Original Article

MOLECULAR METABOLISM

Hepatic gene therapy rescues high-fat diet responses in circadian *Clock* mutant mice

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ABSTRACT

Objective: Circadian *Clock* gene mutant mice show dampened 24-h feeding rhythms and an increased sensitivity to high-fat diet (HFD) feeding. Restricting HFD access to the dark phase counteracts its obesogenic effect in wild-type mice. The extent to which altered feeding rhythms are causative for the obesogenic phenotype of *Clock* mutant mice, however, remains unknown.

Methods: Metabolic parameters of wild-type (WT) and *Clock* $^{\Delta 19}$ mutant mice (MT) were investigated under *ad libitum* and nighttime restricted HFD feeding. Liver circadian clock function was partially rescued by hydrodynamic tail vein delivery of WT-*Clock* DNA vectors in mutant mice and transcriptional, metabolic, endocrine and behavioral rhythms studied.

Results: Nighttime-restricted feeding restored food intake, but not body weight regulation in MT mice under HFD, suggesting *Clock*-dependent metabolic dysregulation downstream of circadian appetite control. Liver-directed *Clock* gene therapy partially restored liver circadian oscillator function and transcriptome regulation without affecting centrally controlled circadian behaviors. Under HFD, MT mice with partially restored liver clock function (MT-LR) showed normalized body weight gain, rescued 24-h food intake rhythms, and WT-like energy expenditure. This was associated with decreased nighttime leptin and daytime ghrelin levels, reduced hepatic lipid accumulation, and improved glucose tolerance. Transcriptome analysis revealed that hepatic *Clock* rescue in MT mice affected a range of metabolic pathways.

Conclusion: Liver *Clock* gene therapy improves resistance against HFD-induced metabolic impairments in mice with circadian clock disruption. Restoring or stabilizing liver clock function might be a promising target for therapeutic interventions in obesity and metabolic disorders.

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Keywords Circadian clock; *Clock* gene; High-fat diet; Liver; Transcription; Gene therapy

1. INTRODUCTION

In response to the Earth's rotation around its axis, most species have evolved endogenous *circadian* clocks, enabling them to adapt behavior and physiology to the 24-h rhythm of day and night [1,2]. At the molecular level, these clocks are based on transcriptional-translational feedback loops built from a set of clock genes/proteins including the two transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ANRT-like 1 (BMAL1 or ARNTL), which together drive rhythmic expression of three *Period* and two *Cryptochrome* genes through binding to *E-box* enhancer motifs. BMAL1 and CLOCK further regulate many other *E-box*-controlled genes in a tissue-specific manner, thereby translating the circadian clock rhythm into physiologically meaningful signals [3,4]. Clocks are found in all body tissues and are synchronized via a master pacemaker located in the hypothalamic suprachiasmatic nucleus (SCN) [5,6] that is entrained by the external light rhythm. Together, peripheral clocks and SCN-controlled sleep-wake and food intake rhythms regulate the expression of many metabolically relevant genes [7]. Interestingly, peripheral clocks not only respond to SCN signaling but also are reset by the timing of food intake. Therefore, mistimed feeding rhythms — as frequently occur in modern industrialized societies — can promote internal clock desynchrony and the development of metabolic disorders [8—13].

The liver is the largest metabolic organ of the body. Transcriptome analyses have identified more than 3,000 rhythmic transcripts [14] and chromatin immunoprecipitation/DNA sequencing experiments revealed more than 2,000 DNA binding sites for BMAL1 in the murine liver [15]. Circadian regulation has been shown for several metabolic processes such as xenobiotic detoxification [16,17], mitochondrial function [18], and lipid and glucose metabolism [14,19,20]. Mice with hepatocyte-specific abrogation of clock function through deletion of *Bmal1* display impaired glucose homeostasis but normal body weight regulation [13].

In contrast, mice carrying a dominant negative mutation in the gene encoding the BMAL1 partner CLOCK (*Clock* $^{\Delta 19}$) are overweight and,

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Received March 3, 2017 • Revision received March 16, 2017 • Accepted March 22, 2017 • Available online 29 March 2017

http://dx.doi.org/10.1016/j.molmet.2017.03.008

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under high-fat diet conditions, develop symptoms of the metabolic syndrome [21]. This obesogenic phenotype is associated with deregulated feeding rhythms and over-eating during the normal rest phase. In addition, mistimed feeding is associated with the development of obesity in mice and humans [13,22–24]. Moreover, in wild-type mice, restricting access to a HFD to the nighttime improves clock gene rhythms and normalizes body weight regulation [25,26]. Appetite regulation and energy expenditure are centrally controlled and, thus, are difficult targets for clinical interventions [27–29]. On the other hand, metabolic feedback signals from the periphery such as leptin and ghrelin, but also liver-derived factors such as fibroblast growth factor 21 (FGF-21) [30] and ketone bodies [31], reach the brain and modulate neuronal circuits to adjust energy metabolism [32]. This bottom-up communication from peripheral metabolic tissues to central regulatory circuits is impaired during obesity [33,34]. Thus, targeting the circadian regulation of metabolic feedback signals by manipulating peripheral clock function may provide a feasible means of restoring homeostatic set points in metabolic disorders. To test this hypothesis, we investigated the effects of liver *Clock* gene therapy on feeding behavior and energy metabolism in *Clock*$^{\Delta 19}$ mutant mice.

2. MATERIALS AND METHODS

2.1. Mice

Adult male C57BL/6J (WT) and congenic homozygous *Clock*$^{\Delta 19}$ (MT) [35] mice were bred under standard laboratory conditions with a temperature of 21 ± 2 °C, a relative humidity of 50 ± 5% and a 12-h:12-h light–dark cycle (LD; light phase: 200 ± 50 lux). Before mice were allocated to the experiments, they were group-housed with *ad libitum* access to standard rodent chow (58% carbohydrates, 33% protein, 9% fat; Ssniff, Germany) and water. After adaptation to single housing, 10-week old mice were assigned to the different experiments with either *ad libitum* access to standard rodent chow or regulated access (*ad libitum vs*. nighttime only) to HFD (45% kJ fat, Ssniff EF D12451) for 10 weeks. Food intake, body weight, locomotor activity, and energy expenditure were measured; then, animals were sacrificed by cervical dislocation for molecular characterization. All experiments were ethically assessed and licensed by the Office of Consumer Protection and Food Safety of the State of Lower Saxony and the Ministry of Energy Change, Agriculture, Environment and Rural Areas of the State of Schleswig–Holstein and performed according to international guidelines on the ethical use of experimental animals.

2.1.1. Feeding regimes

The effects of timed food access under HFD were analyzed in WT and MT mice (n = 12/group). Both genotypes received a HFD for 10 weeks either *ad libitum* (WT-*ad libitum*; MT-*ad libitum*) or restricted to the 12-h dark phase (restricted feeding: RF; WT-RF, MT-RF). Body weight and food intake were measured weekly. An additional cohort was analyzed for energy expenditure at 4 weeks into the feeding regime, followed by activity analysis one week later. In a second experiment, we restored the function of the liver *Clock* gene (liver rescue: LR; WT-LR, MT-LR) by hydrodynamic tail vein delivery (see below) and analyzed the HFD effects in these mice. 10-week old WT (n = 24), WT-LR (n = 13; data not shown), MT (n = 26) and MT-LR mice (n = 24) received a HFD *ad libitum* for ten weeks. Body weight and food intake were measured weekly; activity, energy expenditure, and other metabolic parameters (hormone levels, glucose tolerance) were investigated in an additional cohort in the middle of the feeding experiment after 5 weeks of HFD.

2.1.2. Activity analysis

Locomotor activity of single-housed mice was recorded either by custom-made infrared (IR) detectors placed on the cage lid or by running-wheels. IR recordings were performed for one week under standard LD conditions either in week 2 of the RF-paradigm (Figure 1: n = 12/group) or starting at week 5 after tail vein injection (Figure 4: n = 8/group). Wheel-running activity of adult *ad libitum* chow-fed WT, MT, and MT-LR was recorded for two weeks under standard LD conditions followed by a 4-week period in constant darkness (DD; Figure 3, n = 12 per group). IR and running-wheel measurements were analyzed using ClockLab software (Actimetrics, Evanston, USA).

2.1.3. Energy expenditure

Oxygen consumption and carbon dioxide production were investigated under standard housing conditions (LD, 21 °C) and the introduced feeding paradigm using an open-circuit indirect calorimetry system (TSE PhenoMaster Systems, Bad Homburg, Germany) after 2 weeks on RF-paradigm (Figure 1; n = 12 per group) or after 4 weeks on HFD (Figure 4; n = 24–26 per group). Mice were allowed to acclimatize to the system for 2 days, and then data were acquired in intervals of 15 min for 3–4 days. Energy expenditure was normalized to the individual body weight [36].

2.2. Liver *Clock* gene therapy

Hepatic *Clock* gene expression was manipulated by hydrodynamic tail vein injection [37]. In brief, either WT or MT mice were injected with a vector encoding the full-length, hemagglutinin (HA)-tagged wild-type *Clock* (GenBank AF000998), *secreted embryonic alkaline phosphatase* (*SEAP*), or *lacZ* cDNA under the control of a chimeric promoter composed of the mouse alpha fetoprotein enhancer II and a minimal mouse albumin (*Alb1*) promoter aimed at restricting expression specifically to hepatocytes (*pLIVE*; MIR 5420, Mirus Bio) [38]. Firefly *Luciferase* was under the control of the *Bmal1* promoter [39]. All vectors were diluted in TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio, Madison, USA) and injected into the tail vein at a constant rate over 5–7 s. The total volume was set to 10% of the total body weight. Pressing a moist paper towel on the injection point inhibited bleeding. Mice were returned to their home cage after observation for 5–10 min. Mouse *Clock* cDNA was cloned from a cDNA library of C57BL/6J mice into *pLIVE* vector followed by plasmid preparation (Endo-free Plasmid Maxi Kit, Qiagen, Hilden, Germany). The validation of delivery efficiency was performed using a liver-specific lacZ-expressing reporter plasmid (*pLIVE-lacZ* vector, MIR 5520, Mirus Bio). A working solution containing a total of 35 μg of *Clock* expressing plasmid per injection was used for all subsequent experiments. For the *ex vivo* visualization of liver clock rhythms, we co-injected the *Bmal1-Luc* plasmid together with the *Clock* plasmids. An *Alb1-SEAP* vector (*pLIVE-SEAP*; MIR 5620, Mirus Bio) was used for controls. Mice were allowed to recover for two days before the subsequent feeding experiments.

2.3. X-gal staining

Nine days after tail vein injection of the *lacZ* reporter plasmid, chow-fed mice were killed by cervical dislocation, and the medial liver lobe was removed and embedded in paraffin. 8-μm sections were prepared using a microtome (Leica, Eisfeld, Germany) and fixed in 0.2% glutaraldehyde (5 min at room temperature) followed by PBS washing and an overnight incubation in x-Gal staining solution at 37 °C. On the following day, sections were washed in PBS, dehydrated, mounted on slides, and analyzed using a camera-equipped microscope (Leica). Stained areas were calculated using NIH Image software (NIH,

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