Coordination between the circadian clock and androgen signaling is required to sustain rhythmic expression of Elovl3 in mouse liver

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ELOVL3 is a very long-chain fatty acid elongase, and its mRNA levels display diurnal rhythmic changes exclusively in adult male mouse livers. This cyclical expression of hepatic Elovl3 is potentially controlled by the circadian clock, related hormones, and transcriptional factors. It remains unknown, however, whether the circadian clock, in conjunction with androgen signaling, functions in maintaining the rhythmic expression of Elovl3 in a sexually dimorphic manner. Under either zeitgeber or circadian time, WT mouse livers exhibited a robust circadian rhythmicity in the expression of circadian clock genes and Elovl3. In contrast, male Bmal1−/− mice displayed severely weakened expression of hepatic circadian clock genes, resulting in relatively high, but nonrhythmic, Elovl3 expression levels. ChIP assays revealed that NR1D1 binds to the Elovl3 promoter upon circadian change in WT mouse livers in vivo, and a diminished binding was observed in male Bmal1−/− mouse livers. Additionally, female mouse livers exhibited constant low levels of Elovl3 expression. Castration markedly reduced Elovl3 expression levels in male mouse livers but did not disrupt circadian variation of Elovl3. Injection of female mice with 5α-dihydrotestosterone induced Elovl3 rhythmicity in the liver. In AML12 cells, 5α-dihydrotestosterone also elevated Elovl3 expression in a time-dependent manner. In contrast, flutamide efficiently attenuated this induction effect. In conclusion, a lack of either the circadian clock or androgen signaling impairs hepatic Elovl3 expression, highlighting the observation that coordination between the circadian clock and androgen signaling is required to sustain the rhythmic expression of Elovl3 in mouse liver.

Elovl3 (elongation of very long-chain fatty acids 3), also known as Cig30, was initially identified as a thermogenesis-related gene after its expression in brown adipose tissue was found to be highly elevated in response to cold stimulation (1). Accumulating reports indicate that significant Elovl3 expression also occurs in white adipose tissue, liver, and triglyceriderich glands, such as the sebaceous and meibomian glands (2–5). As a member of the Elovl gene family, Elovl3 encodes an enzyme that functions in the synthesis of C20–C24 saturated and mono-unsaturated very long-chain fatty acids (VLCFAs). 5 It was previously demonstrated that Elovl3−/− mice exhibit a clear skin phenotype with an impaired barrier function resulting from changes in the synthesis of C20–C24 saturated and mono-unsaturated VLCFAs, triglyceride synthesis, and sebum formation (5). Male Elovl3−/− mice also display a diminished capacity to accumulate fat within brown adipose tissue (6). Additionally, male and female Elovl3−/− mice possess reduced hepatic lipogenic gene expression and triglyceride content and are also resistant to diet-induced obesity (7). These findings indicate that ELOVL3 acts as an important regulator of triglyceride and lipid droplet formation in skin, adipose tissue, and liver. To further determine the physiological significance of Elovl3, an increasing number of studies aim to clarify its upstream regulatory mechanisms (2–4, 8, 9). Interestingly, it was observed that VLCFAs enhance adipogenesis through the co-regulation of ELOVL3 and PPARγ in 3T3-L1 adipocytes (9). It was also found that vitamin D/vitamin D nuclear hormone receptor modulates the fatty acid composition in mouse subcutaneous white adipose tissue through the direct inhibition of Elovl3 expression (2). Additionally, several elegant reports provided evidence that Elovl3 expression exhibits a robust circa-

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5 The abbreviations used are: VLCPA, very long-chain fatty acid; CCG, clock-controlled gene; RORE, REV-ERBs/RORs response element (RORE); ZT, zeitgeber time; CT, circadian time; DHT, 5α-dihydrotestosterone; DD, constant darkness; LD, light-dark; qPCR, quantitative real-time PCR; ANOVA, analysis of variance.
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Dian rhythmicity in mouse livers, where Clock mutant mice possess a constant but relatively high level of hepatic Elovl3 expression (3, 4, 10).

The circadian clock system is ubiquitous in nearly all mammalian organs, tissues, and cells (11), where it orchestrates numerous physiological functions and behaviors within the body (12). The central pacemaker of the circadian clock resides in the suprachiasmatic nucleus of the hypothalamus (13), which regulates the subsidiary circadian oscillators in peripheral tissues and cells via humoral and neuronal cues in a hierarchical manner (14, 15). Molecularly, the suprachiasmatic nucleus and peripheral circadian oscillators share an interlocked transcriptional-translational feedback loop involving a set of canonical circadian clock genes, including Bmal1, Clock, Per1/Per2/Per3, Cry1/Cry2, Nr1d1, and Dbp (16). In addition to maintaining oscillations of circadian clock, the proteins encoded by circadian clock genes (BMAL1, CLOCK, NR1D1, and DBP) also maintain the rhythmic expression of clock-controlled genes (CCGs) through binding to promoter E-box, REV-ERBs/RORs response element (RORE), and D-box elements. Using cDNA microarray or Northern blot analysis, prior reports have shown that a diurnal cyclical expression of Elovl3 exists in male mouse liver (3, 4, 10). Clock mutation results in nonrhythmic expression and a marginal increase in the levels of Elovl3 expression in male mouse livers (4, 10). These findings suggest that Elovl3 is a potential CCG. Despite one previous study providing plausible evidence that the circadian clock regulates Elovl3 expression through NR1D1 inhibition (4), additional studies are required to understand and verify this proposed mechanism.

Sexual dimorphism is a common feature of male and female mouse liver. Existing evidence indicates that distinct sex hormone (androgens and estrogens) signaling, and the resulting growth hormone signaling, in the male and female liver are major driving factors underlying this hepatic sexual dimorphism (17, 18). In a recent study, a total of 6612 differentially expressed genes exhibiting at least a 1.5-fold change were identified between male and female mouse livers (19). Intriguingly, two aforementioned studies demonstrated that Elovl3 belongs to the family of hepatic sexually dimorphic genes, with high and rhythmic expression being observed in male mouse livers and undetectable or low expression in female mouse livers (3, 4). This suggests that androgen signaling might play an essential role in determining the hepatic sexual dimorphism of Elovl3 expression; however, evidence is lacking regarding whether androgen supplementation elicits hepatic Elovl3 expression in female mouse livers in vivo or if androgen treatment of hepatocytes increases Elovl3 expression in vitro. Further studies are urgently required to address these issues.

Here, we demonstrate that under zeitgeber time (ZT) or circadian time (CT) conditions, male Bmal1−/− mice exhibited nonrhythmic expression of Elovl3 in liver while maintaining Elovl3 expression at relatively high levels. This was in contrast to observations of male WT mouse livers, where robust circadian rhythmicity in Elovl3 expression was observed to exhibit anti-phase circadian variations with respect to Nr1d1. ChIP assays indicated that Nr1d1 was recruited to a putative RORE site at the Elovl3 promoter in a circadian manner in male WT mice livers in vivo, and this was attenuated in Bmal1−/− mice. In addition, we confirmed that Elovl3 is a hepatic sexually dimorphic gene exhibiting high and rhythmic expression in male mice and low and constant expression in female mice. Although it did not profoundly alter the expression of circadian clock genes, castration greatly decreased Elovl3 expression in mouse liver. 5α-Dihydrotestosterone (DHT) treatment of female mice not only vastly elevated Elovl3 expression at CT0 and restored its circadian rhythmicity in female mouse liver in vivo, but in vitro, it also significantly increased the expression of Elovl3 in AML12 cells in a time-dependent manner. Flutamide treatment also efficiently reduced Elovl3 expression induced by DHT in AML12 cells. Our current study therefore provides novel findings that extend our current understanding of how the circadian clock and androgen signaling synergistically regulate rhythmic Elovl3 expression in mouse liver, highlighting the significance of circadian clock and androgen signaling in coordinating hepatic lipid metabolism.

Results
Loss of Bmal1 results in hepatic triglyceride accumulation and elevated and arrhythmic Elovl3 expression

To investigate the physiological role of the circadian clock in regulating murine hepatic Elovl3 mRNA expression and its associated hepatic lipid metabolism, we used Bmal1−/− mice and their WT siblings (control). PCR genotyping of tail biopsies, immunohistochemistry, and Western blot analysis were initially used to confirm complete deficiency of BMAL1 protein in Bmal1−/− mice compared with WT (Fig. S1, A–C). As expected, Bmal1−/− mice completely lost circadian locomotor activity in constant darkness (DD), whereas day–night rhythms were observed under light–dark (LD) cycles due to the masking effect of the environmental lighting cycle (Fig. S1D). Additionally, Oil Red O staining for neutral lipids was increased in livers of male Bmal1−/− mice (Fig. 1A), and the hepatic triglyceride content was nearly double that of WT mice (Fig. 1B).

We then examined the temporal expression profiles of several circadian clock genes (Bmal1, Nr1d1, and Dbp) and Elovl3 in the livers of male WT and Bmal1−/− mice. As shown in Fig. 1 (C and D), Bmal1−/− mice exhibited undetectable hepatic Bmal1 expression compared with the robust circadian rhythmicity of Bmal1 observed in WT mice under both ZT and CT conditions (Cosinor analysis, p < 0.001). Indeed, in WT mice, both the Nr1d1 and Dbp transcripts exhibited a cyclical expression pattern (Cosinor analysis, p < 0.001) that was opposite that of the Bmal1 expression profile (Fig. 1, C and D). In contrast, in WT mice hepatic Elovl3 mRNA exhibited a similar expression pattern to that of Bmal1, with a trough of expression at ZT12 (Fig. 1, C and D). In Bmal1−/− mice, the loss of BMAL1 not only greatly inhibited hepatic Nr1d1 mRNA expression but also led to its nonrhythmic expression (Fig. 1, C and D). Interestingly, the expression of Dbp was significantly attenuated and phase-shifted in Bmal1−/− mouse liver under ZT conditions (Fig. 1C); however, Dbp expression maintained its circadian rhythmicity (Cosinor analysis, p < 0.01). The expression of Dbp was remarkably suppressed and completely lost its circadian rhythmicity in Bmal1−/− mice under CT conditions (Fig. 1D). It should be noted that the diurnal rhythmic expression profile of
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Elovl3 was completely abolished in the livers of Bmal1\(^{-/-}\) mice, with significant, but constant, levels of expression being noted, where elevated levels were observed at ZT4, ZT8, and ZT12 compared with those of WT mice (Fig. 1C). Similarly, the expression levels of Elovl3 were constant, with elevated levels observed at CT8, CT12, and CT16 compared with those of WT mice (Fig. 1D).

Diurnal recruitment of NR1D1 to the Elovl3 promoter in mouse liver in vivo

NR1D1, also known as REV-ERB\(\alpha\), is a nuclear hormone-related protein that functions as a transcriptional repressor of its target genes (20, 21). A computational algorithm (http://jaspar.genereg.net/)\(^6\) (44) identified a putative RORE site within the Elovl3 promoter between \(-831\) and \(-841\) (Fig. 2A). To determine whether NR1D1 binds to the Elovl3 putative RORE site in mouse liver with a circadian change, we performed ChIP assays using a NR1D1 antibody in the livers of male WT and Bmal1\(^{-/-}\) mice using samples collected at two representative time points (CT8 and CT20). Bmal1-RORE was used as a validated positive control (22), and a DNA region located in the first intron of Elovl3 was used as a negative control (Fig. 2A). ChIP results revealed NR1D1 binding to the genomic Elovl3-RORE of WT mouse livers with a circadian change (Fig. 2B) that was similar to that of Bmal1-RORE, with strong binding at CT8 and weak binding at CT20. Additionally, NR1D1 recruitment was diminished at both Elovl3-RORE and Bmal1-RORE in Bmal1\(^{-/-}\) mice, consistent with the dramatic reduction of Nrd1d1 mRNA levels observed in Bmal1\(^{-/-}\) mice.

Sexually dimorphic pattern of Elovl3 expression in mouse liver

To determine whether there is sexual dimorphism in hepatic Elovl3 expression, we determined the expression profile of Elovl3, as well as three other canonical clock genes (Bmal1, Per2, and Dbp), in male and female WT mouse livers at two representative time points (CT0 and CT12) using a quantitative real-time PCR (qPCR) assay. The results are shown in Fig. 3. All clock genes examined exhibited robust circadian changes in their mRNA expression in both male and female mouse livers (Fig. 3). Additionally, Per2 and Dbp exhibited the expected anti-phase circadian variations with respect to Bmal1 (Fig. 3). It should be noted that hepatic Elovl3 expression exhibited a clear sexual dimorphism, with a constant low level of expression.

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observed in female mice and profound circadian changes in hepatic expression detected in male mice, along with significantly higher levels of expression (Fig. 3).

Castration reduces the circadian variations in Elovl3 in male mouse livers

To test whether androgen signaling participates in maintaining the sexually dimorphic pattern of Elovl3 expression, we measured the mRNA expression of Elovl3 and the other three canonical circadian clock genes (Bmal1, Per2, and Dbp) in castrated (Cast) or control (Cont) mouse livers at CT0 and CT12 (Fig. 4). As shown in Fig. 4A, control mice maintained circadian changes in serum testosterone, with high levels at CT0 and low levels at CT12. Castration significantly decreased serum testosterone concentration compared with that of the control group and resulted in a loss of rhythmicity (Fig. 4A). Castration clearly did not alter Per2 and Dbp transcription, with respect to either circadian variation or expression level (Fig. 4B). Additionally, the circadian changes in Bmal1 transcription were unaffected, despite the observed significant decrease in Bmal1 expression levels at CT0 due to castration (Fig. 4B). Interestingly, castration also markedly decreased Elovl3 mRNA expression levels at both CT0 and CT12 compared with levels observed in the control mice, although the circadian changes in Elovl3 transcription were unchanged.

DHT injection results in Elovl3 rhythmic expression in female mouse livers

To further determine whether DHT supplementation could elicit circadian changes in Elovl3 transcription in female mouse livers, we examined the expression of Elovl3 and three other circadian clock genes (Bmal1, Per2, and Dbp) at CT0 and CT12 in female mice injected with either sesame oil (Cont) or DHT (Fig. 5). All three circadian clock genes (Bmal1, Per2, and Dbp) displayed robust circadian changes in expression in the livers obtained from either control or DHT mice (Fig. 5). DHT injection did not alter the expression levels of Bmal1 and Per2, despite the observed significant decrease in Dbp expression at CT12 after DHT supplementation. Surprisingly, DHT injection elicited prominent circadian variations in Elovl3 expression, with a sharp increase in Elovl3 expression at CT0 following DHT treatment compared with that observed in the control group and a significant reduction in expression at CT12 compared with that observed at CT0 (Fig. 5).

DHT treatment increases Elovl3 expression in AML12 cells via androgen receptor signaling

To assess whether the stimulatory effect of androgen signaling on Elovl3 expression occurred directly in mouse hepatocytes, we examined the expression levels of Elovl3 and circadian
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Figure 4. Effect of castration on serum testosterone concentrations and the mRNA expression profiles of circadian clock genes and Elovl3 in male mouse livers. Serum and liver samples from control (Cont) and castrated (Cast) mice were collected at the indicated times (CT0 and CT12). A, serum testosterone concentrations were measured using an ELISA kit. B, total RNA was extracted from the livers of castrated and control mice, and the mRNA levels were quantified by qPCR. A two-way ANOVA with Bonferroni’s post-test was performed to investigate the effects of castration and time on the expression of the indicated genes. Asterisks indicate significant differences. *, p < 0.01; **, p < 0.01; ***, p < 0.001. Error bars, S.E.

Figure 5. Effect of DHT injection on the mRNA expression profiles of circadian clock genes and Elovl3 in female mouse livers. Total RNA was extracted from the livers of DHT-injected and control (Cont) female mice collected at CT0 and CT12, and the mRNA levels were quantified by qPCR. A two-way ANOVA with a Bonferroni’s post-test was performed to investigate the effects of castration and time on the expression of the indicated genes. Asterisks indicate significant differences. *, p < 0.01; **, p < 0.01; ***, p < 0.001. Error bars, S.E.

clock genes in AML12 cells after treatment with 1 μM DHT. As shown in Fig. 6A, the mRNA expression of circadian clock genes (Bmal1, Per2, Dbp, and Nr1d1) did not significantly change following DHT treatment. Only minor reductions in Dbp and Nr1d1 mRNA expression were observed at 2 h compared with levels at 0 h (control). DHT treatment, however, gradually increased Elovl3 mRNA expression levels in a time-dependent manner (Fig. 6B). Additionally, flutamide, a selective antagonist of the androgen receptor, efficiently blocked the induction effect of DHT on Elovl3 expression (Fig. 6C), providing evidence that DHT stimulates hepatic Elovl3 expression through androgen receptor signaling.

Discussion

Several prior reports have shown that Elovl3 transcripts exhibit a robust circadian rhythmic expression in male mouse livers (3, 4, 10). Additionally, it was reported that mutation of the Clock gene completely abolished the daily rhythmicity of Elovl3 expression (4, 10). The existing evidence suggests that the circadian clock system in the mouse liver is at least somehow linked with the rhythmic expression of Elovl3. Here, we further dissected the role of the circadian clock in regulating Elovl3 cyclic transcription using Bmal1+/− mice. It is widely accepted that BMAL1 is a core transcriptional activator controlling the positive limb of circadian oscillators. In the livers of Bmal1+/− mice, Dbp mRNA levels are low and lose the rhythmicity normally seen under CT conditions (23). Mice with a liver-specific disruption of Bmal1 also exhibit a nearly complete loss of liver expression of Nr1d1 and Dbp (24). Additionally, two recent reports indicated that both Bmal1 global and liver-specific knockout mice accumulated more neural lipids in the liver compared with that observed in their control group, demonstrating the significance of the circadian clock gene Bmal1 in regulating liver lipid homeostasis (22, 25). In agreement with these previous findings, we confirmed the elevated amount of triglyceride and severely reduced Nr1d1 and Dbp expression levels in the livers of Bmal1+/− mice (Fig. 1, A–D).
Importantly, our results revealed that Elovl3 expression completely lost its circadian rhythmicity and was expressed at relatively high levels in the livers of Bmal1−/− mice (Fig. 1, C and D), in agreement with previous findings in Clock mutant mice (4, 10). Interestingly, a prior study reported that Elovl3−/− mice gained less triglyceride in their livers after feeding with a regular or high-fat diet (7), which is consistent with our finding of an increased amount of triglyceride in Elovl3 expression in Bmal1−/− mouse livers. Our current findings using Bmal1−/− mice have therefore provided further evidence that the cycling of Elovl3 expression is at least in part under the control of the circadian clock, suggesting that the circadian clock may coordinate hepatic lipid metabolism through orchestrating Elovl3 expression.

It is established that the transcriptional activity of the CLOCK-BMAL1 heterodimer reaches its maximal level during the second half of the light phase (26). Our current study, however, showed that Elovl3 mRNA expression did not coincide with the diurnal changes in CLOCK-BMAL1 activity, and this was in agreement with other previous studies (3, 4, 10). Instead, Elovl3 mRNA expression displayed a circadian rhythm pattern, which was in anti-phase to two CLOCK-BMAL1–dependent genes, specifically Nr1d1 and Dbp (Fig. 1, C and D). Therefore, it is reasonable to speculate that Elovl3 rhythmic expression may be indirectly under the control of CLOCK-BMAL1 activity. Identifying the intermediates that transmit CLOCK-BMAL1–dependent information to the pathway controlling Elovl3 gene expression is necessary. NR1D1, a component of the additional loop of the circadian clock, usually functions as a transcriptional repressor (20, 21). It has been demonstrated that NR1D1 represses Bmal1 transcription (27). Additionally, Nr1d1 is a CCG under the direct regulation of the CLOCK-BMAL1 heterodimer through its E-box elements (28). Intriguingly, a prior report has shown that overexpression of REV-ERBα significantly reduces Elovl3 promoter–driven luciferase activity (4). Considering the presence of a putative RORE within the Elovl3 promoter (Fig. 2A) and the anti-phase expression profile of Elovl3 with respect to NR1D1 peak activity, it is reasonable to deduce that NR1D1 may transmit CLOCK-BMAL1 information to orchestrate the rhythmic expression of Elovl3 in WT mouse livers through binding to the Elovl3-RORE. Consistent with this, our data indicated that the non-rhythmic and low expression of Nr1d1 in Bmal1−/− mouse liver, under both ZT and CT conditions, is accompanied by consistent and relatively high levels of Elovl3 (Fig. 1, C and D), providing further evidence that Nr1d1 represses Elovl3 expression. Indeed, our ChIP assay revealed that there is a circadian change of NR1D1 binding to Elovl3-RORE in WT mouse livers, with strong binding at CT8 and weak binding at CT20 (Fig. 2B), and the recruitment of NR1D1 to Elovl3-RORE is attenuated in Bmal1−/− mouse livers (Fig. 2B). Therefore, our current data provide strong evidence that NR1D1, acting as a repressor, regulates hepatic Elovl3 rhythmic expression through binding to Elovl3-RORE, and diminished recruitment of NR1D1 results in elevated expression of Elovl3 in Bmal1−/− mouse livers.

Regarding the identity of additional intermediates, SREBP1 has been proposed to be responsible for the activation of Elovl3 in mouse liver (4). SREBP1 acts as a transcriptional integrator of circadian and nutritional cues within the liver. Prior reports indicated that SREBP1-mediated transcription is altered in Bmal1−/− and Nr1d1−/− mice (29, 30). Conversely, the daytime, food-induced resetting of the clock in WT mice has been shown to result in a 12-h phase shift in SREBP1 activation and a rescue of its rhythmic activity in Cry1−/−/Cry2−/− mice (31, 32). Previous findings have shown that the overexpression of SREBP1a and -1c, but not SREBP2, robustly enhanced Elovl3 promoter–driven luciferase activity in AML12 cells (4). Therefore, SREBP1 may act as an activator capable of stimulating Elovl3 expression in mouse liver under the dual regulation of the circadian clock and nutritional cues. PPARα is an important
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transcription factor for many target genes related to fatty acid oxidation and de novo lipogenesis. PPARα has previously been identified as a direct target gene of the CLOCK-BMAL1 heterodimer via an E-box–dependent mechanism (33). PPARα has also been demonstrated to act as a potent inducer of Elovl3 expression in rodent brown adipocytes (34). Therefore, PPARα may act as a bridge connecting the circadian clock and Elovl3 transcription in mouse liver. A prior report, however, demonstrated that there were no significant differences in Elovl3 mRNA expression in the livers of WT and PPARα−/− mice at different times during the day, negating the possibility that it acts as a mediator between the circadian clock and Elovl3 transcription in mouse liver (3).

Sexually dimorphic gene expression is a common phenomenon found between male and female livers. One elegant report showed that circadian clock system is necessary to sustain sex dimorphism in mouse liver metabolism (45). Specifically, the hepatic Elovl3 mRNA expression becomes constitutively low, and daily variations are completely abolished in double mutant Cry1−/−Cry2−/− (Cry−/−) male mice compared with WT. In addition, the hepatic Elovl3 expression in Cry−/− males exhibits a feminized pattern of Elovl3 expression which is similar to WT females and Cry−/− females. Using a Northern blotting approach, two prior studies have shown that Elovl3 belongs to the sexually dimorphic gene family in mouse liver, with rhythmic expression occurring in male mouse livers and undetectable expression being observed in female mouse livers (3, 4). Consistently, our qPCR data indicated that the Elovl3 expression levels were high and cyclical in male mouse livers, whereas expression levels were low and maintained at a constant level in female mouse livers (Fig. 3). The only discrepancy between our study and the two prior studies is that we detected low levels of Elovl3 expression, whereas the other studies did not detect Elovl3 transcripts in female mouse livers. We hypothesized that this inconsistency may also arise from the sensitivity of the two different methods. Specifically, qPCR is more sensitive than Northern blotting and can detect low levels of Elovl3 expression in female mouse livers. In support of this, one other previous report identified significant levels of Elovl3 transcripts in female mouse livers. We hypothesized that this inconsistency may also arise from the sensitivity of the two different methods. 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In conclusion, our current data suggest that the rhythmic expression of Elovl3 is at least in part under the control of the circadian clock system through cyclic recruitment of NR1D1 to the Elovl3 promoter. Additionally, we confirmed that the hepatic expression of Elovl3 is sexually dimorphic, with a high and rhythmic expression occurring in male mice and a low and constant expression observed in female mice. Finally, we extended the current understanding of androgen regulation of Elovl3 expression in mouse liver by providing evidence that androgen supplementation may restore rhythmic Elovl3 expression in female mouse liver through androgen receptor signaling. Therefore, we propose that coordination between the circadian clock and androgen signaling is required to sustain rhythmic Elovl3 expression in mouse liver. Although the underlying mechanism requires further investigation, our study may provide an opportunity to gain new insights into the importance of the circadian clock and androgen signaling in determining the sexual dimorphism and rhythmic expression of hepatic genes, and our results also provide novel insights into hepatic lipid homeostasis.

Experimental procedures

Animal experiments and behavior analysis

Male and female C57BL/6J mice aged 8–10 weeks were purchased from the Laboratory Animal Center of the Fourth Military Medical University (Xi’an, China). Bmal1+/− mice of the original mixed background (C57BL/6J and 129SV) were obtained from the National Resource Center of Model Mice (Nanjing, China) (23). Bmal1+/− mice were back-crossed with
C57BL/6J mice for at least five generations. For the reproductive disorders in Bmal1−/− mice, Bmal1−/− mice were generated through breeding of Bmal1−/− mice. Tail biopsies were collected for genotyping using multiplex PCR with specific primers (Common-F, 5'-GCCCATAGCTGATTTGAAG-3'; WT-R, 5'-GCCACATCAGCTTAAA-3'; and Mut-R, 5'-GCCTGAAGAAGGAGATCAGC-3'). WT littermates generated through the breeding of Bmal1+/− mice were used as a control group for the Bmal1−/− mice. Mice were housed individually in light-tight, ventilated closets in a temperature- and humidity-controlled facility with ad libitum access to food and water, unless otherwise stated. All mice were maintained under a 12-h/12-h LD cycle before the indicated experiments were performed. All animal procedures were approved and performed under the control of the Guidelines for Animal Experiments by the Committee for Ethics on Animal Care and Experiments of Northwest A&F University.

For the behavior analysis experiment, a subset of male Bmal1−/− mice and their male WT littermates (n = 5 for each genotype) were placed individually into an isolated LD box equipped with a passive IR sensor for 2 weeks. Following this, the mice were then released into DD under free-running conditions. CT indicates the phase of the animal's endogenous circadian rhythm while under free-running conditions, whereas CT0 marks the beginning of the subjective day, and CT12 marks the beginning of the subjective night. Locomotor activity was recorded every 5 min with the IR sensor and analyzed using ClockLab software (Actimetrics, Wilmette, IL).

For experiments involving WT and Bmal1−/− mice under ZT or CT conditions, male WT and Bmal1−/− mice aged 8–10 weeks were housed individually in light-tight, ventilated closets under a 12-h/12-h LD cycle for at least 2 weeks with ad libitum access to food and water. For the ZT condition experiment, male WT and Bmal1−/− mice were euthanized at six time points (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20; n = 3 per group for each time point). Liver samples were then collected for total RNA extraction. For the CT condition experiment, male WT and Bmal1−/− mice were housed as described above and then released into DD under free-running conditions. At the start of the second day under CT conditions, male WT and Bmal1−/− mice were euthanized at six time points (CT0, CT4, CT8, CT12, CT16, CT20; n = 3 per group for each time point). Liver samples were then collected for total RNA extraction.

For the male and female mouse experiment, male and female WT mice aged 8–10 weeks were housed individually in light-tight, ventilated closets under a 12-h/12-h LD cycle for at least 2 weeks with ad libitum access to food and water. The mice were then released into DD under free-running conditions. Beginning on the second day under CT conditions, male and female WT mice were euthanized at two time points (CT0 and CT12; n = 3 per group for each time point). Liver samples were then collected for total RNA extraction.

For the castration experiment, male WT mice aged 8–10 weeks were castrated or sham-operated (control) (n = 6 mice/group). All operative procedures were performed under pentobarbital anesthesia (50 mg/kg body weight, by intraperitoneal injection). An incision was first made in the wall of the abdome. The testis with the epididymis was then removed following seminal duct ligation. After this operation, the mice were housed individually in light-tight, ventilated closets under a 12-h/12-h LD cycle for 2 weeks. The mice were then released into DD under free-running conditions. At the start of the second day under CT conditions, the mice from the castrated and control groups were euthanized at two time points (CT0 and CT12; n = 3 per group for each time point). Blood samples were then collected for serum testosterone level measurement using an ELISA kit, and liver samples were collected for total RNA extraction.

For the DHT (HY-A0120, MedChemExpress, Monmouth Junction, NJ) treatment experiment, female WT mice aged 8–10 weeks were randomly divided into two groups (DHT and control; n = 6 mice/group). First, 9 mg of DHT was dissolved into 30 ml of sesame oil to prepare a DHT solution (300 μg/ml). The mice in the two groups were housed individually in light-tight, ventilated closets under a 12-h/12-h LD cycle for 2 weeks. At the start of the second week during the LD cycle, mice in the DHT treatment group were injected subcutaneously with DHT (2 mg/kg body weight) every day at ZT12 (a total of seven injections), whereas the control mice were injected with a corresponding volume of sesame oil based on their body weights (37). After 2 weeks under the LD cycle, the mice were released into DD under free-running conditions. The DHT and control mice received one further injection of DHT or sesame oil, respectively, at CT12 on the first day under DD conditions. At the start of the second day under CT conditions, mice in the DHT and control groups were euthanized at two time points (CT0 and CT12; n = 3 per group). Liver samples were then collected for total RNA extraction.

**Immunohistochemistry**

Immunohistochemistry procedures were performed as described previously (38). Liver specimens from male WT and Bmal1−/− mice were collected at ZT0 and ZT12 and fixed with 4% paraformaldehyde and then embedded in paraffin-wax using standard protocols. Five-micrometer-thick sections were deparaffinized with xylene and ethanol, and then antigen-retrieval was performed by pressure cooking in a citric acid salt mixture (1.8 mM citrate and 8.2 mM sodium citrate, pH 6.0) for 15 min. Sections were then immersed in PBS containing 0.2% Triton X-100. Prior to diaminobenzidine (DAB) labeling, immunohistochemical staining of the slices was performed using an UltraSensitive™ SP (rabbit) IHC kit (Fuzhou Maixin Biotech, Fuzhou, China). Briefly, the sections were pretreated with 3% hydrogen peroxide solution (Reagent A in the IHC kit) for 15 min and then blocked with 10% goat serum (Reagent B in the IHC kit) for 1 h at 37 °C. The primary antibody to BMAL1 (Abcam, ab93806, Cambridge, UK) was diluted in PBS (1:1000 dilution) containing 1% BSA, 1% fetal bovine serum, and 0.1% Triton X-100. Following overnight incubation at 4 °C, the sections were washed extensively with PBS containing 0.3% Tween 20. The samples were then incubated with a goat anti-rabbit secondary antibody conjugated to biotin (reagent C in the IHC kit), diluted in the same PBS solution as the primary antibody, and then incubated for 1 h at 37 °C and washed again. For DAB labeling, the sections were incubated with horseradish.
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Table 1

| Gene       | Forward primer (5'–3')                          | Reverse primer (5'–3')                          | Product size (bp) |
|------------|-----------------------------------------------|-----------------------------------------------|------------------|
| mBmal1 RORE| AGCGGATTTGGCTGAAAGAT                             | ACCTCCGCGCGCAGCTTACT                        | 72               |
| mBmal1 RORE| TACCTTCAGACTGGAAG                               | AAAAAAGGGTTACCCCTCTGG                       | 124              |
| mElovl3 intron1 | GATATAGCCTGGCCACACA                | COTCTGGGAGATTTAGGCG                       | 136              |

Table 2

| Gene       | Accession number | Forward (5'–3')                          | Product size (bp) |
|------------|------------------|-----------------------------------------------|------------------|
| mBmal1      | NM_007489.4      | AGCGGATTTGGCTGAAAGAT                             | 147              |
| mNr1d1      | NM_145434.4      | TGGGATTGGTCTACTGTTGGAAG                       | 114              |
| mPer2       | NM_011066.3      | GAAAGCTCTGCCACCCATAAGA                       | 186              |
| mDbp        | NM_016974.3      | AAGCTGTGTACCCCTGGGCTG                        | 175              |
| mElovl3     | NM_007033.2      | GCCTGGACTCTCTGCTCTTCAG                       | 117              |
| mTbp        | NM_013684.3      | TGTATGATGCTGCACCTCCCTCCCTCGTTCGT            | 128              |
| m36b4       | NM_007475.5      | CTCCTGAGACCCCTGATAG                           | 223              |

The preparation of lysates from liver tissues and Western blotting procedures was performed as described previously (39). Protein extracts were prepared from equal amounts of liver tissue collected from male WT and Bmal1−/− mice at ZT0 and ZT12 and lysed in Laemmli SDS buffer supplemented with protease inhibitors (Roche, Basel, Switzerland). The protein concentration was determined using a bicinchoninic acid assay. Protein detection kit (KeyGen Biotech, Nanjing, China). Equal amounts of total protein (25 μg) from each sample were separated by 12% SDS-PAGE and electrically transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA). The membranes were blocked in a 10% nonfat milk powder solution for 1 h in TBS containing 0.5% Tween 20 at room temperature, after which the membranes were incubated overnight at 4°C with the anti-BMAL1 antibody (1:2000 dilution; Abcam) or an anti-β-actin antibody (1:2000 dilution; Sungene Biotech, Tianjin, China) diluted in TBST. On the second day, the membranes were washed and then incubated with horse-radish peroxidase–conjugated secondary antibody (1:4000 dilution; Zhong Shan Jinqiao Biological Technology Co., Beijing, China) diluted in TBST for 1 h at room temperature. The peroxidase activity was detected using a WesternBright ECL horseradish peroxidase substrate kit (Advansta, Menlo Park, CA). Finally, the immunoreactive bands were visualized using a gel imaging analyzer (Tanon Biotech, Shanghai, China).

Oil Red O staining and hepatic triglyceride assay

Male WT and Bmal1−/− mice aged 8–10 weeks were housed individually in light-tight, ventilated closets under one 12-h/12-h LD cycle for at least 2 weeks with ad libitum access to food and water. The liver samples of mice were collected at ZT10 (n = 8 for each genotype). Oil Red O staining was performed according to a previous report with minor modifications (22). Briefly, frozen sections (8 μm) were prepared from snap-frozen liver tissues and fixed in 10% buffered formalin for 10 min. The sections were then stained with freshly prepared 0.5% Oil Red O in isopropyl alcohol at 37°C for 25 min. After rinsing with 60% isopropyl alcohol, the sections were further counterstained with hematoxylin for 5 s. Hepatic lipids were extracted according to the methods of Folch et al. (40). The extract was dissolved in isopropyl alcohol and subsequently quantified using Wako kits (Wako Pure Chemical Industries, Ltd.).

ChIP assay

ChIP assay was performed using a SimpleChIP® enzymatic chromatin IP kit (Cell Signaling, catalog no. 9003) according to the manufacturer’s protocol. Briefly, livers from WT and Bmal1−/− mice were harvested immediately at CT8 and CT20 after euthanasia (n = 4 for each time point of genotype). The shredded mouse liver was resuspended in cold PBS containing 1 mM proteinase, followed by treatment with 1% formaldehyde (for chromatin cross-linking) for 20 min at room temperature. Chromatin with a length of ~150–900 bp was obtained after digestion with micrococcal nuclease and shearing with ultrasonication. For each reaction, 10 μg of fragmented chromatin was immuno precipitated with rabbit anti-NR1D1 (Cell Signaling, catalog no. 13418) or normal rabbit IgG (control, Cell Signaling, catalog no. 2729) by overnight incubation at 4°C. Protein G magnetic beads were then added to each ChIP sample, and samples were then incubated for 2 h at 4°C with shaking to allow precipitation of the immunocomplexes. After elution, decross-linking, and purification, the purified DNAs were used as a template for qPCR with specific primers (Table 1).

RNA extraction and quantitative real-time PCR

Liver tissues or AML12 cell samples were harvested at the indicated time points. Total RNA was extracted using TRIzol reagent (TaKaRa, Dalian, China), and the RNA samples were treated with RNase-free DNase (TianGen, Beijing, China). The cDNAs were generated using a PrimeScript RT Reagent Kit (TaKaRa). The primer sets used for qPCR are listed in Table 2. All primer sets were designed to span introns to avoid amplifying products from genomic DNA. qPCR was performed on the
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CFX96 RT-qPCR system (Bio-Rad) using the SYBR Premix Ex TaqII kit (TaKaRa) with a 20-μl reaction volume containing 10 ng of cDNA and a 200 nM concentration of the specific primers, as described previously (41). Melting peaks were determined using a melting curve analysis to ensure the amplification and the generation of a single product. All reactions were performed in triplicate and displayed amplification efficiencies between 80 and 120%. The 2−ΔΔCt method was used to quantify gene expression. Tbp and 36b4 were used as internal reference genes, and the geometric average of these two reference genes was used to normalize the relative expression according to a previous report (42).

Cell culture and treatment

The hepatocyte cell line AML12, generated from the liver of TGFα-transgenic mice, was kindly provided by Stem Cell Bank, Chinese Academy of Sciences (43). The cells were plated (5 × 103 cells/dish) on 35-mm collagen-coated dishes (Thermo Fisher Scientific) in Dulbecco's modified Eagle's medium/Ham's F-12 (Thermo Fisher Scientific) supplemented with 10% FBS (Gibco), 1× insulin-transferrin-selenium liquid medium supplement (ITS, Sigma-Aldrich), 0.1 μM dexamethasone (Sigma-Aldrich), and 1× antibiotic-antimycotic (containing penicillin, streptomycin, and amphotericin B; Thermo Fisher Scientific) in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. Cells were cultured for 24 h to reach confluence. For the DHT treatment experiment, cells were then treated with 1 μM DHT. Cell samples were collected for total RNA extraction at 0, 2, 4, 8, and 12 h after the DHT treatment. For the flutamide-blocking experiment, AML12 cells were incubated in the presence of DHT (1 μM) with or without co-administration of flutamide (100 nM), and cell samples were collected for total RNA extraction at 12 h following treatment.

Data analysis and statistics

Data are expressed as the means ± S.E. of at least three independent experiments, each performed with triplicate samples. The circadian rhythmicity in gene expression was determined by the single Cosinor method using Time Series Single 6.3 (Expert Soft Tech, Richelieu, France). Rhythmicity was defined by a confidence region for the mesor using a t distribution with the level of significance taken as ±5%. Other statistical analyses were performed using Student’s t test, a one-way ANOVA, or a test, as indicated, using SigmaPlot version 12.0 (Systat Software, San Jose, CA). Differences were considered significant at p < 0.05.

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