indicating a circadian rhythm as analyzed using the JTK_CYCLE (P=0.0057, Fig. 5A). Lgr4 expression level in Lgr4
m/m mice was very low and the amplitude was dammed (Fig. 5B). To determine whether Lgr4 expression also exhibits circadian rhythms in vitro, we synchronized mice primary hepatocytes with 50% horse serum and then collected mRNA samples every 4 h over a period of 48 h. As shown in Fig. 5B, Lgr4 presented a rhythmic expression pattern based on analysis with the JTK_CYCLE (P=0.0056, Fig. 5C). We also measure the expression levels of Bmal1 (Arntl) as positive control, we also found it presented a rhythmic expression pattern (P=0.0296 Fig. 5D).

LGR4 ablation impairs the rhythmic expression of MTTP

To determine the molecular mechanism underlying the impaired triglyceride rhythm observed in Lgr4
m/m mice, we next measured the circadian rhythms of genes related to triglyceride metabolism in liver tissue using real-time PCR. Interestingly, out of genes involved in the metabolism and synthesis of triglycerides, such as Srebp1c, Lpl, and so on, we found that MTTP, which plays a role in the absorption of lipids and the circadian rhythms of plasma lipid levels (Preitner et al. 2002, Pan & Hussain 2007, Pan et al. 2010), displayed the most significant changes in its circadian rhythms among the genes we examined in Lgr4
m/m mice at mRNA level (Fig. 6A). MTTP protein showed diurnal variations in the WT but not in Lgr4
m/m livers, although analyzing the quantitative signals of western blotting failed to detect circadian rhythms at protein level (Fig. 6B). We next analyzed the expression rhythms of Mttp in primary hepatocytes, and similar results were obtained. Mttp mRNA levels showed a circadian periodicity of ~28 h in the hepatocytes of WT mice (P=0.0059), but no rhythm was detected in the hepatocytes of Lgr4
m/m mice (P=0.2891, Fig. 6B and Table 3), which may explain the dampened circadian rhythms of plasma triglyceride in Lgr4
m/m mice compared with their WT littermates. Consistent with the overall lower but unaltered rhythms of plasma cholesterol seen in Lgr4
m/m mice, we also detected a lower level of genes of the SREBP2 pathway but did not find significant changes in the circadian rhythm of these genes in Lgr4
m/m mice (Fig. 6C, D, and E).

Discussion

GPCRs play critical roles in most biological processes and represent the most important group of drug targets...
(Klabunde & Hessler 2002), but little is known about their role in the regulation of circadian rhythms. In Drosophila, the receptor for the neuropeptide, pigment-dispersing factor (PDF), exerts functions on circadian rhythms through regulating cAMP levels in the SCN (Mertens et al. 2005). In mammals, cryptochrome proteins can directly interact with the Gs subunit of GPCRs to inhibit the rhythmic accumulation of cAMP in peripheral tissues.
circadian rhythms (Seaman et al. 1973). Rodents exhibit nocturnal feeding behavior, and plasma lipid levels in mice were high at night and low during the day (Pan & Hussain 2007). We consistently detected similar circadian rhythms in plasma triglyceride levels in WT mice and Lgr4 gene mutant (Lgr4m/m) mice, which was different from plasma triglyceride.

It has recently been suggested that plasma lipid rhythms are a result of the interplay between the circadian clock and metabolism. Almost all of components of the circadian clock, such as CLOCK (DeBruyne et al. 2007), BMAL1 (Shimba et al. 2011), CRYs, PERs (Grimaldi et al. 2010), and REV-ERBs (Cho et al. 2012), have been reported to regulate the plasma lipid rhythms. To obtain further insights into the crosstalk, numerous screens were performed to find circadian clock output genes (Storch et al. 2002, Reddy et al. 2006, Eckel-Mahan et al. 2012). Eckel-Mahan and colleagues established a database entitled ‘CircadiOomics’ that provides a consolidated model of how the daily metabolome, transcriptome, and proteome might work together (Eckel-Mahan et al. 2012). In this database (http://circadiomics.igb.uci.edu/), we found that the LGR4 expression level is high during the light phase and low during the dark phase in liver tissue, which is consistent with the results of this study. However, the loss of rhythmic activity of LGR4 did not affect the rhythms of clockwork components in peripheral tissue. This suggests that LGR4 is regulated by the circadian clock and may be an output gene of clockwork. Taking these findings together, they suggest that LGR4 may participate in the circadian modulation of metabolism.

In plasma, most lipids, such as triglycerides, phospholipids, and cholesterol, are transported by forming protein–lipid complexes known as lipoproteins. Triglycerides are transported to the plasma by lipoproteins containing ApoB, which requires the chaperone protein MTTP (Shelness & Sellers 2001, Hussain et al. 2003). Pan and colleagues reported that MTTP is rhythmic and is important for the daily variations in plasma lipid levels (Pan & Hussain 2007, Pan et al. 2010). We found a similar expression pattern of MTTP in WT mice, which was impaired in the absence of LGR4. This finding suggests that the loss of MTTP rhythms due to the lack of LGR4 activity at least partially explains the arrhythmic plasma lipid phenotype observed in Lgr4m/m mice, although the detailed mechanisms underlying this phenomenon are still unknown. We compared the gene expression profiles of liver tissue of WT and Lgr4m/m mice (data not shown) and found that genes involved in the regulation of Mttp, such as Pcsca, Rxra, Foxa2, were all upregulated in Lgr4m/m mice. These genes can all upregulate the expression of Mttp (Kang et al. 2003, Wolfrum & Stoffel 2006). This may also suggest that Lgr4 might be involved in the negative regulation of Mttp. Further studies are needed to fully understand the mechanisms involved in the crosstalk between the clock, LGR4, and MTTP.

In summary, this study established a molecular link between circadian physiology and plasma lipid metabolism. Although it is possible that other unknown mechanisms might be involved, our results suggest that LGR4 regulates the circadian rhythms of plasma lipids through MTTP. Lack of LGR4 causes an arrhythmic plasma lipid phenotype in mice. Considering that LGR4 is a member of the GPCR family, this study provides a potential means of regulating metabolism through the regulation of the circadian functions.

**Table 3** The circadian parameters of the Mttp expression levels in primary hepatocyte of WT mice and Lgr4 gene mutant mice (Lgr4m/m) calculated using JTK_CYCLE analysis

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<th>Gene</th>
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<tr>
<td>Lgr4m/m</td>
<td>Mttp</td>
<td>0.2891</td>
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(Zhang et al. 2010). In this study, we found that the daily rhythms of the RER and plasma lipid levels were impaired in Lgr4m/m mice, and we further observed that the expression levels of LGR4 were rhythmic in both liver tissue and cultured primary hepatocytes. We also detected altered MTTP expression rhythms in the absence of LGR4, which may be the reason for the altered plasma triglyceride circadian rhythm in Lgr4m/m mice.

Lipid homeostasis has long been known to exhibit circadian rhythms (Seaman et al. 1965, Schlierf & Dorow 1973). Rodents exhibit nocturnal feeding behavior, and thus, the plasma lipid levels in mice were high at night and low during the day (Pan & Hussain 2007). We consistently detected similar circadian rhythms in plasma triglyceride levels in WT mice and ob/ob mice in this study, but these rhythms were impaired in the absence of LGR4. It is worth noting that the plasma cholesterol level was lower with unaltered circadian rhythm in Lgr4m/m mice, which was different from plasma triglyceride.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-13-0042.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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