Association of Expression Levels of Clock Genes and Autophagy-Related Genes under Abnormal Light/Dark Cycle Stimulation in NAFLD Mice

Zhu Zhu
Zhengzhou University First Affiliated Hospital
Zhengyang Wang
Zhengzhou University First Affiliated Hospital
Bo Qin
Zhengzhou University First Affiliated Hospital
Songfeng Zhao
Zhengzhou University First Affiliated Hospital
Huafei Wang
Zhengzhou University First Affiliated Hospital
Lulu He
Zhengzhou University First Affiliated Hospital
Jin Xie
Zhengzhou University First Affiliated Hospital
Long Qiu
Zhengzhou University First Affiliated Hospital
Yanran Wang
Zhengzhou University First Affiliated Hospital
Li Wang
Zhengzhou University First Affiliated Hospital
Bing Chen
Zhengzhou University First Affiliated Hospital
Peipei Liu
Zhengzhou University First Affiliated Hospital
Jinlin Liu
Jilin University
Xin Tian (tianx@zzu.edu.cn)
Zhengzhou University First Affiliated Hospital https://orcid.org/0000-0002-6080-2924

Research

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Abstract

Background: Environmental disorders of the circadian rhythms can lead to metabolism-related diseases or exacerbate pathological conditions. Non-alcoholic fatty liver disease (NAFLD) has emerged with a growing occurrence. In the present study, we attempted to indicate whether circadian clock may influence lipid deposition and the expression levels of autophagy-related genes in liver of mice.

Methods: High-fat diet and abnormal light/dark cycles were employed to induce a mouse model of NAFLD with circadian rhythm sleep disorder. Herein, liver samples were obtained at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 time-point to detect the rhythmic expressions of circadian genes, autophagy-related genes, and Rev-erbα.

Results: Abnormal exposure to light aggravated lipid deposition in liver of mice and exacerbated disorders related to 24-h expression levels of clock genes, autophagy-related genes, and Rev-erbα. Besides, Rev-erbα could transcriptionally control the expression levels of autophagy-related genes.

Conclusions: The long-term high-fat diet combined with abnormal light/dark cycle stimulation aggravated the development of NAFLD and disturbed the expressions levels of autophagy-related genes. An abnormal circadian expression may lead to NAFLD aggression. Besides, the abnormal expression levels of clock genes may create an association between circadian rhythm sleep disorder and autophagy.

Background

Circadian clocks, which are intrinsic and time-tracking systems in different living organisms, can adjust behavior and physiology to adapt environmental changes during 24 h [1]. Circadian clocks exist in almost all of the organs and tissues, and are divide into master clock and peripheral clocks. The hypothalamic suprachiasmatic nucleus (SCN) clock is master clock, which can function autonomously. The SCN clock can reset by external light cues. A 24-h light/dark cycle may be a principal regulator that is unambiguously synchronized to Zeitgeber time (A German word termed external cues of the endogenous clock)[2]. Peripheral clocks, including liver clock, heart clock, fat clock, etc., are synchronized by the SCN clock to make temporally coordinated physiology [3]. The fundamental mechanisms of circadian rhythms are similar in the master clock and peripheral clocks, which are regulated by a series of interplaying clock genes at the transcriptional and post-translational feedback loops. Circadian locomotor output cycles kaput (Clock) and brain and muscle encoding Arnt-like protein 1 (Bmal1) form heterodimers, promoting the expression levels of downstream genes. Conversely, the period (Per1, Per2, and Per3) and cryptochrome (Cry1 and Cry2) inhibit the clock output [3]. The circadian clock genes can produce metabolic rhythms and regulate metabolic processes (e.g., lipid metabolism).

Non-alcoholic fatty liver disease (NAFLD) is the most frequent cause of liver diseases and a more potential for end-stage liver disease both in developed and in developing countries [4]. It is associated with excessive triglyceride (TG) level, which is taken as a systemic lipid metabolic disorder into account [5, 6]. Autophagy is a conservative process in eukaryotes, and using this process, cytoplasmic lipid

droplets are isolated in double membrane vesicles and transport to lysosomes for degradation [7]. It regulates intracellular lipid stores. Singh et al. demonstrated that autophagy could be repressed in cultured hepatocytes, promoting TG storage in lipid droplets [8]. Numerous scholars pointed out that defective hepatic autophagy is closely associated with NAFLD [8–10].

To date, NAFLD has affected within 30% of the global population, and it has emerged with a growing occurrence [11, 12]. In order to explore the origin of rapid increase in the incidence of NAFLD, we concentrated on circadian clocks in the current study. Since the human life style has dramatically changed worldwide during the past decades, and modern life style is globally forcing more and more people to enter the asynchrony state, it is highly essential to find out an association between the social time and the internal circadian clock. Notably, environmental disorders of the circadian rhythms can lead to metabolism-related diseases or exacerbate pathological conditions. Genome-wide gene expression studies demonstrated that the circadian clock plays a significant role in liver physiology [13, 14]. The liver clock regulates hepatic metabolism, including fatty acid, lipid, glucose, etc. [15, 16]. In addition, autophagy activity and autophagy-related genes showed daily rhythms in mice [17]. The ULK1, a critical positive regulator for autophagy initiation, is an autophagy-related gene. It maintains its rhythmicity both in zebrafish liver and mice liver [17, 18], and it controls the lipid metabolism in adipocytes [19]. Besides, microtubule-associated protein light chain 3 (LC3) is a homologue of yeast Atg8p and is associated with the autophagosome membranes. Cytosolic LC3-I and membrane-bound LC3-II are two forms of LC3. LC3-II is associated with the number of autophagosomes, playing a significant circadian oscillatory role in liver and other tissues of mice, including heart, kidney, and skeletal muscle [17, 20]. It is involved in autophagosome formation, and it is transcriptionally regulated Bmal1. In addition, Atg5 is an essential molecule for autophagosome formation, and its rhythmic expression levels can be found in wild-type flies [21]. Meanwhile, inhibition of the Atg5 in hepatocytes could increase TG level [8]. Lamp1, a lysosomal-associated membrane protein 1, is involves in intracellular lipid autophagic clearance [8]. Moreover, the rhythmic expression of lamp1 was found in the liver of mice and in human retinal pigment epithelial cells [22, 23].

However, whether circadian rhythm disruption leads to disruption of autophagy and ultimately affects the occurrence and development of NAFLD. In the present study, we utilized abnormal light/dark cycle to induce mice circadian asynchrony. Additionally, we assessed the degree of lipid deposition in the liver of mice and detected 24-h expression levels of circadian clock genes and autophagy-related genes in the liver of mice. Besides, we preliminary evaluated how abnormal expressions of circadian clock genes could lead to changes in expressions of autophagy-related genes, and this may ultimately lead to the occurrence and development of NAFLD.

Materials And Methods

1. A mouse model of NAFLD

We adopted 72 8-week-old C57 BL/6J mice, which were provided by Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Since the high fat diet is more likely cause TG accumulation in liver [24], we used the Western-type diet (containing 0.15% cholesterol and 21% fat) for feeding mice for 6 weeks under light/dark cycle leading to circadian rhythm sleep disorder in mice [25]. All mice were randomly divided into three groups: (1) mice that were subjected to normal diet and regular light/dark cycle (C57 ND group); (2) mice that received high-fat diet under normal light/dark cycle (C57 WD group); (3) mice that received high-fat diet under abnormal light/dark cycle (C57 WD + DD/LD group).

2. Cultivation Of Primary Hepatocytes

Primary hepatocytes were isolated from C57 ND mice in our vitro experiments. The cells were collected according to the collagenase perfusion method [26]. Arterial blood of the mouse liver was blocked and replaced by 5 ml/min EDTA solution (including 5 mM glucose, 0.5 mM EDTA, 4.15 mM NaHCO3, 136 mM NaCl, 5.4 mM KCl, 0.65 mM NaH2PO4, 0.85 mM Na2HPO4, and 1 mM HEPES) for 6 min. Then, the liver was perfused with 1 mg/ml collagenase type IV. The liver was isolated and cut into pieces. Tissue fragments were filtered, centrifuged, and washed. The cells were collected and incubated in William's E medium, containing 10% fetal bovine serum (FBS). After 4 h, the old medium was replaced with a new William's E medium, containing 10% FBS to remove the dead cells. These cells at passage 0 were directly utilized herein.

3. Short Interfering RNA (SIRNA) Transfection

The Rev-erbα siRNA was synthesized at RiboBio Co., Ltd. (Guangzhou, China). Besides, 100 nM siRNA (Rev-erbα siRNA or Control siRNA) and 50 nM LipofectamineTM 3000 (Thermo Fisher Scientific, Waltham, MA, USA) were incubated at room temperature for 5 min. The Rev-erbα siRNA or Control siRNA were mixed with LipofectamineTM 3000 for 15 min to promote the formation of Rev-erbα siRNA/Lipofectamine complex and Control siRNA/Lipofectamine complex. Then, these complexes were added into the serum-free William's E medium, incubated for 6 h. Replace with a fresh William's E medium for another 48 h. The interference was detected via quantitative reverse transcription polymerase chain reaction (RT-qPCR).

4. Histological Analysis

Histological examination was performed to assess fatty liver degeneration in mice. The liver samples were fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Then, the samples were embedded into paraffin and processed routinely. Finally, the samples were stained with hematoxylin and eosin (H&E) as described previously [27].

5. RNA Extraction And Reverse Transcription

Total RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration was determined by UV spectrophotometry. RNA

integrity was assessed using agarose gel electrophoresis. Then, 1 μg RNA was reversely transcribed into cDNA using ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to manufacturer's protocol.

6. RT-QPCR

The cDNA was amplified by ABI QuantStudio5 (Applied Biosystems, Foster City, CA, USA). The qPCR reaction mixture included 2.5 μl cDNA, 1 μl of 100 nM upstream primers, 1 μl of 100 nM downstream primers, 10 μl SYBR Green Master mix (Bio-Rad Laboratories, Hercules, CA, USA), and 20 μl H2O. The relative expression levels of target genes were calculated using the 2ΔΔCt method and normalized to 18S. The qPCR primers are presented in Table 1.

Table 1
The primer sequences.

Gene names	Primers	Primer sequences (5’-3’)
Real time-PCR		
Bmal1	Sense	CACTGACTACCAAGAAAGTATG
	Antisense	ATCCATCTGCTGCCCTGAGA
Clock	Sense	ATGAGCACCAAGACCATTCC
	Antisense	GCTTCAGTGCTCCCAACTTC
Per2	Sense	CAGACTCATGATGACAGAGG
	Antisense	GAGATGTACAGGATCTTCCC
Cry2	Sense	GCTAGAGTGACGGAGATGCC
	Antisense	ACTACCACCTCACTGGGACA
ULK1	Sense	TGGGGAGAAGGTGTGTA
	Antisense	TACTCTACAACAAGGGGCACA
LC3Ⅱ	Sense	CCCAGTGATTATAGAGCGATACAAGGGGGAG
	Antisense	CTGCAAGCGCCGTCTGATTATCTTGATGAG
Atg5	Sense	GGCACACCCCTGAAATGGCATTATCC
	Antisense	CCTCAACCGCATCCTTGGATGGAC
Lamp1	Sense	GCCCTGGAATTGCAGTTTGG
	Antisense	TGCTGAATGTGGGCACTAGG
Rev-erb α	Sense	TACATTGGCTCTAGTGGCTCC
	Antisense	CAGTAGGTGATGGTGGGAAGTA
GAPDH	Sense	ACAGCCGCATCTTCTTGTGCAGTA
	Antisense	GGCCTTGACTGTGCCGTGAATTT
ChIP		
Arbp	Sense	GAGGTGGCTTTGAACCAGAG
	Antisense	TCTTTGTCTCTGTCTCGGAAAA
Bmal1	Sense	GGAAAGTAGGTTAGTGGTGCGAC
	Antisense	AAGTCCGGCGCGGGTAAACAGG
ULK1	Sense	GCCTGGACTACAGGAAACCC

Gene names	Primers	Primer sequences (5’-3’)
	Antisense	AACTGGCTGGCTTCAGACTC
Atg5	Sense	GCTGCTGACAGAGCAAAGTG
	Antisense	GGGTTTGAGACAAGCTCTCG
RNA interfering		
mRev-erbα siRNA	Sense	CUUCGUUGUUCAACGUGAATT
	Antisense	UUCACGUUGAACAACGAAGTT

7. Chromatin Immunoprecipitation (CHIP) Assay

The ChIP assay was conducted using an EZ-ChIP™ kit (Millipore, Billerica, MA, USA) according to manufacturer’s instructions. The liver tissue was collected at ZT8, cut into 1 ~ 3 mm3 pieces, and moved into a 50 ml centrifuge tube. Cross-link proteins to DNA by adding 27 μL of 37% formaldehyde to the sample and incubate for 15 min at room temperature with shaking. Add glycine to a final concentration of 125 mM to the medium and incubate with shaking for 5 min at room temperature. Rinse cells twice with 10 mL cold phosphate-buffered saline (PBS). Add 5 mL of cold PBS, scrape dishes thoroughly with a cell scraper and transfer into 50 mL tube. Add 3 mL PBS to dishes, scrape again and transfer the remainder of the cells to the 50 mL tube. Centrifuge at 4 °C for 5 min at 1,000 rpm. Carefully aspirate off supernatant and resuspend the pellet in ChIP lysis buffer, and incubate for 10 min on ice. The chromatin solutions were precipitated with 5 μg Rev-erbα antibody (Cell Signaling Technology Inc., Danvers, MA, USA). Each reaction was performed at 4 ℃ for 12 h. Adopt Protein A Agarose to capture Rev-erbα complex. Elute the complex from Rev-erbα antibody and reverse protein-DNA crosslinks. The qPCR was performed to assess the enrichment of Rev-erbα protein.

8. Statistical Analysis

The data were presented as mean ± standard deviation (SD). Single cosinor method was employed to analyze circadian rhythm as previously described [28, 29]. The following equation was formulated for cosine function: $Y(t) = M + A*\cos(x*t + \mu)$. The daily rhythm characteristics included mesor (midline estimating statistic of rhythm corresponding to the mean level), amplitude (half of the peak-to-trough difference of the fitted cosine function), and acrophase (the crest time of rhythm given in degrees (°C), where 360 °C is corresponding to a 24-h cycle) were estimated by the above-mentioned function. Differences between the values of each pair of parameters were compared by one-way analysis of variance (ANOVA), and $P \leq 0.05$ was considered statistically significant.

Results

1. The abnormal lipid accumulation in hepatic cells after undergoing high-fat diet and abnormal light/dark cycle stimulation

To assess whether high-fat diet-induced NAFLD in mice, hematoxylin and eosin (H&E) staining was performed to evaluate lipid accumulation in the liver cells. As displayed in Fig. 1, there was no abnormal lipid accumulation in control mice that received normal diet under regular light/dark cycle. However, for mice that received high-fat diet for 6 weeks, lipid accumulation was noted in several liver cells. According to entrainment of the circadian system under light/dark cycle [30], we herein conducted the experiments on the base of high-fat diet and abnormal light/dark cycle stimulation. Figure 1 illustrates under high-fat diet and abnormal light/dark cycle, more lipid droplets were accumulated in the liver cells. The above-mentioned results suggested that high-fat diet and abnormal light/dark cycle could aggravate the fatty liver degeneration in mice.

2. The rhythmic expression levels of circadian genes in liver tissues

Circadian clock genes can modulate liver lipid metabolism. Therefore, the rhythmic expression levels of clock genes (Bmal1, Clock, Per2, and Cry2) were detected in C57 ND, C57 WD, and C57 WD + LD/DD groups at ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20 (Table 2). As shown in Fig. 2, the circadian oscillation of Bmal1 in C57 WD + LD/DD group was attenuated. The expression level of Per2 was significantly increased in C57 WD + LD/DD group compared with that in C57 ND group. The mesors of Per2 were remarkably elevated in C57 WD + LD/DD group compared with those in control group. Moreover, the amplitudes of Per2 were notably increased in C57 WD + LD/DD group compared with those in C57 ND group. Furthermore, the peak periodic values of Clock, Per2, and Cry2 were altered in C57 WD + LD/DD group compared with those in C57 ND group.

Table 2
Circadian rhythmic parameters of clock genes,ULK1, LC3 II, Atg5 and Lamp1 in mouse liver.

Gene	Mesor	Amplitude	Acrophase ZT (h)
C57 ND			
Bmal1	1.07 ± 0.24	0.57 ± 0.03	22.17 ± 0.34
Clock	1.09 ± 0.30	0.61 ± 0.17	21.40 ± 0.13
Per2	2.93 ± 0.82	2.85 ± 0.54	10.88 ± 0.12
Cry2	2.83 ± 0.91	2.01 ± 0.40	8.54 ± 0.08
ULK1	0.85 ± 0.31	0.76 ± 0.27	1.88 ± 0.07
LC3 II	1.25 ± 0.08	0.97 ± 0.36	21.05 ± 0.17
Atg5	2.19 ± 1.05	2.17 ± 0.28	17.89 ± 1.77
Lamp1	5.79 ± 1.24	6.85 ± 3.98	9.54 ± 0.04
Rev-erb α	1.20 ± 0.33	0.94 ± 0.33	4.15 ± 0.04
C57 WD			
Bmal1	0.99 ± 0.24	0.67 ± 0.20	19.32 ± 0.14
Clock	0.67 ± 0.18	0.50 ± 0.09	21.28 ± 0.06
Per2	4.43 ± 1.89	3.33 ± 1.15	11.27 ± 0.12
Cry2	2.68 ± 1.12	1.90 ± 0.76	8.92 ± 0.12
ULK1	0.77 ± 0.43	0.63 ± 0.32	1.96 ± 0.22
LC3 II	0.95 ± 0.11**	0.91 ± 0.66	21.24 ± 0.05
Atg5	1.56 ± 0.58	1.41 ± 0.21*	17.65 ± 0.05
Lamp1	4.33 ± 1.81	4.16 ± 1.60*	9.85 ± 0.09
Rev-erb α	1.30 ± 0.37	1.07 ± 0.34	2.62 ± 1.47
C57 WD DD/DL			
Bmal1	-	-	-
Clock	0.80 ± 0.09	0.54 ± 0.08	19.05 ± 0.02**
Per2	6.41 ± 1.51**	3.87 ± 0.38*	7.71 ± 0.03**
Cry2	2.53 ± 0.99	1.88 ± 0.34	5.58 ± 1.48**

n = 4. *, $p < 0.05$, **,$p < 0.01$ versus C57 ND mice

Gene	Mesor	Amplitude	Acrophase ZT (h)
ULK1	-	-	-
LC3 II	-	-	-
Atg5	2.63 ± 0.91	1.54 ± 0.49	12.27 ± 0.08**
Lamp1	2.58 ± 0.27**	2.19 ± 0.92**	16.04 ± 0.25**
Rev-erb α	0.38 ± 0.18**	0.35 ± 0.16*	0.50 ± 0.07**
n = 4. *, p < 0.05, **,p < 0.01 versus C57 ND mice			

3. Circadian rhythmic expression patterns of autophagy-related genes in liver tissues

Autophagy is a highly conserved intracellular degradation system, and recently was shown to display circadian rhythms in mice. In the present study, we detected the expression levels of autophagy-related genes (ULK1, LC3Ⅱ, Atg5, and Lamp1) in liver tissues of mice (Table 2). As shown in Fig. 3, ULK1 and LC3Ⅱ in C57 WD + LD/DD mice were lost its circadian rhythms. The mesors of LC3Ⅱ in C57WD group and Lamp1 in C57 WD + LD/DD group were decreased compared with those in C57 ND group. Additionally, the amplitudes of Atg5 in C57 WD group were significantly attenuated compared with those in C57 ND group. Besides, the amplitudes of Lamp1 in both C57 WD and C57 WD + LD/DD groups were markedly reduced compared with those in control group. Moreover, the peak values of Atg5 and Lamp1 were dramatically decreased in C57 WD and C57 WD + LD/DD groups, respectively, compared with those in control group. Meanwhile, the peak periodic values of Atg5 and Lamp1 in C57 WD + LD/DD groups were different from those in control group.

4. Diurnal expression of Rev-erbα gene and its relationship with autophagy-related genes

The mechanism of the abnormal light/dark cycle stimulation causing changes in the circadian rhythm of autophagy-related genes have not been fully elucidated. The circadian clock gene Rev-erbα, known as nuclear receptor 1D1, plays a circadian oscillatory role in liver, heart, and skeletal muscle. Notably, Rev-erbα regulates the expression levels of several circadian target genes and plays regulatory roles in lipid metabolism [31]. Moreover, Rev-erbα deficiency resulted in deactivation of the Stk11–Ampk–Sirt1–Ppargc1-α signaling pathway, whereas autophagy was up-regulated, resulting in both impaired mitochondrial biogenesis and increased clearance [32]. Additionally, Huang et al. pointed out that the circadian clock can directly regulate the expression levels of autophagy-related genes through Rev-erbα in zebrafish liver [18]. In the current research, we attempted to indicate whether Rev-erbα could induce changes in rhythmic expression levels of autophagy-related genes in C57 WD + LD/DD group. We first examined the expression level of Rev-erbα in C57ND, C57WD and C57 WD + LD/DD groups (Table 2). As shown in Fig. 4, the mesors were significantly decreased in C57 WD + LD/DD group compared with those in C57ND group. The amplitudes of Rev-erbα were markedly inhibited in C57 WD + LD/DD group than

those in C57 ND group. Moreover, the peak periodic values in C57 WD + LD/DD group were markedly altered compared with those in control group (Fig. 4A). The rhythmic expression level of Rev-erbα changed in C57WD + LD/DD group.

In order to explore the relationship between Rev-erbα and autophagy-related genes, the ChIP assay was performed to indicate whether Rev-erbα could transcriptionally regulate the expression levels of autophagy-related genes. However, Rev-erbα promoted the expression level of Bmal1 as high as ~ 9.3-fold. Besides, Rev-erbα upregulated the expression levels of autophagy-related genes (ULK1 and Atg5) within ~ 3.45- and ~ 4.78-fold, respectively (Fig. 4B).

To further verify the effects of Rev-erbα on the expression levels of ULK1 and Atg5, siRNA was used to detect the expression levels of ULK1 and Atg5. In primary liver cells, when the expression level of Rev-erbα decreased to ~ 1.56-fold, the expression levels of ULK1 and Atg5 were reduced to ~ 0.96- and ~ 1.19-fold, respectively (Fig. 4C). The above-mentioned results indicated that the alteration of expression levels of autophagy-related genes induced by circadian rhythm sleep disorders may be accomplished through Rev-erbα.

Discussion

In the present study, we showed that long-term high-fat diet combined with abnormal light/dark cycle stimulation may lead to or aggaravate NAFLD and change circadian expression patterns of circadian clock genes and autophagy-related genes. The pathogenic mechanisms of NAFLD have not been well understood. However, circadian clock participates in lipid and glucose metabolism, and is associated with metabolic syndrome. The relationship between the pathogenesis of NAFLD and impairment of circadian has been previously reported by a number of scholars. For instance, shift workers with irregular sleep time are more susceptible to obesity and associated disorders such as NAFLD [33]. The circadian clock genes, such as Clock, Per2, and Rev-erbα regulate liver lipid metabolism, and circadian rhythm disorder leads to lipid accumulation in liver cells [34–36]. Moreover, a regular eating time and a proper sleep time can reduce the risk of metabolic syndrome and NAFLD [37]. In the present study, high-fat diet and abnormal light/dark cycle stimulation led to the accumulation of lipid and the abnormal expression levels of circadian clock genes in the liver of mice. However, the specific relationship between circadian rhythm disorder and the pathogenesis of NAFLD has not yet been fully elucidated.

Circadian rhythms are closely related to metabolism, while how such daily rhythms organize lipid metabolism in liver cells has not been fully understood. Apart from canonical lipolysis, autophagy, a highly conserved biological degradation process, contributes to lipid drops degradation. Consistent with our study, autophagy-related genes displayed circadian rhythms in both mice and in zebrafish [18, 38], and its periodic induction may provide a new association between daily rhythms and lipid metabolism. Autophagy has shown robust circadian clocks in the liver of mice, and it is accompanied by cyclic induction of autophagy-related genes involved in various steps of autophagy [17]. In the zebrafish liver, circadian rhythms directly regulate autophagy-related genes via Rev-erbα [18]. The master regulators of

autophagy, TFEB and TFE3, exhibited a circadian expression manner [39]. In the present research, Rev-erbα showed alteration of biological rhythm during 24 h that resulted from nutrient and light stimulation. Furthermore, we demonstrated that Rev-erbα could transcriptionally regulate the expression levels of ULK1 and Atg5. Meanwhile the changes in mRNA levels of Rev-erbα, ULK1, and Atg5 were consistent with results of ChIP assay. Therefore, it can be concluded that Rev-erbα may create an association between biological rhythm and autophagy, however, further research needs to be conducted to confirm this finding.

Selective autophagy termed ‘lipophagy’ may create a novel association between circadian clock and development and progression of NAFLD. In recent years, the function of autophagy in NAFLD has attracted scholars’ attention. Liver-specific knockout of the autophagy gene Atg7 in mice has displayed a remarkable lipid accumulation, which could mimick the human NAFLD condition [8]. Treatment with activators of autophagy could lower TG level in the liver and blood [40]. Tanaka et al. demonstrated that rubicon, an autophagy-inhibiting protein, could repress autophagy and prompt lipid accumulation in hepatocyte in NAFLD [41]. The present research revealed that the greater the changes in rhythmic expression levels of autophagy-related genes, the more lipid accumulated in the liver cells.

Conclusions

In summary, the current study revealed that high-fat diet and light stimulation may result in lipid accumulation in liver cells of mice, and alter expression levels of circadian clock genes and autophagy-related genes. Furthermore, Rev-erbα may create an association between circadian rhythms and autophagy.

Abbreviations

NAFLD	Non-alcoholic fatty liver disease
ZT	Zeitgeber time
Rev-erbα	nuclear receptor subfamily 1, group D, member 1
SCN	suprachiasmatic nucleus
Clock	Circadian locomotor output cycles kaput
Bmal1	brain and muscle encoding Arnt-like protein 1
Per1	Period1
Per2	Period2
Per3	Period3
Cry1	Cryptochrome1
Cry2	Cryptochrome2
TG	triglyceride
ULK1	unc-51 like kinase 1
LC3	microtubule-associated protein light chain 3
Atg5	autophagy related 5
Lamp1	lysosomal-associated membrane protein 1
ND	normal diet
WD	high-fat diet
DD/LD	abnormal light/dark cycle
FBS	fetal bovine serum
siRNA	Short interfering RNA
RT-qPCR	reverse transcription polymerase chain reaction
ChIP	Chromatin immunoprecipitation
PBS	phosphate-buffered saline
SD	standard deviation
H&E	hematoxylin and eosin

Declarations

Compliance with ethical standards

All procedures were in accordance with the ethical standards of the institution and approved by the Animal Care and Ethics Committee of Zhengzhou University, Zhengzhou, China.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Conflicts of interest

The authors have no competing interests to disclose.

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Author's contributions

Study design: ZZ, ZYW, BQ and XT. Study conduct: ZZ, ZYW, BQ, SFZ, JLL and XT. Data collection: ZZ, HFW, LLH and JX. Data analysis: ZYW, LQ, YRW and LW. Data interpretation: ZZ, BC, PPL and XT. Drafting manuscript: ZZ, BQ and XT. Approving the final version of the manuscript: All authors. ZZ, ZYW, BQ and XT take responsibility for the integrity of the data analysis. The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figures

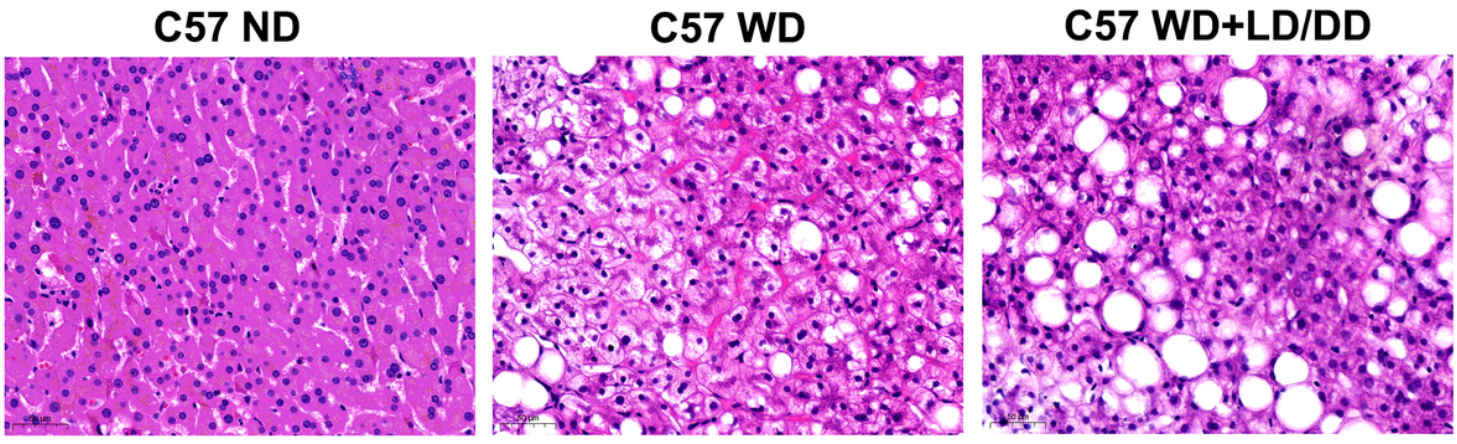

Figure 1

Representative images of hematoxylin and eosin (H&E) staining of the liver tissues from C57 ND, C57 WD, and C57 WD+LD/DD mice. The liver tissues of C57 ND, C57 WD, and C57 WD+LD/DD mice were obtained at ZT8 time-point. We performed H&E staining of the liver tissues, and lipid droplets were significantly

observed in C57 WD and C57 WD+LD/DD mice. Moreover, the lipid accumulation was markedly increased in C57 WD+LD/DD mice compared with that in C57 WD mice (Original magnification, ×100). All data were shown as mean ± standard deviation (SD) (n=4 for each group).

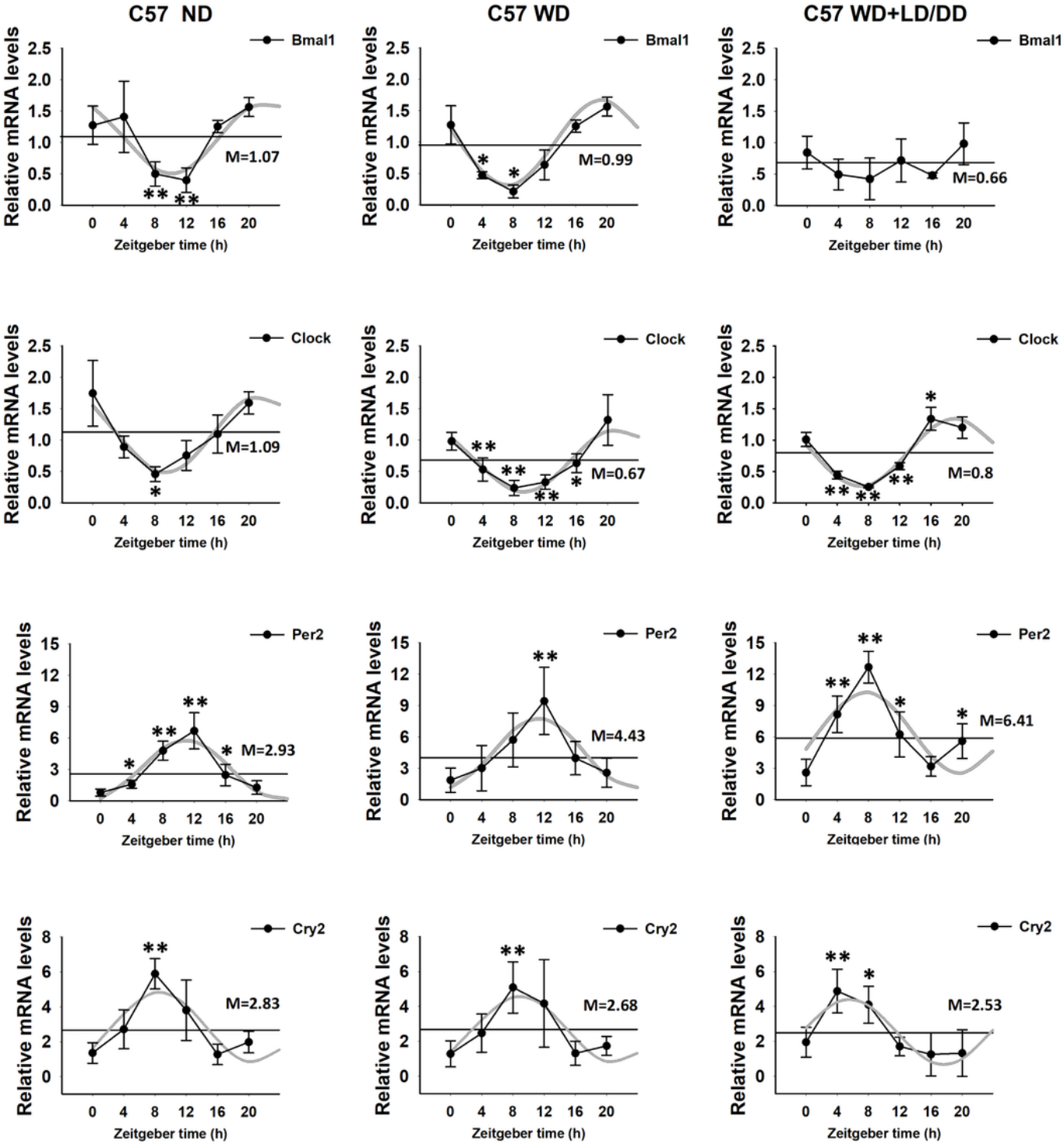

Figure 2

Expression levels of circadian clock genes in liver tissues of C57 ND, C57 WD, and C57 WD+LD/DD mice. The liver tissues of C57 ND, C57 WD, and C57 WD+LD/DD mice were obtained at ZT0, ZT4, ZT8, ZT12,

ZT16, and ZT20 time-point. The expression levels of circadian clock genes, such as Bmal1, Clock, Per2, and Cry2 were detected by RT-qPCR. These four clock genes showed 24-h circadian oscillations in C57 ND, C57 WD, and C57 WD+LD/DD mice, except for Bmal1 in C57 WD+LD/DD mice. The expression level of Per2 was remarkably increased in C57 WD+LD/DD mice compared with that in C57 ND mice. The mesors of Per2 were increased in C57 WD+LD/DD mice compared with those in control mice. The amplitudes of Per2 were heightened in C57 WD+LD/DD mice compared with those in C57 ND mice. The peak periodic values of Clock, Per2, and Cry2 were markedly altered in C57 WD+LD/DD mice compared with those in control mice. The mRNA levels were normalized to GAPDH. All data were expressed as mean ± SD at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 time-point (n=4 for each group), ∗,P< 0.05, ∗∗,P< 0.01 versus ZT0.

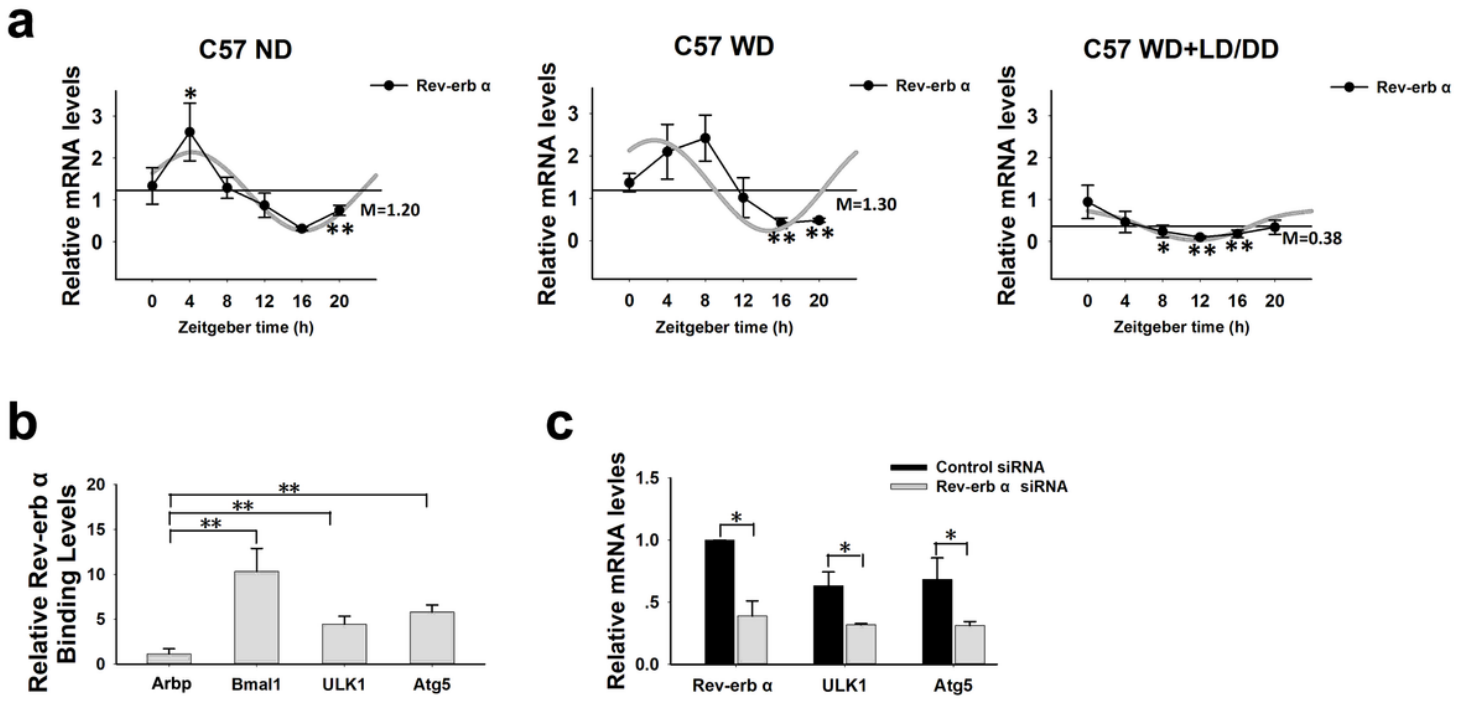

Figure 3

Circadian expression levels of autophagy-related genes in liver tissues of C57 ND, C57 WD, and C57 WD+LD/DD mice. Circadian rhythms of ULK1 and LC3Ⅱ in C57 WD+LD/DD mice were lost. The mesors of LC3Ⅱ in C57WD mice were remarkably reduced compared with those in C57 ND mice, and those of Lamp1 in C57 WD+LD/DD mice were significantly decreased compared with those in C57 ND mice. The amplitudes of Atg5 in C57 WD mice were notably repressed than those in C57 ND mice, and the amplitudes of Lamp1 in both C57 WD mice and C57 WD+LD/DD mice were remarkably reduced compared with those in control mice. The peak expression levels of Atg5 in C57 WD mice and Lamp1 in C57 WD+LD/DD mice were significantly decreased compared with those in control mice. The peak periodic values of Atg5 in C57 WD+LD/DD mice and Lamp1 in C57 WD+LD/DD mice were markedly different from those in control mice. The mRNA levels were normalized to GAPDH. All the data were presented as mean ± SD at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 time-point (n=4 for each group), ∗,P< 0.05, ∗∗,P< 0.01 versus ZT0.

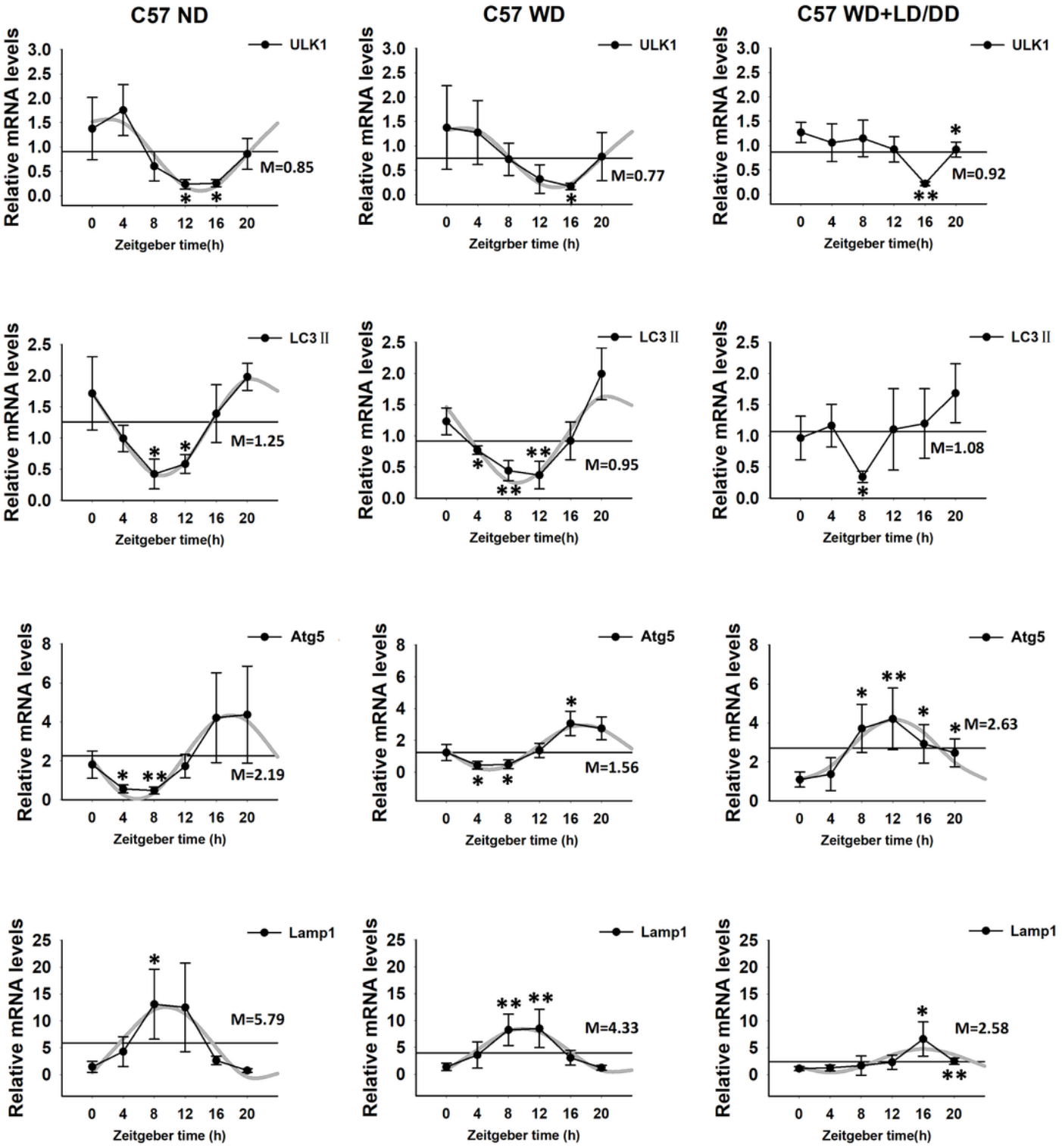

Figure 4

Circadian expression level of Rev-erbα and its relationship with autophagy-related gene. (A) 24-h expression levels of Rev-erbα in C57ND, C57WD, and C57 WD+LD/DD mice. The mesors in C57 WD+LD/DD mice were significantly decreased compared with those in C57 ND mice. The amplitudes of Rev-erbα in C57 WD+LD/DD mice were notably repressed compared with those in C57 ND mice. The peak periodic value in C57 WD+LD/DD mice was significantly altered compared with that in control group. The

mRNA levels were normalized to GAPDH. All the data were presented as mean ± SD at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 time-point (n=4 for each group), ∗,P< 0.05, ∗∗,P< 0.01 versus ZT0. (B) ChIP assay was performed to indicate whether Rev-erbα could transcriptionally regulate the autophagy-related genes. There was no significant enrichment in Rev-erbα to promote RORα as negative control, and Bmal1 was promoted as high as ~9.3-fold as positive control. Besides, the autophagy-related genes, ULK1 and Atg5, demonstrated ~3.45- and ~4.78-fold enrichment of Rev-erbα recruitment to their promoter, respectively. The mRNA levels were normalized to GAPDH. All the data were presented as mean ± SD at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 time-point. n=4 for each group, ∗,P< 0.05, ∗∗,P< 0.01 versus control. (C) Primary liver cells were isolated form mice, and we utilized siRNA to decrease the expression of Rev-erbα. When the expression of Rev-erbα decreased to ~1.56-fold, the expressions of ULK1 and Atg5 were reduced to ~0.96- and ~1.19-fold, respectively. The mRNA levels were normalized to GAPDH. All the data were presented as mean ± SD (n=4 for each group), ∗,P< 0.05, ∗∗,P< 0.01 versus control siRNA.