OBSTETRICS

Conditional postnatal deletion of the neonatal murine hepatic circadian gene, *Npas2*, alters the gut microbiome following restricted feeding

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BACKGROUND: We have recently shown in both non-human primates and in rodents that fetal and neonatal hepatic expression of the circadian transcription factor, *Npas2*, is modulated by a high fat maternal diet and plays a critical role in establishing life-long metabolic homeostasis. Similarly, we and others have also established the importance of the maternal and early postnatal diet on establishment of the early gut microbiome.

OBJECTIVE: We hypothesized that altered circadian gene expression solely in the neonatal liver would result in gut microbiome dysbiosis, especially with diet-induced metabolic stress (ie, restricted feeding). Using a murine model in which we conditionally knock out *Npas2* in the neonatal liver, we aimed to determine the role of the circadian machinery in gut dysbiosis with restricted feeding.

STUDY DESIGN: We collected fecal samples from liver *Npas2* conditional knockout (n = 11) and wild-type (n = 13) reproductive-aged mice before (study day 0) and after the restricted feeding study (study day 17). Extracted DNA was sequenced using the MiSeq Illumina platform using primers specific for the V4 region of the 16S ribosomal DNA gene. The resulting sequences were quality filtered, aligned, and assigned taxonomy. Principal coordinate analysis was performed on unweighted and weighted UniFrac distances between samples with a permutation analysis of variance to assess clustering significance between groups. Microbial taxa that significantly differ between groups of interest was determined using linear discriminate analysis effect size and randomForrest.

RESULTS: Principal coordinate analysis performed on weighted UniFrac distances between male conditional knockout and wild-type cohorts revealed that the gut microbiome of the mice did not differ by genotype at the start of the restricted feeding study but did differ by virtue of genotype at the end of the study ($P = .001$). Moreover, these differences could be at least partially attributed to restricted feeding—associated alterations in relative abundance of the *Bacteroides* genus, which has been implicated as crucial to establishing a healthy gut microbiome early in development.

CONCLUSION: Here we have provided an initial key insight into the interplay between neonatal establishment of the peripheral circadian clock in the liver and the ability of the gut microbiome to respond to dietary and metabolic stress. Because *Npas2* expression in the liver is a target of maternal high-fat diet—induced metabolic perturbations during fetal development, we speculate that these findings have potential implications in the long-term metabolic health of their offspring.

Key words: gut microbiome, mouse model, *Npas2*, peripheral circadian clock, restricted feeding

The study of how circadian biology is established, and when and how it is modified, is of importance to obstetrical and neonatal providers because intact circadian rhythms are responsible for the coordination of metabolism with key behaviors such as sleep and feeding.[1]

With increasing attention on the role of the human microbiome in health and disease, it is evident that many aspects of our lifelong metabolic health are influenced not only by our genetics but also rather by the totality of our genomic and metagenomic inheritance.[2,3] This includes both changes on the genome (or epigenetics) such as posttranslational histone modifications and DNA methylation as well as the microbiome's metagenome.[4,5] While it has previously been shown that there is bidirectional communication between the host circadian biology and the gut microbiome,[3,6-8] the timing and mechanisms by which this occurs remains unknown.

Establishing circadian clock function in early life: fetal disruption of *Npas2* and its long-term health effects

Previous work in our nonhuman primate model of maternal high-fat diet feeding led to the discovery that the circadian gene, neuronal PAS domain protein 2 (*Npas2*), acted as an epigenomically modified regulator of fetal hepatic metabolism.[9,10] Fetuses exposed to a maternal high-fat diet (HFD) in utero demonstrated a significant increase in the acetylation of histone H3 at lysine 14 (H3K14ac); these alterations persisted in juvenile animals.[9-11]

Using differential display chromatin immunoprecipitation approaches to determine where this modification was enriched throughout the fetal hepatic genome, we found differential occupancy of the *Npas2* promoter region by H3K14ac H3 between control and HFD-exposed offspring.[9] Further analysis using chromatin immunoprecipitation followed by site-specific quantitative polymerase chain reaction revealed an increase of H3K14ac occupancy in the *Npas2* promoter in animals exposed to a HFD in utero,[10] a finding that persisted in juvenile animals despite later consumption of a control diet.[3] This alteration in promoter occupancy associated

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with differential *Npas2* transcription between the control diet and HFD-exposed offspring[10] and was accompanied by persistent metabolic dysfunction and nonalcoholic fatty liver disease through at least 3 years of age.[12-18]

These findings not only implicate a lasting role of a high-fat maternal diet in regulating fetal and postnatal *Npas2* but also provide additional evidence of the importance of the liver circadian clock in maintaining optimal metabolic health in the offspring, starting in utero and persisting into their juvenile years. However, precisely when, where, and how epigenomic reprogramming of the offspring liver exerts such broad metabolic disarray is poorly understood.

When and where: generation of neonatal liver-specific *Npas2* conditional knockout (cKO) mice and overall study design

To first separate the impact of the role of immediate neonatal vs circadian gene expression in the liver on the lifelong regulation of metabolic homeostasis, we generated a novel cKO mouse model in which *Npas2* is specifically deleted in the neonatal (but not the fetal) liver.

Previous studies have characterized the metabolic phenotype of an *Npas2* knockout (KO; whole-body knockout) mouse model, and under normal metabolic conditions, no phenotype was reported.[19-22] However, under a restricted feeding regimen, *Npas2* KO mice lose a greater body weight percentage when food is restricted over a 2 week period, suggesting a morbid maladaptation and abnormal satiety signals.[23,24]

In one study the weight loss was so severe that the KO mice wasted, resulting in the death of 20% of the mice.[23] However, in these previous studies, *Npas2* was knocked out in all tissues, including the brain (ie, the light-dark responsive master clock in the superchiasmatic nucleus), which is presumed to be the master pacemaker of the circadian clock. To determine whether neonatal *Npas2* peripheral expression in the liver is necessary for adaptive feeding, we exposed *Npas2* cKO mice to a restricted feeding regimen, which is the only previously published condition to elucidate a unique role for *Npas2* in regulating metabolic homeostasis.

How: circadian interactions and the gut microbiome

We and others have recently shown that the neonate is not born sterile and that the primate and human gut microbiome is persistently altered with maternal HFD exposure.[25,26] Emerging evidence has indicated that the host circadian clock is closely intertwined with the gut microbiome.[8,27-33]

Work in the germ-free mouse has demonstrated that the peripheral circadian clock, particularly in the liver, is potentially regulated by the gut microbiome because mice devoid of any microbes demonstrate altered patterns of hepatic circadian gene expression.[34,35] However, given the poor overall metabolic health of gnotobiotic animals, discerning cause from effect is problematic.

Similarly, the composition of the gut microbiome has been shown to exhibit a circadian rhythmicity that is abolished in *Clock* and *Bmal1* knockout mice,[36,37] indicating a bidirectional dependency between circadian clock rhythmicity and gut microbiome composition. Subsequent studies have further indicated that the impact of circadian clock disruption on the gut microbiome manifests most prominently when the animals are placed under a dietary stress, like a high-fat diet challenge.[38] However, whether the dependency of gut microbiome on host circadian clock gene expression is due to the central clock in the brain or through the liver has not been explored. Although evidence points to the hepatic clock as the likely regulator of the gut environment, the impact of the central clock cannot be ruled out because the gut and the central nervous system are closely interconnected through the many neural projections via the gut-brain axis.

To determine how isolated postnatal disruption of the liver circadian clock has an impact on the later composition of the gut microbiome, in the current study we generated a conditional knockout mouse model whereby *Npas2* was specifically deleted in hepatocytes during neonatal life and henceforth. Considering the past work of us and others demonstrating the impact of the dietary stress on circadian clock disruption, we hypothesized that under normal conditions, the gut microbiome would be unchanged but would be differentially altered during a period of a restricted feeding.

The aims of the current study were therefore to address the mechanistic role of neonatal *Npas2* expression of metabolism and gut dysbiosis later in life.

Materials and Methods

Npas2 hepatic cKO generation

The *Npas2* floxed allele was designed to delete exon 3 through Cre-mediated recombination. Deletion of exon 3 eliminates the coding region for the basic helix-loop-helix DNA binding domain of *Npas2* as well as the Aryl hydrocarbon receptor nuclear translocator-like-—binding domain, encoding for a nonfunctional NPAS2 protein.[39,40]

Mice heterozygous for the *Npas2* floxed allele ($Npas2^{fl/+}$) mice were crossbred to generate mice homozygous for the floxed *Npas2* allele ($Npas2^{fl/fl}$). These and subsequent mice were housed at the Alkek Building for Biomedical Research mouse facility at Baylor College of Medicine, and all animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (AN-4826).

$Npas2^{fl/fl}$ mice were bred with B6.Cg-Tg(Alb-cre)21Mgn/J mice purchased from Jackson Laboratories (*Alb-cre*; Jackson Laboratories, Bar Harbor, ME), which are homozygous for the transgenic *cre* gene with an albumin promotor generating mice heterozygous for the floxed *Npas2* and *Alb-cre* transgenic alleles ($Npas2^{fl/+}$/*Alb- cre*). In *Alb-cre* mice, *cre* is expressed in the postnatal liver.[41] $Npas2^{fl/+}$/*Alb- cre* females were mated to $Npas2^{fl/fl}$ males to generate hepatocyte specific *Npas2* cKO ($Npas2^{fl/fl}$/*Alb- cre*) mice. $Npas2^{fl/fl}$ (wild-type; WT) offspring were used as controls in this study (Supplemental Figure 1).

Restricted feeding study

Mice are nocturnal animals and preferentially feed at night during the dark. To

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