Editorial

JOURNAL OF HEPATOLOGY

Hedgehog signaling keeps liver clock in check

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Most organisms, including humans, have developed an autonomous time-keeping system hard-wired by biological clocks that generate daily fluctuations in physiological and cellular processes. These biological clocks are reset daily by light to adjust physiology to the day/night cycle generated by the rotation of the Earth around its axis. In organs involved in glucose and lipid metabolism, such as the liver, biological clocks are also reset by feeding cues, which allow the integration of systemic and nutritional signals to adapt fuel production and utilization according to the feeding/fasting cycle. In a ground-breaking report, published in 2019, Chinese researchers reported the generation of a monkey harboring deletion of the *BMAL1* circadian gene, using CRISPR/Cas9 editing of monkey embryos.[1] Not surprisingly, these monkeys exhibit insomnia and psychiatric disorders, producing a horrifying ethical minefield and widespread protests. In fact, it is already well known that circadian disruption is a risk factor for metabolic, psychiatric and age-related disorders, in an intertwined fashion.[2–4] At the molecular level, in every single cell circadian rhythmicity is generated by an oscillator that comprises a feedback loop in which the expression of so-called clock genes is suppressed periodically by their protein products and completes one cycle each day. The core clock genes form interacting positive (*BMAL1, CLOCK*) and negative (*PERIOD, CRYPTOCHROME*) limbs: a heterodimer of the CLOCK and BMAL1 proteins rhythmically activates the transcription of clock genes *PER1, PER2, PER3, CRY1, CRY2*. The PER and CRY proteins form a complex that translocates back into the nucleus, interacts directly with the CLOCK:BMAL1 heterodimer and represses its transcriptional activity.[5] The CLOCK:BMAL1 heterodimer activates an auxiliary loop promoting the expression of the nuclear receptors RORα, REV-ERBα (encoded by *NR1D1*) and REV-ERBβ (encoded by *NR1D2*), that in turn, control the rhythmic transcription of *BMAL1*, as the REV-ERBα/β protein binds to ROR/REV-ERB-response elements (RORE) of the *BMAL1* promoter and represses *BMAL1* transcription, preventing the binding and activating action of RORα. This stabilizing loop is important for precise control of the cell-autonomous biological clock, as well as hepatic lipid metabolism, and ultimately for the harmonization of metabolism with circadian behavioral and physiological rhythms.[6,7]

Excessive lipid accumulation in the liver, otherwise termed hepatic steatosis or non-alcoholic fatty liver disease (NAFLD), represents a pandemic whose prevalence (25% in the general population) parallels the worldwide increase of obesity.[8] NAFLD can often evolve into non-alcoholic steatohepatitis (NASH), a potentially progressive liver disease that can lead to cirrhosis, hepatocellular carcinoma and death. NASH is characterized by steatosis, hepatocellular ballooning and inflammation, and has a global prevalence ranging from 3% to 5%.[8] Of concern, NAFLD can occur in several cases even in the absence of obesity, so-called "lean" NAFLD.[8] The pathogenic mechanisms and the contribution of environmental and genetic factors to the risk of developing a progressive course of NAFLD/NASH are not totally understood. In the liver, the clock machinery is essential for the maintenance of metabolic homeostasis. All metabolic processes, ranging from carbohydrates, to lipid, to amino acid metabolism, to drug metabolism are tightly dependent on a functioning biological clock in hepatocytes.[9] It is now clear that biological clock dysfunction accelerates the development of NAFLD, cirrhosis and liver cancer, and in turn, these disorders also disrupt clock function.[9,10] The hepatic clock strictly regulates the pharmacokinetics of xenobiotics through each of its phases, including absorption and uptake, distribution, metabolism and elimination. Most of these studies took advantage of valuable mice models, such as global clock-gene-knockout mice, or mice with liver-specific knockout of clock genes or clock-controlled genes. Moreover, targeted reporter mutant mice expressing PER2::LUC bioluminescence similar to endogenous PER2 expression, became widely useful in chronobiology research as a real-time reporter of circadian dynamics in different tissues, particularly in the liver.[11]

Compelling recent evidence demonstrates that, in addition to the circadian clock, dysregulation of the hedgehog (Hh) signaling pathway accelerates the development of NAFLD, cirrhosis and liver cancer.[12] Hh is a morphogenetic factor that is critical for embryonic development and limb patterning. Re-activation of this pathway occurs during adult tissue injury, as part of the regeneration and repair processes in various tissues, including the liver. Hh ligands (*i.e.* Sonic Hh, Indian Hh, Desert Hh) bind to the cognate cell surface receptor Patched (Ptch), which causes the release of its inhibitory action and de-represses the

Received 5 February 2019; accepted 11 February 2019
☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2019.01.022.
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co-receptor Smoothened (Smo). Active Smo then transactivates downstream transcription factors, Glioma-associated oncogene (Gli1, 2, 3), which lead to the expression or repression of various target genes.[12] Healthy adult hepatocytes do not produce Hh ligands but hepatic synthesis of Hh ligands drastically increased during NAFLD and NASH.[12,13] Although excessive Hh pathway activation is believed to promote the progression of NAFLD to NASH and beyond, some basal level of Hh activity may be necessary to prevent NAFLD. Therefore, transient Hh signaling activation might constitute a compensatory response to hepatic fat accumulation. This conceptual framework has been validated in a large number of genetic and dietary mice models for the disease.[12] For instance, conditional liver-specific deletion of Smo in adult mice downregulated the Hh pathway and induced NAFLD,[14] through the induction of lipogenic factors. Vice versa, activation of the Hh pathway (through SUFU knockdown, small molecule Shh agonists or Gli overexpression) reversed NAFLD in the liver of genetic models of obesity-related NAFLD.[14] In humans, the level of activation of the Hh pathway correlates with the severity of liver damage.[12] Even if the role of the Hh pathway in the initial stages of NAFLD – simple steatosis - is not completely understood, Hh appears consistently activated and is one of the best-studied pathways for regulating NASH-related fibrosis progression.

In the present issue, Marbach-Breitrück *et al.*[15] report the results of an original study investigating the interplay between Hh signaling and the biological clock in the regulation of lipid metabolism in the liver. They assessed time-related patterns of expression of Hh signaling pathway and core clock components in liver and hepatocytes from controls and mice with ablation of Smoothened (SAC-KO) and crossbreeds with PER2:: LUC reporter mice. Several Hh pathway genes (*Ihh*, *Ptch1*, *Smo*, *Hhip1*, *Fu*, *SuFu*) showed circadian fluctuations in the liver and hepatocytes *in vitro*, the genes encoding Gli transcription factors (*Gli1*, *Gli3*) exhibited only tiny fluctuations and the serum levels of Indian, the ligand encoded by *Ihh*, oscillated with 24-hour periodicity.[15] Bmal1 downregulation in hepatocytes provoked important alterations in the expression of Hh pathway genes (upregulation of *Gli1* and *Ihh*, downregulation of *Ptch1*) and, in turn, altered expression of clock genes in SAC-KO mice was demonstrated and corroborated *in vitro* using RNA interference against *Gli1* and *Gli3*. Rhythmicity of many metabolic pathways, including hepatic lipid metabolism, was affected by *Smo* knockout in SAC-KO hepatocytes, as evidenced using genome-wide screening.[15]

Interestingly, nutritional challenge represented by high-fat diet or starvation applied at diverse zeitgeber time differently impacted the biological clock-Hh signaling interplay and these results could supply a mechanistic explanation for the dissimilar effect of differently timed eating/fasting on the liver.

This point could be of particular importance considering, on one side, the relevance of feeding time as a risk factor for dysmetabolism in humans, independently of the amount or content of food intake, and, on the other side, the positive metabolic effects of prolonged fasting or very low energy intake and time restricted feeding. In particular, the relationship between food intake and melatonin secretion onset impacts body composition, with augmented body fat and reduced glucose tolerance linked to eating at a later circadian time.[10,16,17] Furthermore as the Hh signaling pathway inhibits autophagy in mammalian cells,[18] established Hh inhibitors, such as the FDA-approved Vismodegib (a potent Smo antagonist) may be studied in the future in combination with chronomodulated nutritional approaches to treat obesity and NAFLD/NASH.

Overall, the results of this interesting study point to a tight bi-directional interplay between Hh signaling and the biological clock, mainly operated by the Gli transcription factors, as well as to a pivotal role of this interplay in the homeostasis of lipid metabolism and NAFLD pathogenesis. A future perspective could be represented by the investigation of the role played by Hh signaling for the modulation of cellular processes controlled by the biological clock, such as the pathways involved in bile acid turnover, autophagy, inflammation in the liver as well as gut microbiota dynamics, which are lead actors in the onset of hepatic steatosis-progression to NASH, and its impact on the metabolic effects of timed eating/fasting patterns, *i.e.* chrononutrition.

Financial support

We acknowledge funding support from the 5 × 1000 voluntary contribution and from the Italian Ministry of Health (No. RC1803ME40) (G.M.), and from the European Social Fund and European Regional Development Fund – Project MAGNET (No. CZ.02.1.01/0.0/0.0/15_003/0000492) (M.V.).

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.02.009.

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