Maternal obesity disrupts circadian rhythms of clock and metabolic genes in the offspring heart and liver

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Early life nutritional adversity is tightly associated with the development of long-term metabolic disorders. Particularly, maternal obesity and high-fat diets cause high risk of obesity in the offspring. Those offspring are also prone to develop hyperinsulinemia, hepatic steatosis and cardiovascular diseases. However, the precise underlying mechanisms leading to these metabolic dysregulation in the offspring remain unclear. On the other hand, disruptions of diurnal circadian rhythms are known to impair metabolic homeostasis in various tissues including the heart and liver. Therefore, we investigated whether maternal obesity perturbs the circadian expression rhythms of clock, metabolic and inflammatory genes in offspring heart and liver by using RT-qPCR and Western blotting analysis. Offspring from lean and obese dams were examined on postnatal day 17 and 35, when pups were nursed by their mothers or took food independently. On P17, genes examined in the heart either showed anti-phase oscillations (Cpt1b, Pparα, Per2) or had greater oscillation amplitudes (Bmal1, Tnf-α, Il-6). Such phase abnormalities of these genes were improved on P35, while defects in amplitudes still existed. In the liver of 17-day-old pups exposed to maternal obesity, the oscillation amplitudes of most rhythmic genes examined (except Bmal1) were strongly suppressed. On P35, the oscillations of circadian and inflammatory genes became more robust in the liver, while metabolic genes were still kept non-rhythmic. Maternal obesity also had a profound influence in the protein expression levels of examined genes in offspring heart and liver. Our observations indicate that the circadian clock undergoes nutritional programming, which may contribute to the alternations in energy metabolism associated with the development of metabolic disorders in early life and adulthood.

Keywords: Circadian clock, maternal obesity, metabolic programing, offspring

INTRODUCTION

It has been postulated that obesity begets obesity. Indeed, maternal obesity and excessive gestational weight gain have been identified as risk factors to induce obesity in the offspring (Silverman et al., 1998). This becomes a serious concern for public health as currently, an estimated 23.3% of Chinese women of childbearing age are overweight and 6.9% are obese, with the rates in large cities even higher (Yin, 2008). The increased incidence of obesity has been linked to an increased economic burden. Research in the Asia-Pacific region indicates that the estimated annual cost burden of overweight and obesity in China is as high as $7.4 billion; the combined direct and indirect costs of obesity may be over 4.1% of the country’s gross domestic product (Wu et al., 2014). Maternal obesity has a strong association with obesity in the offspring, both in childhood (Olson et al., 2009; Whitaker, 2004) and in adulthood (Jaaskelainen et al., 2011; Reynolds et al., 2010). It is worse that the rising level of pediatric obesity leads to an increased risk of various metabolic disorders, such as juvenile type 2 diabetes, non-alcoholic fatty liver disease and hypertension (Barker et al., 1993; Samuelsson et al., 2008). In this sense, understanding how the intrauterine environment contributes to the development of offspring obesity is of particular interest and importance. The Barker hypothesis firstly provides a framework linking the in utero environment to long-term health outcomes in offspring (Barker, 2007). To date, one generally accepted thesis is that in response to an adverse intrauterine environment, the fetus adapts
its physiological development to maximize its immediate chances for survival. These adaptations include resetting metabolic rate, remodeling endocrine systems and down-regulating growth, commonly manifest in an altered birth phenotype (Sandovici et al., 2012). In this developmental programing framework, various hormones (e.g. leptin and insulin), nutrients (e.g. glucose, free fatty acids and triglycerides) and inflammatory cytokines are implicated to transmit signals from mother to offspring (Jones et al., 2009; Nicholas & Hartmann, 1991; Segovia et al., 2014; Trottier et al., 1998). Moreover, maternal abnormal energy status causes perturbations in epigenetic modifications, which contribute to the susceptibility to obesity in the offspring (Suter et al., 2014). Although many such advances have helped us better understand fetal programing, much of the molecular mechanisms leading to metabolic dysregulation remain to be elucidated.

Metabolism is finely controlled by the circadian clock, and disruption of their integration promotes obesity and metabolic syndrome (Asher & Schibler, 2011; Shi et al., 2013). Circadian misalignment caused by shifted work (chronic) and jet lag (rapid) impacts energetics, particularly in metabolic tissues where peripheral circadian clocks locate, such as heart, liver, skeletal muscle and brown adipose (Bass & Takahashi, 2010; Richards & Gumz, 2012). For example, shift work at a young adult age is associated with elevated long-term cortisol levels and increased BMI, which may contribute to the increased cardiovascular risk (Suwazono & Nagawa, 2014). Conversely, nutritional factors, such as feeding, fasting or nutrient composition of diet can modulate rhythmicity and clock gene expression. Feeding mice a high fat diet, for example, has been shown to cause three main changes in circadian rhythms: lengthened period, blunted feeding rhythm and alterations in the expression of circadian clock genes (Kohsaka et al., 2007). More specifically, although little is known about fetal circadian rhythms in relation to developmental physiology, it is proposed that circadian rhythms are established in the fetus by virtue of maternal cues, probably to prepare the fetus for the postnatal environment.

Given that the circadian clock is tightly linked to energy metabolism and is entrainable by food, here we investigate whether perinatal overnutrition affects the circadian expression rhythms of clock and metabolic genes in the heart and liver of the offspring.

MATERIALS AND METHODS

Animal experiments

All animal procedures in this investigation conform to the established international ethical standards (Portaluppi et al., 2010) and the approved regulations set by the Laboratory Animal Care Committee at Nanjing Normal University (permit number 2090658, date issued 20 April 2008). Four-week-old C57BL/6J virgin female mice were housed in a 12-h light/12-h dark cycle (light/dark, 12:12) in a temperature- and humidity-controlled environment and fed ad libitum. Zeitgeber time zero (ZT0) referred to lights on. After acclimation for 1 week, mice were weight-matched and divided into two groups (n = 20/group). Mice were fed with either a normal diet ND (ND, D12450B, fat content 10%; Research Diets Inc., New Brunswick, NJ, USA) or a high-fat diet (HFD, D12492, fat content 60%) for 6 week until the mating. Mice continued to be fed on the same diet during pregnancy and lactation (until lactational day 16). On lactational day 16, both groups were fed on ND to eliminate the possibility that offspring could eat the dropped HFD before weaning. The day of parturition was set as postnatal day zero (P0), and pups were weaned on P21 and were fed on ND until sacrifice. Schematic of experimental design is shown in Figure S1(A). Experiments were performed on P17 and P35 male pups. Three to five pups were selected randomly from at least three different litters to eliminate the influence of litter. The exact number of offspring collected at each time point for circadian experiment is presented in Figure S1(B). At sacrifice, animals were killed at 4-h intervals from ZT 1 to ZT 21 using a rising concentration of CO2 and cervical dislocation. Liver and heart were dissected and immediately frozen in liquid nitrogen and stored at −70°C for later analyses. Serum was obtained by centrifugation of blood samples and stored at −20°C.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was isolated from the heart and liver using Trizol reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was reverse transcribed into complementary DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for total complementary DNA content. mRNA levels were quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) using SybrGreen Master Mix (Applied Biosystems, Foster City, CA). Samples were amplified using the Lightcycler Nano System (Roche, Basel, Switzerland). Primers for quantitative polymerase chain reaction (qPCR) were either adopted from our previously published paper (Tao et al., 2011) or designed by using the Primer3Web software (http://bioinfo.ut.ee/primer3/). Before the formal experiment, melting curve analysis for each pair of primers was performed to guarantee the high specificity of the reaction (Figure S2). The primers used for the amplification were presented in Table S1. The relative expression levels of the mRNAs were calculated using the comparative 2−ΔΔCt method (Livak & Schmittgen, 2001) and were expressed as fold changes to mean expression of the respective gene at ZT5 in pups of ND dams.
Western blot
Tissue samples were homogenized and the cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 0.1 mM dithiothreitol, 0.002 mg/ml leupeptin, 1 mM NaVO₃, 0.05 mM phenylmethyl-sulfonylfluoride, and 0.002 mg/ml aprotinin. The protein concentration was quantified with a DC protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded and separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated overnight with appropriate primary antibodies. Bound antibodies were then visualized using horseradish peroxidase-conjugated secondary antibodies. Quantitative analysis was performed by using the NIH Image J 1.32j software (Bethesda, MD, USA). Anti-PPARα, anti-TNF-α and anti-FAS antibodies were purchased from Proteintech (Chicago, IL); anti-PER2 antibody was obtained from Abcam (Cambridge, MA, USA); anti-GAPDH antibody was purchased from Kangcheng (Shanghai, China).

Measurement of the body weight
The body weight of 5- to 11-week-old female mice (dams) was measured on a weekly basis. Postnatal offspring were weighed at P2, P17 and P35.

Serological analysis
Serum triglyceride and total cholesterol levels of dams fed on HFD for 6 weeks were determined enzymatically using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) with an Epoch Microplate Reader (Biotex, Winooski, VT), according to manufacturers’ protocols.

Data analysis
Statistical analysis was performed by using the Origin 8 software (version 8.6, OriginLab Corporation, Northampton, MA). Groups of data were presented as mean ± SD (standard deviation). To test for the presence of circadian rhythms in clock and metabolic gene expressions in offspring heart and liver on P17 and P35, time series data were first analyzed by the one-way ANOVA. Detailed F and p values are shown in Tables S2, S6 (for mRNA) and S4 (for protein). They are also demonstrated in the “Results” section. To ascertain the difference in the levels of gene expression between ND and HFD groups at the different time points, data were analyzed by two-way ANOVA with mother diet (ND, HFD) and ZT as factors. Detailed F and p values are shown in Tables S3, S7 (for mRNA) and S5 (for protein). When significant differences were found, Bonferroni’s test for multiple comparisons was performed. Other comparisons, such as the body weight difference between ND and HFD pups were performed using unpaired two-tailed Student’s t-test. A value of p < 0.05 was considered as statistically significant.

RESULTS
Regulation of the body weight and serum lipids by HFD in adult female mice
To investigate the effect of maternal diet-induced obesity on offspring development, we first created diet-induced obese female mice as described previously. Age-matched, adult female mice (5-week old) were fed either a ND or a HFD for 6 weeks. One week after HFD feeding, the body weight of HFD-fed female mice began to increase faster than that of ND-fed control mice (Figure S3A). Such difference became statistically significant since the second week. We next measured the serum lipid concentrations in these two groups at the age of 11 weeks. As expected, significant increases in serum triglyceride (Figure S3B) and total cholesterol levels (Figure S3C) were observed in HFD-fed female mice compared with ND-fed animals. These data suggest that HFD-fed female mice developed obesity, hyperlipidemia and hypercholesterolemia, which are characteristics of metabolic syndrome.

Effects of maternal obesity on postnatal offspring development
We next investigated the effect of maternal obesity on postnatal offspring development. To our surprise, pups from obese dams showed a decrease in the body weight on P2 (Figure 1), but their development rapidly caught up and their body weight surpassed the controls on P17 and P35. These data coincided with previous findings and suggest that the suckling period plays an important role in the observed body weight gain (Luzzo et al., 2012; Melo et al., 2014). Notably, the milk of obese dams has more energy content (Neville et al., 1991), which may contribute to faster body weight gain in their offspring during lactation.

FIGURE 1. Changes in mean body weight (mean±SD) of ND offspring (n=20) and HFD offspring (n=19) at different developmental stages (P2, P17 and P35). **p<0.01 HFD pups versus ND pups at the same postnatal day.
Circadian profile of gene expression in the offspring heart on P17
Long-chain fatty acids are important oxidizable substrates that are used by heart for muscle contraction (Glatz et al., 2001). After birth, a dramatic increase in fatty acid oxidation occurs in hearts of several species (Taegtmeyer et al., 2010). During this transition, induced expression of carnitine palmitoyltransferase 1b (Cpt1b) and peroxisome proliferator-activated receptor α (Pparα) genes is expected to play a key role. As shown in Figure 2(A) and Table S2, the mRNA expression of Cpt1b and Pparα in the heart of control animals on P17 exhibited a daily rhythm with a peak of expression in both cases during the dark cycle (peaked at ZT17 and ZT21, respectively) (Cpt1b F-value = 4.00, p = 0.013; Pparα F-value = 2.42, p = 0.076). However, although Cpt1b and Pparα also displayed a rhythmic expression profile in pups from obese dams, the peak of their expression was reversed to the light phase (Cpt1b F-value = 14.22, p < 0.001; Pparα F-value = 8.63, p < 0.001). The overall oscillation patterns were in anti-phase between these two groups (detailed F and p values are shown in Table S3).

To determine whether maternal HFD affects the core circadian clock, we analyzed the rhythmic expression of the mRNA transcripts encoding brain and muscle arnt-like protein 1 (BMAL1) and period 2 (PER2). RT-qPCR analysis indicated that the expression of these genes follows a circadian pattern in control pups and reaches the highest levels at ZT21. In the 17-day-old offspring of dams fed a HFD, Bmal1 expression in the heart shared a similar oscillation pattern with the controls, except that its expression level was abnormally high at ZT1. For Per2, the anti-phase oscillation pattern was again observed in these animals.

Obesity is now considered as a chronic inflammatory disease. It is interesting to evaluate the effect of maternal obesity on inflammatory cytokine expression in the offspring. We found that the mRNA expression levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), two classic inflammatory cytokines, oscillated in a moderate manner in the heart of control animals (Tnfα F-value = 2.74, p = 0.052; Il-6 F-value = 1.65, p = 0.20). However, in pups from obese dams, Tnfα and Il-6 were induced and displayed a trend towards circadian oscillation, although the amplitudes were not significant due to the great variation within group animals (Tnfα F-value = 2.03, p = 0.132; Il-6 F-value = 0.96, p = 0.473).

In addition to quantify mRNA expression levels, we also performed Western blotting analyses to verify the changes of protein expression levels induced by maternal obesity. As shown in Figure 2(B), Tables S4 and S5, both PPARα and PER2, but not TNF-α, displayed rhythmic oscillations in the control offspring heart on P17, with the peak of PER2 occurring at the dark phase. However, maternal obesity reversed their oscillation phases, which was similarly seen in the changes of mRNA expression levels. The protein expression levels of TNF-α were induced by the maternal obesity and showed a more significant fluctuation. Overall, effects of maternal obesity on the mRNA and protein expression levels of examined genes were comparable in this setting.

Circadian profile of gene expression in the offspring liver on P17
We further explored the impact of maternal obesity on the expression levels of the metabolic enzyme fatty acid synthase (FAS) and the transcriptional regulator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which integrates the circadian clock and cellular metabolism (Liu et al., 2007), in the offspring liver on P17 (Figure 3A). As expected, Fas exhibited a rhythmic expression pattern in control animals which peaked at ZT9, the late stage of light phase (F-value = 74.27, p < 0.001). In contrast, such a cyclic expression was severely impaired in pups from obese dams (F-value = 4.28, p = 0.013). Pgc-1α did not oscillate remarkably in pups from ND groups (F-value = 2.10, p = 0.113), but exhibited a significantly rhythmic and lower expression in HFD pups (F-value = 6.34, p = 0.002). For the diurnal expression of core clock components including BMAL1 and PER2, we found that similar rhythmic patterns were preserved in the liver of ND and HFD pups (ND pups: Bmal1 F-value = 5.27, p = 0.004; Per2 F-value = 7.54, p < 0.001; HFD pups: Bmal1 F-value = 48.32, p < 0.001; Per2 F-value = 13.53, p < 0.001). However, Bmal1 possessed a more robust oscillation. Finally, the mRNA expression levels of TNF-α and IL-6 were down-regulated at most examined time-points in HFD pups. For these animals, Tnfα still showed a rhythmic expression pattern, while Il-6 oscillation was almost abolished (ND pups: Tnfα F-value = 3.20, p = 0.031; Il-6 F-value = 2.74, p = 0.052; HFD pups: Tnfα F-value = 5.365, p = 0.005; Il-6 F-value = 0.85, p = 0.533).

Western blotting analyses revealed that maternal obesity blunted the rhythmic expression of FAS, PER2 and TNF-α proteins in offspring liver on P17 (Figure 3B, Tables S4 and S5). Furthermore, the peak time was shifted from the light phase towards the late dark phase for the oscillations of FAS protein.

Circadian profile of gene expression in the offspring heart on P35
At 35 days of age, when pups could take food independently, HFD offspring still exhibited significant alterations in the circadian expression profile of diverse genes regulating metabolism, clock and inflammation. In particular, the acrophase of Cpt1b and Pparα oscillations was shifted in the heart of HFD pups associated with a significant decrease in the expression levels of both genes at ZT1, when the dark phase was transited to light phase (Figure 4A, Tables S6 and S7). Similar observations were obtained for the PPARα protein.
FIGURE 2. Rhythmic expression profile of genes involved in fatty acid β-oxidation, circadian clock and inflammation in the heart of 17-day-old pups. (A) The heart was dissected every 4 h from pups born to dams fed a ND (open circles) or a HFD (closed circles) diet during pregnancy and lactation, and the expression levels of the transcripts were determined by RT-qPCR. Data are shown as the relative abundance of each transcript in relation to those of the endogenous Gapdh amplified within the same sample and under the same experimental conditions. *p < 0.05, **p < 0.01 HFD pups versus ND pups at the same time-point. Each point corresponds to the mean ± SD expression levels of three to five pups born to at least three different dams. (B) Western blot analysis for PPARα, PER2, TNF-α and GAPDH protein expression levels. GAPDH serves as an internal control. **p < 0.01 HFD pups versus ND pups at the same time-point.
FIGURE 3. Daily expression profile of FAS, PGC-1α and of the core clock and inflammatory genes in the liver of 17-day-old pups born to dams fed either on a ND (closed circles) or a HFD (open circles) diet during pregnancy and lactation. (A) The liver was harvested at 4-h intervals over a 24-h period and the expression levels of the transcripts were determined by RT-qPCR. Data were shown as the relative abundance of each transcript in relation to those of the endogenous Gapdh amplified within the same sample and under the same experimental conditions. Each point corresponds to the mean ± SEM expression levels of three to five pups born to at least three different dams. *p < 0.05, **p < 0.01 HFD pups versus ND pups at the same time-point. Each point corresponds to the mean ± SD expression levels of three to five pups born to at least three different dams. (B) Western blot analysis for FAS, PER2, TNF-α and GAPDH protein expression levels. GAPDH serves as an internal control. **p < 0.01 HFD pups versus ND pups at the same time-point.
FIGURE 4. Long-lasting effects of maternal obesity on the daily expression profile of metabolic, clock and inflammatory genes in the heart of 35-day-old pups. (A) RT-qPCR analysis was carried out using cDNA reversed transcribed from total heart RNA of 35-day-old mice born to dams fed a ND (open circles) or a HFD (closed circles) diet during pregnancy and lactation and weaned on standard chow at 21 days. Data were shown as the relative abundance of each transcript in relation to those of the endogenous Gapdh amplified within the same sample and under the same experimental conditions. **p < 0.01 HFD pups versus ND pups at the same time-point. Each point corresponds to the mean ± SD expression levels of three to five pups born to at least three different dams. (B) Western blot analysis for PPARα, PER2, TNF-α and GAPDH protein expression levels. GAPDH serves as an internal control. *p < 0.05, **p < 0.01 HFD pups versus ND pups at the same time-point.
expression (Figure 4B, Tables S4 and S5). For clock and inflammatory gene expressions, the peak time of Bmali, as well as Tnf-α, was shifted four hours earlier in 35-day-old HFD mice. In addition, oscillations of TNF-α protein expression were attenuated by maternal obesity (Figure 4B, Tables S4 and S5). In contrast, both mRNA and protein expression of PER2 showed a similar rhythmic pattern in both groups. The expression of Il-6 was quite stable and did not show any obvious fluctuation in ND pups (F-value = 1.58, p = 0.211), but it reached a peak at ZT21 in HFD pups (F-value = 26.10, p < 0.001). In general, phase abnormalities observed on P17 were improved in the heart of P35.

Circadian profile of gene expression in the offspring on P35

Apparently, an intact oscillation pattern of Fas and Pgc-1α had already been set up in the offspring on P35 from lean dams (Figure 5A, Fas F-value = 6.24, p = 0.001; Pgc-1α F-value = 20.69, p < 0.001). In contrast, the maternal HFD abolished the circadian expression rhythm of these two genes in offspring (Fas F-value = 3.96, p = 0.017; Pgc-1α F-value = 1.85, p = 0.16). The effects of maternal obesity on clock gene expressions, in this stage, were quite different. For Bmali, its expression levels were significantly higher in HFD pups, especially in the dark phase (Figure 5A and Table S7). However, HFD decreased Per2 expression at most examined time points. Finally, inflammatory genes did not oscillate robustly in the ND group (Tnf-α F-value = 2.34, p = 0.079; Il-6 F-value = 1.02, p = 0.43), while a profound circadian rhythmicity was observed for Tnf-α and Il-6 expression in the HFD group (Tnf-α F-value = 11.88, p < 0.001; Il-6 F-value = 8.51, p < 0.001). Notably, these two genes demonstrated obvious fluctuation mainly during late dark phase.

To our surprise, although maternal obesity impaired the oscillation of Fas mRNA expression, it markedly increased FAS protein expression (Figure 5B, Tables S4 and S5). Also, the expression of FAS protein showed a semidiurnal rather than diurnal rhythm. This may be caused by the post-transcriptional regulation. PER2 protein expression levels were significantly down-regulated at most examined time points by HFD, which was similar to the mRNA expression changes. Regarding the TNF-α protein expression, it was constitutively induced by maternal obesity, which differed from the mRNA expression (induced at ZT21 only).

DISCUSSION

In the present study, we found that feeding a HFD during gestation and lactation induces a long-lasting disruption of the diurnal expression pattern of several genes involved in the regulation of energy metabolism, circadian clock, and inflammation in the offspring heart and liver. Our findings strongly suggest that the circadian clock undergoes metabolic programing during development.

A hallmark of cardiac metabolism before birth is the predominance of carbohydrate use for energy provision. After birth, energy substrate metabolism rapidly switches to the oxidation of fatty acids. In response to such a switch, PPARα is activated by ligand binding of long-chain fatty acids and regulates the expression of multiple genes controlling both fatty acid uptake and oxidation, including CPT1b (Taegtmeyer et al., 2010). Our data demonstrated that the rhythmicity of cardiac expression of Cpt1b and Ppara was in the same phase either in ND pups or in HFD pups, confirming that PPARα is an upstream regulator of CPT1b. Second, the oscillation patterns of Cpt1b and Ppara differ a lot between P17 and P35 groups. For HFD pups, their oscillations are even completely reversed. Such divergence may be due to the entrainment of food intake, which is an important zeitgeber for the circadian rhythms of peripheral clock genes (Damiola et al., 2000; Hara et al., 2001; Schibler et al., 2003; van der Veen et al., 2006). For P17 pups, they are fed by the mother. The mouse mother nurses her infants periodically mainly in her rest time, during the day, and she could feed herself during the night (Sumova et al., 2006). At this stage, peripheral rhythms in the heart of infantile mice are driven by the mother through direct and indirect pathways. It has been reported that circadian rhythms of pups are entrained by maternal cues during the first postnatal week. In contrast, P35 pups can already take food independently. Their peripheral rhythms should be reasonably reset by their food intake time, mainly during the dark phase. More importantly, great difference (anti-phase) in Cpt1b and Ppara rhythmic expression was observed in ND and HFD pups on P17, which was ameliorated in P35 heart. We speculate that both in utero microenvironment and postnatal nutritional signals contribute to induce this difference. A recent study has shown that modulation of mRNA synthesis and degradation rate, as well as epigenetic alterations in histone marks, regulates Ppara expression in the liver of rat offspring born to obese dams (Sookoian et al., 2014). It is interesting to investigate whether similar regulation occurs in the offspring heart using our experimental settings. In addition, the milk of obese dam has higher fat content and provides more energy (Neville et al., 1991). Therefore, food intake time, the nutritional component of the food and prenatal microenvironment collectively affect the oscillation patterns of metabolic genes in the heart.

As the largest metabolic organ, the liver responds to nutritional signals sensitively. This is evidenced by our finding that the peak of Fas rhythmic expression was promptly switched from the light phase (ZT9) on P17 to the dark phase (ZT21) on P35 in ND pups. The peak of Pgc-1α, an important metabolic regulator, also occurred at ZT1 when the dark phase just ended in the liver of P35 pups. However, HFD severely damped the oscillation.
FIGURE 5. Daily expression profile of FAS, PGC-1α and of the core clock and inflammatory genes in the liver of 35-day-old pups born to dams fed either on a ND (closed circles) or a HFD (open circles) diet during pregnancy and lactation. (A) The liver was harvested at 4-h intervals over a 24-h period and the expression levels of the transcripts were determined by RT-qPCR. Data are shown as the relative abundance of each transcript in relation to those of the endogenous Gapdh amplified within the same sample and under the same experimental conditions. Each point corresponds to the mean ± SD expression levels of three to five pups born to at least three different dams. **p < 0.01 HFD pups versus ND pups at the same time-point. Each point corresponds to the mean ± SD expression levels of three to five pups born to at least three different dams. (B) Western blot analysis for FAS, PER2, TNF-α and GAPDH protein expression levels. GAPDH serves as an internal control. *p < 0.05, **p < 0.01 HFD pups versus ND pups at the same time-point.
amplitudes of both Fas and Pgc-1α in either P17 or P35 pups. Of note, mice fed an HFD consume more food during the light period and less food during the dark period, resulting in an attenuated diurnal rhythm of food intake (Kohsaka et al., 2007). Disorganization in the feeding rhythm in dams may contribute to the loss of hepatic rhythmic expression of Fas and Pgc-1α. On the other hand, it was unfortunate that we could not monitor the exact amount and time of food intake of pups. Therefore, the question remains as to whether the food intake behavior is also abnormal in HFD pups and thus affects diurnal expression of metabolic genes in the liver. We have also noticed that the effects of HFD on adult mice and infantile mice are quite different. Kohsaka et al. (2007) reported that HFD feeding increases Fas expression in the mouse liver, which is opposite to our findings. This inconsistency highlights the importance to dissect impacts of early and adult over-nutrition on the changes in the physiology and metabolism of the metabolic organs, therefore programing the onset of metabolic diseases in later life.

The circadian clock in mammals is organized hierarchically at the molecular level (Schibler et al., 2003). CLOCK and BMAL1 are two basic helix-loop-helix transcription factors that activate the transcription of Per and cryptochrome (Cry) genes. PER and CRY proteins in turn inhibit their own expression by repressing Clock/ Bmal1 activity, forming the critical feedback loop within the clock circuitry (Asher & Schibler, 2011; Froy, 2012; Huang et al., 2011). In addition, the orphan nuclear receptors of the ROR and REV-ERB families are also implicated in the control of circadian clock function (Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004; Solt et al., 2011). As the key components of clock machinery, Bmal1 and Per2 oscillate more robustly in the offspring heart, compared with the oscillation of Cpt1b and Pparα, in our study. Actually, circadian oscillations mature very fast in the heart. It has been demonstrated that the exact day that circadian oscillator in mouse heart begins to operate is as early as P3 (Huang et al., 2010). Another report has revealed that Per1, Bmal1 and Dpb mRNA in rat heart starts circadian expression between P2 and P5 (Sakamoto et al., 2002). In the liver, the clock machinery was also quickly established. On P35, the oscillation patterns of Bmal1 and Per2 were already similar to those observed in adult liver. In addition, we found that abnormalities in Bmal1 and Per2 rhythmicity were in general more severe in the early developmental stage in HFD pups. For example, the anti-phase (e.g. Per2 in the heart), as well as amplitude changes (e.g. Bmal1 in the heart and liver), was observed in HFD pups on P17. Contrasting, the maternal obesity mainly affected oscillation amplitudes on P35. These results suggest that the circadian clock has been bona fide nutritionally programed. Recovery to normal diet after weaning helped to partially rescue the defects of circadian clock rhythmicity induced by maternal obesity.

Obesity has been considered as a chronic, low-grade inflammatory disease, characterized by abnormal cytokine production, an altered adipokine profile, and activation of inflammatory pathways (Hotamisligil, 2006). The role of chronic low-grade inflammation in obese mothers has become an emerging focus in the developmental programming field. It is believed that maternal obesity is linked to an enhanced inflammatory response in offspring. Challier et al. (2008) observed macrophage accumulation and increased expression of proinflammatory cytokine expression in placenta from obese women compared to those from lean women. Infiltrating macrophages can secrete inflammatory cytokines into the maternal or fetal systemic circulation, resulting in the programed alterations in offspring metabolism associated with increased adiposity and insulin resistance (Howie et al., 2013; Li et al., 2013; Reynolds et al., 2013). Similarly, when female mice were on P35 during gestation and lactation, increased serum proinflammatory cytokines and hepatic IκB kinase phosphorylation were observed in their offspring (Ashino et al., 2012; Park et al., 2010). Coincided with these findings, we found that the cardiac expression of Tnf-α and IL-6 was higher in HFD pups on P17. In P35 pups, IL-6 expression was also significantly higher at ZT21, indicating a stronger inflammatory response in the heart of these animals. In contrast, both Tnf-α and IL-6 showed lower expression levels and did not oscillate robustly in ND pups. In the liver, the effects of maternal obesity on inflammatory gene expression were different during the development. On P17, Tnf-α and IL-6 expression levels were lower in HFD pups, while Tnf-α was induced on P35. It is therefore interesting to compare inflammatory states in various tissues in offspring with the maternal obesity challenge.

Numerous studies have demonstrated that peripheral oscillators are self-sustained, cell-autonomous and tissue-specific. Various cyclic expression patterns of metabolic and clock genes within and between peripheral tissues may ensure that the circadian clock and metabolism are coupled, thus integrating energy flux with varying physiological demands across the light–dark cycle. For example, it has been reported that components of gluconeogenesis, glycolysis and fatty acid metabolism cycle with a peak during the subjective night in mouse liver (Panda et al., 2002). In our study, we also found that in control pups, the hepatic expression of FAS, a key enzyme involved in fatty acid synthesis, exhibited a robust oscillation and peaked at the time when food was available. Such a sensitive fluctuation is important for the energy homeostasis, when considering that FAS catalyzes the de novo synthesis of fatty acids and produces fat for storage of energy when nutrients are present in excess. In contrast, the hepatic expression of inflammatory markers, such as TNF-α and IL-6, kept stably at the low levels, suggesting that routine food intake behaviors have a moderate effect on the inflammatory response, which should be strictly controlled to
avoid any deleterious outcomes. Therefore, depending on their distinct physiological functions, different genes showed different phases and amplitudes at the same time point.

The results presented here indicate that the circadian clock undergoes metabolic reprogramming, shedding new insights into the pathogenesis of metabolic diseases that might be of special concern for neonatal nutrition abnormalities. Since maternal obesity is a public health concern, we provided new evidences that maternal metabolic conditions can lead to long-term effects and impair the postnatal circadian rhythmicity of genes in the offspring heart and liver. In the future, a comprehensive analysis of the metabolic tissues regarding external cues, such as environmental factors, lifestyle, metabolic functions, and the relationship between parents and offspring, is required. Such a clarification will help us better understand the precise mechanisms of maternal imprinting of metabolic disorders.

DECLARATION OF INTEREST

The authors report no competing interests. The authors alone are responsible for the content and writing of the paper.

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