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말초조직 일주기성이 에너지 대사 조절에
미치는 역할 규명

Roles of peripheral circadian clock
in the regulation of energy metabolism

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생명과학부

장 하 군

ABSTRACT

Roles of peripheral circadian clock in the regulation of hepatic energy homeostasis

Hagoon Jang

Emerging evidence has suggested that the circadian clock is a control tower in the regulation of behavioral and molecular processes under day/night cycle. Also, precise regulation of the circadian clock is crucial for maintaining whole-body energy homeostasis. Thus, dysregulation of the circadian clock is closely associated with obesity and metabolic complications. In metabolic organs, such as adipose tissue, muscle and liver, the circadian clocks affect various metabolic processes including lipogenesis, gluconeogenesis and lipid oxidation. Moreover, unsynchronized cooperation between the hypothalamic central clock and the peripheral clock, in shift workers, is prone to occur metabolic disorders. However, the pathophysiological role of the circadian clock and its metabolic regulatory processes have not been thoroughly elucidated.

In this study, I have demonstrated that unsynchronized circadian clock influences hepatic glucose and lipid metabolisms without altering body weight. With the same amounts of caloric intake during different time periods, the expression patterns of peripheral circadian clock genes showed different by day time feeding and

night time feeding, while that of light-regulated hypothalamic circadian clock genes was not affected. For instance, the expression pattern of core circadian clock genes, such as BMAL1, CLOCK and PER2 in peripheral tissues, was altered by different feeding periods. In addition, hepatic expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation genes was also changed by feeding periods. In conclusion, it is likely that the amounts of food consumed might be a crucial factor to induce obesity compared to feeding time because feeding period restriction has no effects on body weight gain either normal chow diet (NCD) or high fat diet (HFD).

In addition, I have revealed that feeding-induced CRY1 gene expression leads to suppression of hepatic gluconeogenesis. Given that SREBP1c is a well-known transcriptional activator in postprandial state for activating lipogenesis, I found out that SREBP1c stimulated CRY1 gene expression. CRY1 was induced by feeding and insulin challenge. In addition, I have shown that hepatic SREBP1c contributed to repress gluconeogenesis through CRY1 induction. Hepatocytes overexpressing CRY1 inhibited hepatic gluconeogenic genes, such as PEPCK and G6Pase, via lowering FOXO1 protein. Furthermore, CRY1 was elevated in long term insulin action for sustainable suppression of hepatic gluconeogenesis. Intriguingly, SREBP1c-induced CRY1 accelerated FOXO1 degradation via ubiquitination. I discovered that CRY1 would act as a scaffold protein by binding with MDM2, an E3 ubiquitin ligase, and FOXO1. Although SREBP1c is increased in obese diabetic animals, such as *db/db* mice and HFD fed mice, SREBP1c failed to stimulate CRY1

and thereby hepatic gluconeogenesis was not suppressed in obese animals. When I overexpressed CRY1 in the liver of *db/db* mice to test the CRY1 effects on gluconeogenesis suppression. CRY1 overexpression reduced blood glucose level as well as downregulated hepatic gluconeogenic gene expression. Taken together, these data suggest that circadian clock genes actively and dynamically regulate energy metabolism in the liver. Among many circadian clock genes, SREBP1c-induced CRY1 especially contributes to the suppression of hepatic glucose production through FOXO1 degradation in liver. However, body weight gain is mainly determined by the amounts of calorie intake rather than alteration of peripheral circadian clock gene. Therefore, it is likely that appropriate regulation of SREBP1c-CRY1 signaling pathway would be crucial for maintaining whole-body energy homeostasis.

Key words: Obesity, Diabetes, Circadian clock, SREBP1c, CRY1, FOXO1, MDM2, PEPCCK, G6Pase, Hepatic gluconeogenesis, Insulin signaling

Student number: 2008-20377

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BACKGROUND

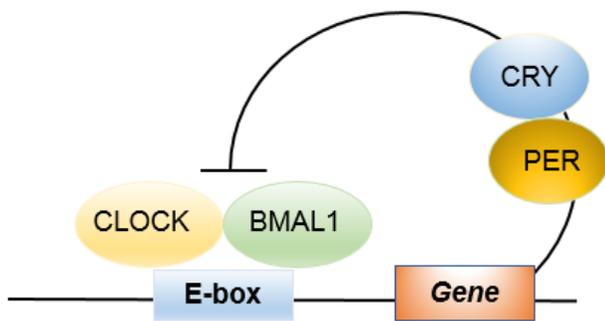
1. Circadian clock and metabolic regulation

(1) Circadian clock

Circadian clocks control behavioral and molecular processes with the day and night cycle. The molecular circadian clock in mammals exists within pacemaker neurons of the suprachiasmatic nucleus (SCN) and peripheral tissues. The central SCN clock regulates standard time for peripheral tissue clock (Ramsey et al., 2007). Various physiological changes, such as sleep-wake cycles, body temperature, blood pressure, and hormone secretion, are associated with the circadian clock. Molecular circadian oscillation is composed of transcription-translational auto-regulatory negative feedback loops; BMAL1, CLOCK, PER, and CRY are key circadian molecules that produce rhythmic oscillations in a cell-autonomous manner (Thresher et al., 1998; Vitaterna et al., 1999). BMAL1 and CLOCK play key roles in inducing PER and CRY. Induced PER and CRY then form a transcriptional repressor complex to suppress BMAL1 and CLOCK, which eventually leads to negative feedback regulation (Figure 1). In addition, BMAL1 and CLOCK also increase the mRNA levels of Rev-erb α and ROR α , which then compete to bind to the retinoic acid-related orphan receptor response elements (ROREs) in BMAL1 promoter and repress or activate the expression of BMAL1, respectively (Raspe et al., 2002; Tini et al., 1995). This alternating promoter occupancy is due to the rhythmic expression of Rev-erb α

Figure 1. Molecular clock: circadian negative feedback loop.

CLOCK and BMAL1, heterodimer initiates transcription of target genes such 'period' genes (PER1, PER2, and PER3) and two cryptochrome genes (CRY1 and CRY2). Negative feedback is achieved by PER/CRY complexes which translocate into the nucleus to suppress their own transcription by blocking the activity of the CLOCK/BMAL1 heterodimer.



and ROR α .

Moreover, these auto-regulatory loops are modulated by various post-translational modifications such as phosphorylation, sumoylation, acetylation, and ubiquitination. For instance, casein kinase phosphorylates the PER proteins for degradation via the Skp1, cullin1, F-box protein (SCF)/ B-TrCP ubiquitin ligase complex. Adenosine monophosphate kinase (AMPK) phosphorylates CRYs and leads to ubiquitin-mediated proteasomal degradation via the SCF/FBXL3 ubiquitin ligase complex (Lamia et al., 2009; Zheng et al., 2014). In addition, cellular NAD⁺ level regulates SIRT1 activity which participates in CLOCK protein deacetylation (Peek et al., 2013; Ramsey et al., 2009).

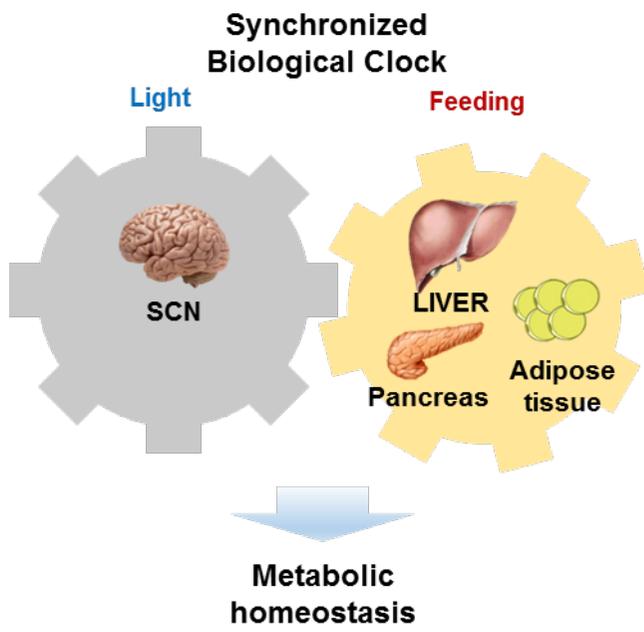
Central SCN circadian oscillation is primarily regulated by light, whereas peripheral circadian oscillation is affected by food intake along with hormones such as insulin and glucagon (Gamble et al., 2014; Hoyle and O'Neill, 2013). These clocks regulate biological processes to maintain whole-body homeostasis with the environmental changes of light and nutrients (Figure 2). Many recent studies have shown that numerous aspects of metabolic regulation, such as circulating hormones, intracellular metabolites, and feeding behaviors, exhibit daily rhythmicity (Brandenberger and Weibel, 2004).

(2) Circadian clock and metabolic regulation

Emerging evidences suggest that the circadian clock is closely associated

Figure 2. Light-dependent central clock and feeding-mediated peripheral clock.

The master pacemaker within SCN, although clock genes are also expressed in other tissues such as liver, adipose tissue, and pancreas. Emerging evidence suggests that peripheral clock synchronized with central clock regulates whole-body energy homeostasis.



with whole-body energy homeostasis. For example, the finding that participation of the orphan nuclear hormone receptors, Rev-erb α and the opposing ROR α , in a short feedback loop by controlling BMAL1 transcription provides a direct evidence for metabolic input into the molecular clock (Raspe et al., 2002; Tini et al., 1995). In addition, peroxisome proliferator-activated receptor α (PPAR α) and peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1 α) also regulate BMAL1 transcription via circadian core feedback loop (Liu et al., 2007). Moreover, microarray analysis reveals that more than half of transcripts show rhythmic gene oscillation, with some variation among different tissues such as liver, skeletal muscle, and brown and white adipose tissue (Yang et al., 2006). The numbers of transcripts rhythmic oscillation are up to 20%, indicating that a large proportion of the transcriptomes seem to be affected by circadian genes. These oscillating genes are involved in biosynthetic and metabolic processes such as cholesterol and lipid metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation, and detoxification pathways (Akhtar et al., 2002; McCarthy et al., 2007). Interestingly, most rate-limiting enzymes in these metabolic pathways are regulated by circadian clocks, implying that the clocks could influence on biosynthetic and metabolic processes whole-body metabolic homeostasis

(3) Circadian clock and metabolic disorder

In the aspect of clinical studies, several evidences suggest that circadian

disruption is associated with metabolic complications across large portions of the human population (Karlsson et al., 2001). Cross-sectional studies have revealed an increased prevalence of metabolic syndrome, such as high body mass index and cardiovascular events, in shift workers. These observations suggest the possibility that chronic disharmony between central and peripheral clocks, combined with light/dark and fasting/feeding, might contribute to body weight gain and metabolic complications (Kalra and Kalra, 2004). In addition, intriguing human behavioral studies propose that nocturnal feeding patterns might be related with metabolic diseases (Ayala et al., 2009). These findings have been recapitulated by recent rodent experimental studies, indicating that diet-induced obesity (DIO) with HFD may lead to increased energy intake only in the rest/light period and not in the active/dark period (Kohsaka et al., 2007). In addition, several rodent models have suggested that circadian clock regulation is associated with metabolic disease. For example, CLOCK-defective mice show increased body weight and hyperphagia with disrupted circadian oscillation (Turek et al., 2005). HFD-fed mice become obese and diabetic concomitantly with altered expression of circadian clock genes (Kohsaka et al., 2007). BMAL1 knockout mice show dysregulation of hepatic glucose homeostasis (Rudic et al., 2004). Furthermore, key metabolic genes, such as PPAR α , PPAR γ , and AMPK, are involved in the regulation of circadian genes, and the circadian clocks in turn modulate whole-body energy metabolism (Lamia et al., 2009; Schmutz et al., 2010; Wang et al., 2008a). In addition to rodent studies, the control of glucose metabolism

by circadian oscillation in humans is a well-known aspect of clinical diabetes management, and a variation of the normal cyclic pattern of glucose tolerance is an important indicator of type 2 diabetes (Allison et al., 2007; Tasali et al., 2008).

Unsynchronized central and peripheral clocks caused by different regulation of light/dark cycle and the feeding/fasting cycle have been implicated in metabolic disorder, along with higher calorie intake in the case of shift-workers (Ellingsen et al., 2007; Karlsson et al., 2001). Under physiological conditions, the central and peripheral clocks are synchronized by the light/dark cycle and the feeding/fasting cycle. However, it has been also demonstrated that disharmonious signaling by these two cues leads to the independent regulation of each circadian oscillation. Although it appears that there is a close relationship between the circadian clock and metabolic regulation, the effects of unsynchronized SCN and peripheral-tissue circadian clocks on metabolic regulation are largely unknown. In addition, it is still elusive whether feeding period alteration might be a key determinant of body weight change without change of total calorie intake.

2. Hepatic lipid and glucose metabolism

(1) Lipogenesis

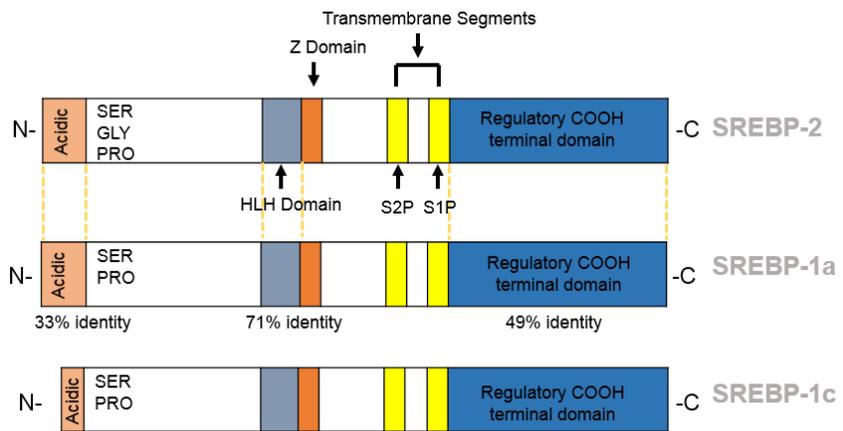
In liver, synthesis of triacylglycerides in liver is nutritionally and hormonally regulated. The ingestion of a high-carbohydrate diet causes a marked elevation of enzymes involved in key metabolic pathways, including glycolysis,

lipogenesis, fatty acid elongation and desaturation steps, and finally triacylglyceride synthesis. Especially, lipogenesis is the process that converts acetyl-CoA to fatty acids. Acetyl-CoA is an intermediate metabolite produced from glucose, a source of primary energy of living organisms. Through lipogenesis and subsequent triacylglyceride synthesis, the excess energy can be efficiently stored in the form of neutral lipid metabolites (Ferre and Foufelle, 2010). Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, and increased malonyl-CoA level leads to produce long chain fatty acids. This reaction is the controlling step in fatty acid synthesis and takes place in the cell cytosol. The overall synthesis of fatty acids is catalyzed by the fatty acid synthase (FASN) complex, a single polypeptide containing seven distinct enzymatic activities (Griffin and Sul, 2004).

Sterol regulatory element binding proteins (SREBPs) including SREBP1a, SREBP1c, and SREBP2 belong to the basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family that regulates de novo lipogenesis and cholesterol biosynthetic pathway (Figure 3) (Brown and Goldstein, 1997). SREBP precursor consists of structurally three functional domains; an NH₂-terminal domain that contains the bHLH-LZ region for DNA binding, two hydrophobic transmembrane spanning domains interrupted by a short loop which are inserted into the lumen of ER, and COOH-terminal domain that recruits the gene regulatory machinery (Figure 3). Precursors of SREBPs are processed by several SREBPs processing apparatus.

Figure 3. Structure of SREBP isotypes.

Sterol regulatory element binding protein (SREBP) was identified as a protein that bound to the sterol regulatory element (SRE). SREBPs are a family of transcription factors that control lipid homeostasis by regulating the expression of enzymes required for cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis. SREBPs are synthesized as precursor forms bound to the endoplasmic reticulum membranes. Upon activation, the precursor undergoes a sequential two step cleavage process to release the N-terminal active domain in the nucleus.



SREBP cleavage-activating protein (SCAP) is a sensor of cholesterol, and escorts the SREBP from the ER to the Golgi, where there are two proteases involved in the cleavage (DeBose-Boyd et al., 1999; Tomita et al., 1998). In the Golgi apparatus, SREBP is released from the Golgi membrane through cleavage by Site-1 protease (S1P) and Site-2 protease (S2P). The NH₂-terminal domain of SREBPs is translocated to the nucleus, where it stimulates target gene transcription by binding to sterol response elements (SREs) or E-BOX in the promoter/enhancer regions of various target genes (Kim et al., 1995).

SREBP1c is the master regulator of de novo lipogenesis in fat tissue and liver (Shimomura et al., 1999a). Insulin, which is released from pancreatic β -cells, stimulates, leading to fatty acid synthesis during nutrient rich status (Kim et al., 1998a). However, glucagon, which is released from pancreatic α -cells during fasting, suppresses SREBP1c mediated lipogenic action (Lee et al., 2014a). SREBP1c regulates lipogenic pathways by stimulating the expression of fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) and acetyl-coenzyme A carboxylase (ACC) (Kim et al., 1998a; Shimomura et al., 1999b). The level of SREBP1c falls in streptozotocin-treated animals by accelerating pancreatic β -cell apoptosis and increases after insulin injection (Shimomura et al., 1999b). Moreover, overexpression of nuclear SREBP1c in livers of transgenic mice prevents the reduction of lipogenic action in fasting (Takahashi et al., 2005). Taken together, accumulating evidences indicate that SREBP1c is a key player in insulin-mediated lipogenic activation in liver.

(2) Glucose metabolism

During fasting, most animals maintain energy balance by shifting from glucose utilization to fat burning. The levels of glycogenolysis and gluconeogenesis are increased to provide glucose as an energy source in many organs and tissues such as the brain and the red blood cell compartment, which are deficient in enzymes for burning free fatty acids (Altarejos and Montminy, 2011; Roach et al., 2012). Fasting also triggers elevation of circulating free amino acids, which are also major precursors for hepatic glucose production. When fasting is prolonged, hepatic gluconeogenesis is blocked to protect against excessive muscle wasting by over-converting protein to glucose, and liver derived ketone bodies become the primary energy source for the brain (Morris, 2005).

During fasting, elevated levels of circulating pancreatic glucagon stimulate the gluconeogenic program through the activation of the protein kinase A (PKA) pathway. Increased cAMP levels activate PKA, then gluconeogenic genes such as phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are upregulated (Montminy et al., 2004). cAMP response element binding protein (CREB), one of major regulators of hepatic gluconeogenesis, binds to promoters for the PEPCK and G6Pase genes to directly stimulate the gluconeogenic program. The significance of CREB in the activation of hepatic gluconeogenesis has been revealed by a study utilizing albumin-ACREB TG mice overexpressing a dominant negative CREB in liver (Herzig et al., 2001). These mice show lower blood glucose levels with

downregulated mRNA levels for hepatic gluconeogenic genes, indicating that CREB is a physiological transcriptional regulator of gluconeogenesis *in vivo*.

In parallel, decrement of insulin signaling upon fasting also stimulates gluconeogenic gene expression through dephosphorylation and nuclear translocation of the forkhead box (FOXO) domain proteins (Matsumoto and Accili, 2005). FOXO1 belongs to a subclass of the forkhead family of transcription factors which have a forkhead box type DNA binding domain. FOXO1 recognizes insulin response element (IRE) located in the promoters of PEPCK and G6Pase. Regulation of subcellular localization of FOXO1 is one of the important regulatory pathways to control its activity, which is modulated by the phosphorylation status of Ser/Thr residues. Insulin and PI3K signaling pathway activates AKT-dependent phosphorylation of FOXO1 and phosphorylated FOXO1 binds with 14-3-3 shuttle protein to translocate to the cytoplasm (Nielsen et al., 2008; Tzivion et al., 2011; Wang et al., 2006). The cytoplasm-localized FOXO1 then undergoes subsequent degradation by an ubiquitin-proteasome pathway. Although the translocation of hepatic FOXO1 from the nucleus to the cytoplasm is a well-defined mechanism mediating a quick decrease in glucose production by insulin, it is largely unknown how insulin provides a sustainable inhibition of hepatic gluconeogenesis during the postprandial state.

Interestingly, SREBP1c appears to be involved in hepatic carbohydrate metabolism. For example, SREBP1c affects the mRNA levels of PEPCK, G6Pase,

and IRS-2 genes and inhibits the interaction between HNF4 and PGC1 α to suppress gluconeogenic genes (Lee et al., 2007; Yamamoto et al., 2004). Although it has been reported that hepatic SREBP1c suppresses hepatic glucose production, the molecular mechanism(s) by which SREBP1c could repress hepatic gluconeogenesis is unclear.

(3) Selective insulin resistance in liver

Under nutrient rich status, pancreatic β -cell secretes insulin, which is one of the major anabolic hormones in human body. Insulin accelerates glucose uptake in fat tissue and muscle as well as inhibits hepatic gluconeogenesis for lowering blood glucose level (Biddinger and Kahn, 2006). Moreover, insulin elevates lipogenic activity to store excess energy as triacylglyceride in liver and fat tissue (Kim et al., 1998a).

Type 2 diabetes are closely associated with hyperinsulinemia, hyperglycemia, and hypertriacylglyceridemia. Insulin resistance is one of the characteristics of type 2 diabetes. In insulin resistant animals, increased insulin is not enough to lower blood glucose, and consequently hyperglycemia reveals (Saltiel and Kahn, 2001). In diabetic subjects, liver, muscle, and adipose tissue are the major insulin resistant organs and the precise contributions of each organ to hyperglycemia and hypertriacylglyceridemia are unclear.

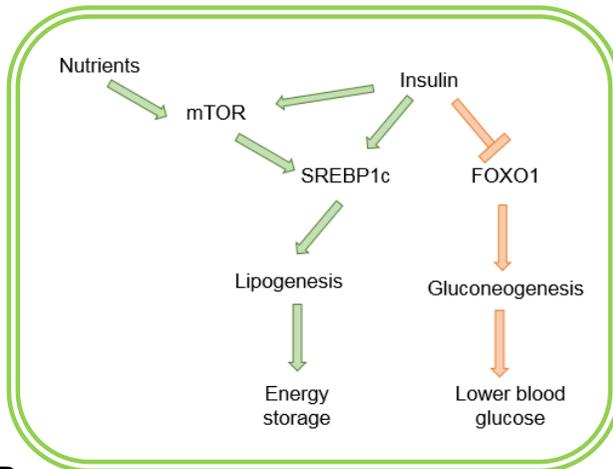
Upon feeding conditions, increased plasma insulin levels elicit two key actions at the level of gene expression, at least, in the liver. First, insulin activates

transcription factor SREBP1c which enhances transcription of genes required for fatty acid and triacylglyceride biosynthesis such as ACC and FASN (Kim et al., 1998a). In liver, the newly synthesized triacylglycerides are secreted in the form of very low density lipoproteins (VLDL), which are delivered to adipose tissues for storage and to muscles for fueling. The uptake of VLDL-derived fatty acids in adipose tissue is facilitated by insulin, which activates lipoprotein lipase on the surface of endothelial cells (Bourgeois et al., 1995; Lewis et al., 1994). Second, insulin stimulates the phosphorylation of FOXO1, a transcription factor that activates gluconeogenesis (Puigserver et al., 2003; Schilling et al., 2006; Xiong et al., 2013). Insulin-mediated FOXO1 phosphorylation prevents FOXO1 from entering the nucleus, which downregulates the expression of genes required for gluconeogenesis, including PEPCK and G6Pase. Blocking of hepatic glucose production by insulin is one of the crucial pathways to maintain blood glucose homeostasis. Interestingly, in diabetic liver, FOXO1 pathway becomes insulin resistant whereas SREBP1c-mediated lipogenesis shows insulin sensitive (Figure 4) (Brown and Goldstein, 2008). Despite extremely high insulin levels, the mRNAs of PEPCK and G6Pase remain high, and gluconeogenesis continues in diabetic liver. Moreover, since nuclear SREBP1c levels are high, fatty acid synthesis is accelerated and triacylglycerides are accumulated, leading to hepatic steatosis (Lee et al., 2014a). Further, elevated hepatic triacylglycerides are secreted via VLDL, raising plasma triacylglyceride levels in obese animals. Consequently, fatty acids derived from these triacylglycerides

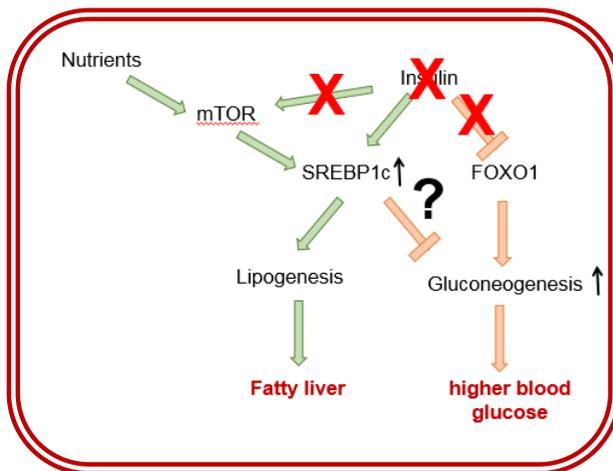
Figure 4. Selective insulin resistance in liver.

(A) Normal insulin response in liver. Insulin activates SERBP1c-mediated lipogenesis whereas inhibits gluconeogenesis. (B) Selective insulin resistance in liver of type 2 diabetes. Hyperinsulinemia still activates lipogenic action while is not able to suppress glucose production.

A Normal condition of insulin signaling



B Selective insulin resistance



aggravate insulin resistant state in muscle and adipose tissue, and the net results become hyperglycemia, hyperinsulinemia, and hypertriacylglyceridemia in type 2 diabetic animals (Figure 4).

An understanding the selective insulin resistance in diabetic liver is important for providing appropriate approaches to type 2 diabetes. Although it has been reported that hepatic SREBP1c is upregulated in obese animals, it is unknown why increased SREBP1c appears to fail to repress hepatic gluconeogenesis. Thus, it is crucial that understanding the molecular mechanisms by which SREBP1c could modulate gluconeogenesis under physiological and pathological conditions.

3. Purpose of this study

Circadian clock has a close correlation with metabolic regulation. Feeding is a major trigger that modulates the peripheral circadian clock while light activates the central circadian clock. These biological clocks are synchronized and synergically regulates various whole-body homeostasis including metabolism, body temperature, and sleep/wake cycle. Emerging evidence for the roles of circadian clock in metabolic tissues, such as adipose tissue, liver and muscle, proposes that the circadian clock actively interacts with metabolic signaling pathway to maintain the whole-body biological clock and energy homeostasis.

In this study, I have focused on body weight change according to feeding period restriction to investigate whether the restriction of feeding periods may affect body weight gain upon NCD and HFD. Whether the modulated peripheral circadian clock or the different amounts of food intake might be the primarily cause of obesity in shift workers is unclear. I have demonstrated that body weight gain in mice is not significantly changed by restricting feeding period to daytime or to nighttime if the animals take same calorie intake. On the contrary, the expression of peripheral circadian clock genes was altered by feeding period restriction, while the expression of light-regulated hypothalamic circadian clock genes was unaffected by both a NCD and HFD. The expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation related genes in the liver was also altered by feeding period restriction. Given that feeding period restriction does not affect body weight gain with both a NCD and HFD, it is likely that the amount of food consumed might be a crucial factor in determining body weight. In the second part, I have aimed to find out the new target gene of SREBP1c to find the signaling pathway that suppresses the gluconeogenesis. Although SREBP1c suppresses hepatic glucose production, the underlying molecular signaling pathway is unclear. To find out missing link between SREBP1c and inhibition of gluconeogenesis, I discovered CRY1 as a new target gene of SREBP1c. With fasting and refeeding experiment, I have shown that CRY1 is increased by feeding and insulin, which suppresses hepatic glucose production by FOXO1 degradation. Taken together, I would like to propose that the interpretation

of the relationship between circadian clock and hepatic glucose/lipid metabolism is important for understanding the pathophysiology of obesity and diabetes.

CHAPTER ONE:

**Feeding period restriction alters expression of
peripheral circadian rhythm genes without body
weight change in mice**

Abstract

Accumulating evidence suggests that circadian clock is closely associated with metabolic regulation. However, it has not been clearly understood whether impaired circadian clock is a direct cause of metabolic dysregulation including body weight gain. In this study, I demonstrate that mice body weight gain is not significantly changed by feeding period restriction; ad libitum, day time feeding and night time feeding. Expression of peripheral circadian clock genes was altered by feeding period restriction, while that of light-regulated hypothalamic circadian clock genes was not affected with either normal chow diet (NCD) or high fat diet (HFD). In liver, the expression pattern of circadian clock genes including BMAL1, CLOCK, and PER2 was changed by different restrictions of feeding period. Moreover, the expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation-related genes was also altered in liver by feeding period restriction. Taken together, these data suggest that restriction of feeding period would modulate expression of peripheral circadian clock genes, which is uncoupled from light-sensitive hypothalamic circadian clock genes, even though feeding period restriction might not be a crucial factor to affect body weight gain under NCD or HFD, implying that the amounts of energy intake seems to be a crucial factor to determine body weight.

Introduction

Various physiological and behavioral oscillations such as sleep-wake cycles, body temperature, blood pressure, and hormone secretion are associated with circadian clock (Bass and Takahashi, 2010). Circadian oscillation is composed of auto-regulatory negative feedback loops; BMAL1, CLOCK, PER, and CRY are key circadian transcription factors that determine rhythmic oscillation in a cell-autonomous manner. BMAL1 and CLOCK play a key role to induce PER and CRY. Then, elevated PER and CRY form a transcriptional repressor complex to suppress BMAL1 and CLOCK, which eventually leads to negative feedback regulation. In addition to the PER and CRY targets, BMAL1 and CLOCK also activate mRNA level of Rev-erb α and ROR α , which thereafter compete for binding to the retinoic acid related orphan receptor response elements (ROREs) as to repress or activate expression of Bmal1, respectively. This alternating promoter occupancy is due to rhythmic expression of Rev-erb α (Green et al., 2008). In addition, these auto-regulatory loops are modulated by various post-translational modifications such as phosphorylation, sumoylation, acetylation, and ubiquitination (Lamia et al., 2009; Nakahata et al., 2009; Shirogane et al., 2005).

Circadian clock exists in the hypothalamic suprachiasmatic nucleus (SCN) as well as peripheral tissues including liver and fat (Bass and Takahashi, 2010). SCN circadian oscillation is primarily regulated by light signal whereas peripheral circadian oscillation is affected by food intake accompanied by hormones such as

insulin and glucagon (Green et al., 2008). Emerging evidences have suggested that circadian clock is deeply associated with whole-body energy homeostasis. For instance, CLOCK defective mice exhibit obesity and hyperphagia with disrupted circadian oscillation (Turek et al., 2005). High fat diet (HFD) fed wild type mice become obese and show altered expression of circadian clock genes with metabolic dysregulation (Kohsaka et al., 2007). Further, liver specific Bmal1 knockout mice lose their hepatic glucose homeostasis (Canaple et al., 2006). Moreover, key metabolic genes such as PPAR α , PPAR γ , and AMPK are involved in the regulation of circadian genes, and circadian clocks in turn modulate whole-body energy metabolism (Canaple et al., 2006; Lamia et al., 2009; Wang et al., 2008b). Under physiological conditions, SCN clock and peripheral clock are synchronized by light-dark cycle and feeding-fasting cycle. Nonetheless, it has been also demonstrated that disharmonious signaling of these two cues, light-dark cycle and feeding-fasting cycle, independently regulates each circadian oscillation (Damiola et al., 2000). Although it appears that there is a close relationship between circadian clock and metabolic regulation, the effects of unsynchronized circadian clocks in SCN and peripheral tissues on metabolic regulation are largely unknown. In addition, it is unclear whether alteration of feeding periods would be a major determinant of body weight change even when there is no change of calorie intake.

In this study, I have investigated whether the restriction of feeding periods may affect body weight gain upon NCD and HFD. Also, I have analyzed gene

expression profiles of circadian clocks from SCN and peripheral tissue under different feeding periods. Our data suggest that feeding period restriction would not influence body weight gain whereas it would differently regulate circadian oscillations in peripheral tissues but not in SCN.

Methods

Animal care and experimental protocol

Four-week-old male C57BL/6N mice were obtained from SAMTAKO BIO KOREA Co., Ltd. Mice were maintained according to the guidelines of Seoul National University Animal Experiment Ethics Committee. They were housed in individual cages for pair feeding in 12 hr light/ 12 hr dark cycles. After a minimum 1-week stabilization period, ad libitum group mice (5 weeks old) were exposed to food freely, and the night time fed mice were pair fed to match the amount of food of day time group with NCD and 60% HFD (Research Diets, Inc.) for 4 weeks (Figure 5). Only those pair-fed mice (night time fed) with similar body weights as those of day time fed mice were subjected to the procedure from 5 weeks of age to 9 weeks of age. Body weight and food intake were measured daily at ZT0 (7 a.m.) and ZT12 (7 p.m.) during the experimental protocol. The average initial body weights in each group of mice were not different. All mice were euthanized, and dissected tissue specimens were immediately stored at -80C until analysis.

Quantitative real-time RT-PCR analysis

Total RNAs were isolated from liver and hypothalamus as described previously (Choe et al., 2007), and cDNA was synthesized using the M-MuLV reverse

transcriptase kit (Fermentas, Glen Burnie, MD). The primers used for the real-time PCR analyses were produced in Bioneer (Korea). The primer sequences used for real-time PCR analyses are provided in supplementary Table 1.

Biochemical analysis

The levels of plasma cholesterol and triacylglycerides were measured using Infinity reagents (Thermo, Melbourne, Australia). Plasma glucose levels were measured with a freestyle blood glucose meter (Therasense; Uppsala, Sweden).

Table 1. q-RT PCR primer sequence.

Gene	Primer Sequence
Clock	5'-TTGCGTCTGTGGGTGTTG-3' 5'-TGCTTTGTCCTTGTCATCTTCT-3'
Bmal1	5'-AACCTTCCCGCAGCTAACAG-3' 5'-AGTCCTCTTTGGGCCACCTT-3'
Per2	5'-TGTGCGATGATGATTCGTGA-3' 5'-GGTGAAGGTACGTTTGGTTTGC-3'
TBP	5'-GGGAGAATCATGGACCAGAA-3' 5'-CCGTAAGGCATCATTGGACT-3'
Srebp1c	5'-GGAGCCATGGATTGCACATT-3' 5'-CAGGAAGGCTTCCAGAGAGG-3'
Fasn	5'-GCTGCGGAACTTCAGGAAAT-3' 5'-AGAGACGTGTCACCTCCTGGACTT-3'
G6pase	5'-ACACCGACTACTACAGCAACAG-3' 5'-CCTCGAAAGATAGCAAGAGTAG-3'
Pepck	5'-AAAAGCCTTTGGTCAACAAC-3' 5'-AAACTTCATCCAGGCAATGT-3'
Cpt1	5'-ACTCCTGGAAGAAGAAGTTCAT-3' 5'-AGTATCTTTGACAGCTGGGAC-3'
Ppara	5'-ATGCCAGTACTGCCGTTTTTC-3' 5'-GGCCTTGACCTTGTTTCATGT-3'

Result

Feeding period restriction does not change body weight gain

To address the question whether body weight gain is sensitively altered by feeding behavior, accompanied with changes of circadian clock genes, I have investigated the effects of feeding period restriction on body weight. Restriction of feeding period has been designed with the following three groups: 1) ad libitum in which mice were freely exposed to food, 2) restriction feeding to day time (RF Day), in which mice could access food only in day time, and 3) pair-feeding in night time (PF Night), in which mice were given the same amount of food as the RF Day group only in night time (Figure 5). Since mice are nocturnal animals that would take most food at night time than during day time, I measured the amount of food intake in day time, and the same amount of food was given to night time feeding (PF Night) group. I have designed the pair-feeding group to investigate the effect of feeding period variation on body weight with the same amount of calorie intake. Moreover, mice were fed with either normal chow diet (NCD) or high fat diet (HFD) in order to test the effects of different nutrition sources on body weight gain upon feeding period restriction.

Interestingly, I observed that RF Day and PF Night groups revealed a similar pattern of body weight gain, regardless of diet source (NCD or HFD) (Figure 6A, 6B, 6D, 6E). Compared to restricted feeding groups such as RF Day and PF Night,

Figure 5. Feeding period restriction scheme for the three feeding groups in this study.

The ad libitum (AL) group was freely exposed to food; the feeding restricted to day time (RF Day) group could access food only during daytime; and the pair-feeding at night (PF Night) group was given the same amount of food as the RF Day group but only at night. Mice were fed with a normal chow diet (NCD) or a high-fat diet (HFD). The light was turned on at ZT0 and turned off at ZT12. At ZT2 and ZT14, mice were sacrificed to prepare tissues and harvest blood samples.

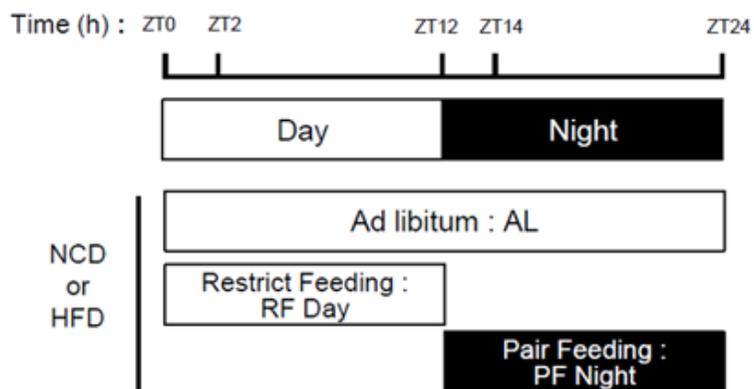
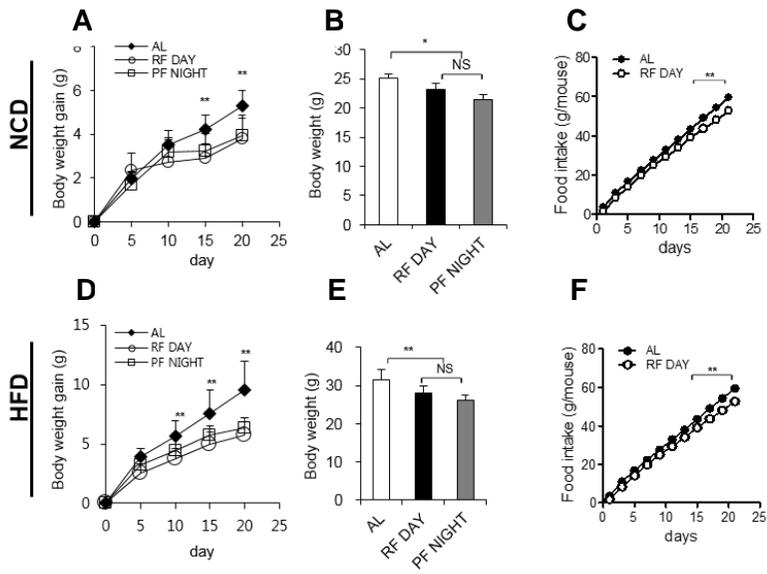


Figure 6. Feeding period restriction does not change body weight gain.

(A and B) Body weight gain or total body weight of AL, RF Day, and PF Night NCD-fed mice. (C) Total food intake in NCD-fed AL and RF Day mice. (D and E) Body weight gain or total body weight of AL, RF Day, and PF Night HFD-fed mice. (F) Total food intake in HFD-fed AL and RF Day mice. Each bar represents mean \pm SD of each group of mice (n=6), *P<0.05, **P<0.01.



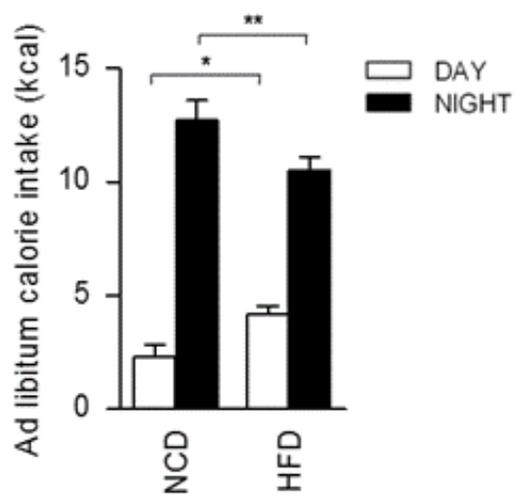
body weight gain of ad libitum group was significantly increased with either NCD or HFD (Figure 6A, 6B, 6D, 6E). When the amounts of cumulative food intakes were measured, ad libitum group gradually consumed more food than RF Day or PF Night group (Figure 6C, 6F). Recently, it has been reported that circadian clock is disrupted by HFD (Kohsaka et al., 2007). Thus, I examined food intake patterns during day time (ZT0-ZT12) and night time (ZT12-ZT24) time periods in ad libitum group. Expectedly, mice ate more food during night time period than day time periods (Figure 7). Interestingly, it seemed that HFD fed mice slightly, but substantially, consumed more food in day time period than NCD fed mice (Figure 7). These data suggest that the amount of food intake rather than feeding periods would be a major determinant of body weight gain, which is both applicable in different nutrition sources such as NCD and HFD.

Day time feeding changes the expression of circadian clock genes in liver but not in hypothalamus

I next investigated the expression of circadian clock genes upon feeding period restriction. In order to examine the expression profiles of circadian clock genes, mice were sacrificed at ZT2 and ZT14, which may reflect different expression pattern of circadian clock genes in 12:12 light-dark cycle. Total RNAs were isolated from liver and hypothalamus as representative peripheral clock and central clock tissues,

Figure 7. Ad libitum calorie intake during day and night with NCD and HFD feeding.

Each bar represents mean \pm SD of each group of mice (n=6), *P<0.05, **P<0.01.



respectively, and analyzed by qRT-PCR. As shown in Figure 8 and 9, the expression patterns of circadian clock genes such as BMAL11, PER2 and CLOCK were altered by feeding period restrictions in liver but not in hypothalamus with either NCD or HFD. Overall, ad libitum group and PF Night group showed the similar expression patterns of circadian clock genes in liver, which would be resulted from the behavior of nocturnal mice that took most food during night time. In contrast, in liver, RF Day group revealed distinct expression pattern of circadian genes from either ad libitum or PF Night group. Unlike liver, the expression patterns of hypothalamic circadian genes were not changed by feeding period restrictions with either NCD or HFD (Figure 8, 9). These data strongly indicate that feeding period restriction would influence expression of circadian clock genes in peripheral tissues, probably via modulating nutritional hormones such as insulin or glucagon, which may not affect expression of circadian clock genes in hypothalamus.

Feeding period restriction alters expression of metabolic genes and plasma metabolites

It is well known that feeding is one of important factors to regulate circadian clock genes as well as hepatic lipid and glucose metabolism (Kohsaka et al., 2007; Lamia et al., 2008). Given that RF Day changed expression patterns of hepatic circadian clock genes, I decided to examine whether RF Day might also influence expression of metabolic genes in liver. I analyzed hepatic gene expression via qRT-

Figure 8. Daytime feeding changes expression of circadian clock genes in the liver but not in the hypothalamus in NCD.

Hepatic and hypothalamic BMAL1, PER2, and CLOCK gene expression profiles in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<,0.05, **P<0.01.

NCD

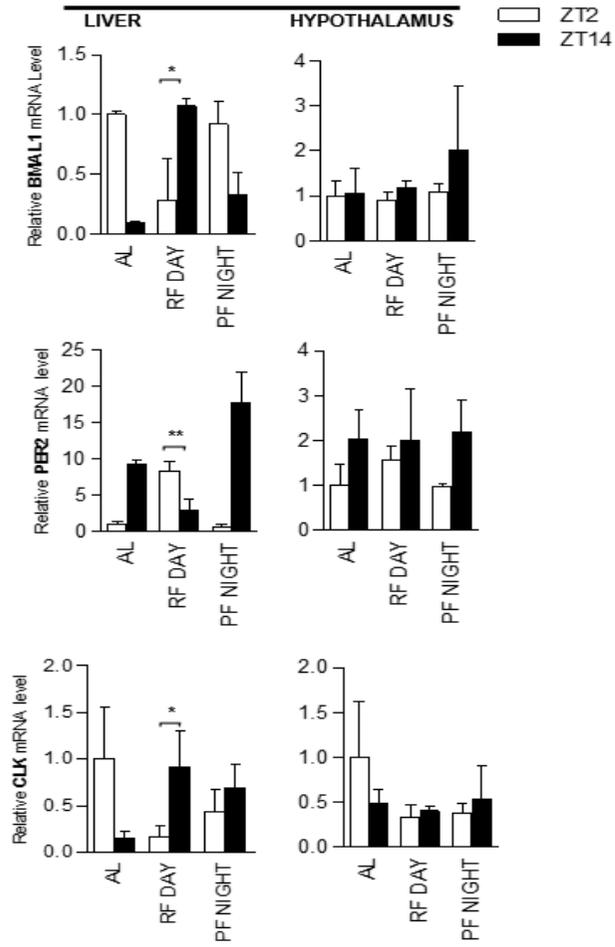
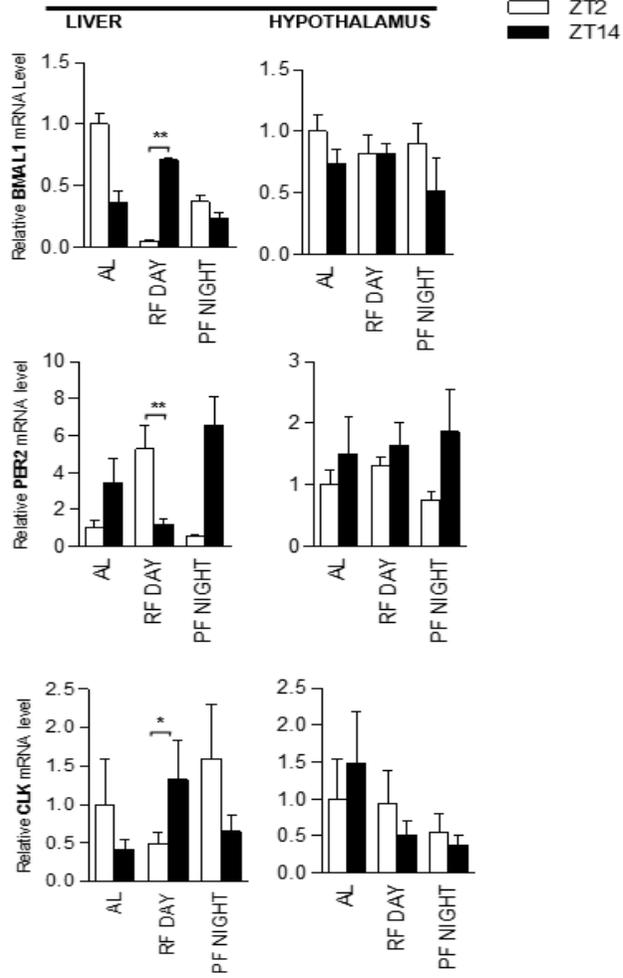


Figure 9. Daytime feeding changes expression of circadian clock genes in the liver but not in the hypothalamus in HFD.

Hepatic and hypothalamic BMAL1, PER2, and CLOCK gene expression profiles in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

HFD



PCR from NCD (Figure 10A, 10B, 10C) or HFD (Figure 11A, 11B, 11C) fed mice at ZT2 and 14. In order to conjecture key metabolic changes, I have investigated expression of several lipid and carbohydrate metabolism genes such as SREBP1c, FASN, PEPCK, G6PASE, PPAR α , and CPT1. Since SREBP1c is a master transcription factor for lipogenesis, it regulates the expression of *FASN* upon nutritional and hormonal changes (Kim et al., 1998a; Kim and Spiegelman, 1996; Kim et al., 1995; Kim et al., 1998b; Kim et al., 2004). Under NCD feeding, expression of SREBP1c mRNA was greatly suppressed in RF Day at ZT14, while it was up-regulated at ZT14 in PF Night (Figure 10A). Despite of this, FASN did not show significant change at ZT2 and 14 in RF Day and PF Night, implying that it may need several hours to reflect SREBP1c target gene expression *in vivo*. Upon HFD feeding, FASN was increased in RF Day at ZT2, while it was not altered in PF Night (Figure 11A). Conversely, the expression of gluconeogenic genes such as PEPCK and G6Pase, which are well known as fasting-induced genes, was not changed at ZT2 and 14 both in RF Day and PF Night in NCD (Figure 10B). When fatty acid oxidation genes such as PPAR α and CPT1 were analyzed, their mRNA levels were not significantly different in RF Day and PF Night groups (Figure 10C). These data propose that expression of several metabolic genes might to be partly altered by feeding period restriction but not as much as that of circadian clock genes in liver.

Since feeding period restriction altered expression of subset of metabolic genes in liver without body weight gain, I have examined serum metabolites,

Figure 10. Feeding period restriction changes the expression of metabolic genes in NCD fed mice.

(A) Expression profiles of lipogenic genes such as SREBP1c and FASN in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (B) Expression profiles of the gluconeogenic genes PEPCCK and G6Pase in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (C) Expression profiles of the fatty acid oxidation genes PPAR α and CPT1 in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

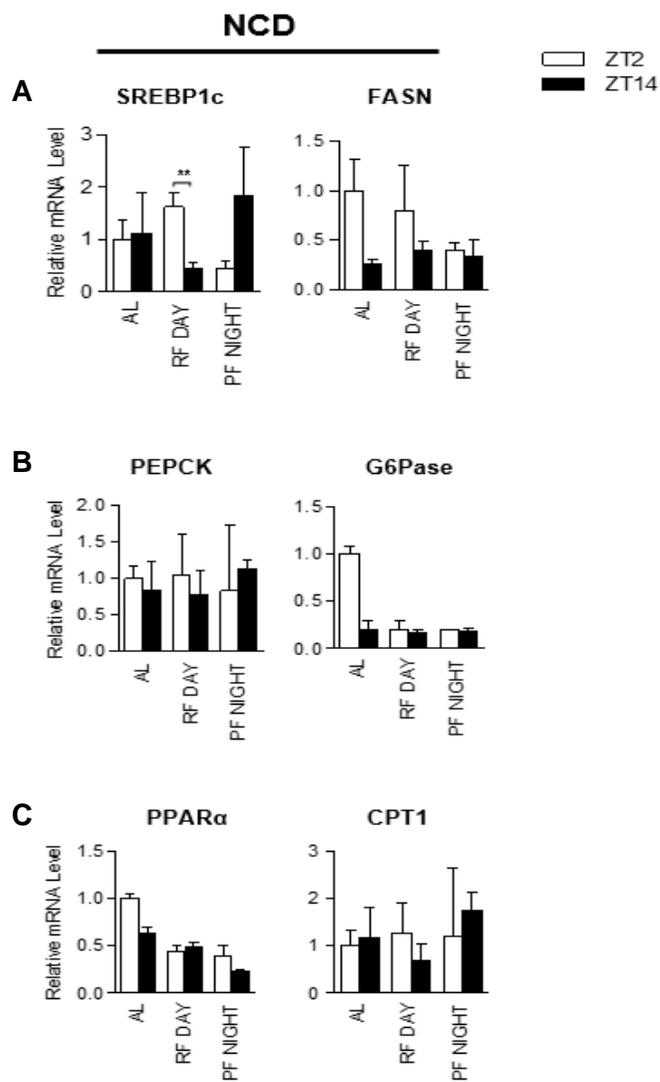
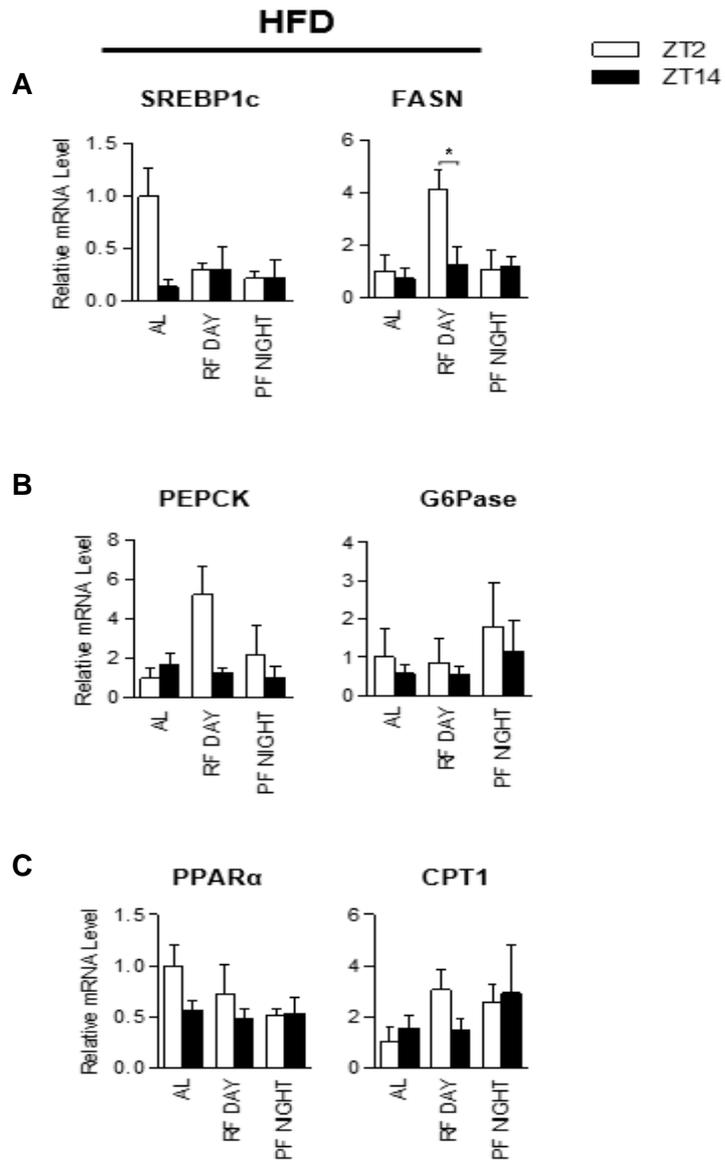


Figure 11. Feeding period restriction changes the expression of metabolic genes in HFD fed mice.

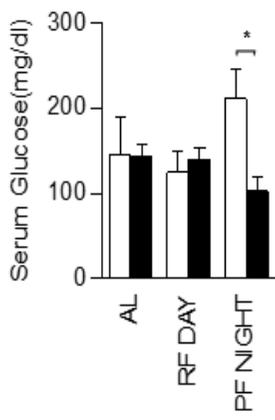
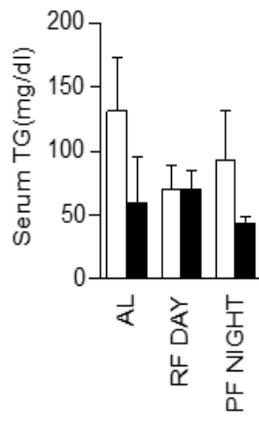
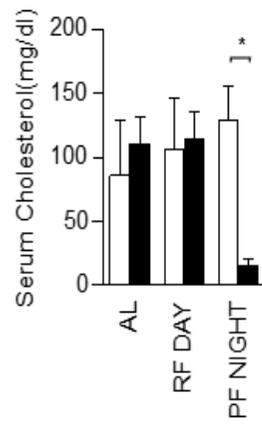
(A) Expression profiles of lipogenic genes such as SREBP1c and FASN in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. (B) Expression profiles of the gluconeogenic genes PEPCCK and G6Pase in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. (C) Expression profiles of the fatty acid oxidation genes PPARa and CPT1 in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.



relevant to whole-body energy state, in different feeding period restriction groups. Although it has been reported that serum metabolite levels are varied throughout feeding-fasting cycle (Morris et al., 2012), our experimental windows limited at ZT2 and 14 did not sensitively reflect whole-body energy state. When I measured serum glucose, triacylglyceride (TG), and cholesterol levels at ZT2 and 14, I failed to observe distinct patterns of those metabolites. Nonetheless, in PF Night group, the levels of serum glucose, TG, and cholesterol were higher at ZT2 than at ZT14 under NCD, implying that there might be a correlation between peripheral circadian oscillation and serum metabolites (Figure 12A, 12B, 12C). On the contrary, HFD challenged animals exhibited disrupted serum metabolite profiles as previously reported (data not shown) (Hatori et al., 2012). These data indicate that feeding period restriction may modulate the profiles of several serum metabolites without changes in body weight gain, concomitant with alteration of circadian clock genes as well as metabolic genes.

Figure 12. Feeding period restriction changes plasma metabolites.

(A) Serum glucose levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (B) Serum TG levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (C) Serum cholesterol levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

A**B****C**

Discussion

Obesity is characterized by increase of adipose tissue and lipid metabolism, which is caused by chronic excess of energy intake than energy expenditure. Very recent reports have suggested that feeding period restriction would be an important factor to influence body weight gain, leading to obesity (Arble et al., 2009; Hatori et al., 2012). Since Arble et al. have reported that day time HFD-fed mice were more obese than night time HFD-fed mice with no differences in calorie intake and activity (Arble et al., 2009), it has been proposed that change of feeding behavior by shifting from night to day time would be an important factor to regulate energy metabolism contribute to. In accordance with this, it has been reported that night-eating syndrome in human might lead to weight gain as a result of excess calories consumption at night (Aronoff et al., 2001; Colles et al., 2007; Tholin et al., 2009). On the contrary, several studies have revealed that there is no correlation of night eating syndrome and body weight gain in human (Gluck et al., 2008; Striegel-Moore et al., 2008; Striegel-Moore et al., 2004). Nonetheless, it has not been clearly understood whether dysregulation of any circadian clock gene expression is an essential factor to alter body weight gain.

In the present work, I have investigated the relationship between body weight gain and feeding period restriction by providing the same amount of food at either night time or day time. I observed that feeding period restriction evidently altered expression pattern of peripheral circadian clock genes, provably via nutrition sensitive hormones. However, the expression of central circadian clock genes in

hypothalamus was not modulated by feeding restriction regardless of NCD or HFD. These data implied that central circadian clock and peripheral circadian clock would be independently regulated, at least, at transcriptional level, upon feeding period restriction (Damiola et al., 2000). Furthermore, hepatic expression of metabolic genes including lipogenesis, gluconeogenesis, and fatty acid oxidation were modulated upon feeding period restriction. Similarly, the levels of serum glucose, triacylglyceride, and cholesterol were also adjusted by feeding period restriction. Despite these changes of peripheral metabolic gene expression, there were no significant body weight differences between RF Day and PF Night groups with NCD or HFD, indicating that the change of peripheral circadian clock genes may not be a key factor to influence body weight. Instead of feeding behavior, it appears that the amount of energy intake would be a crucial factor to determine body weight, which might be closely linked with central circadian genes.

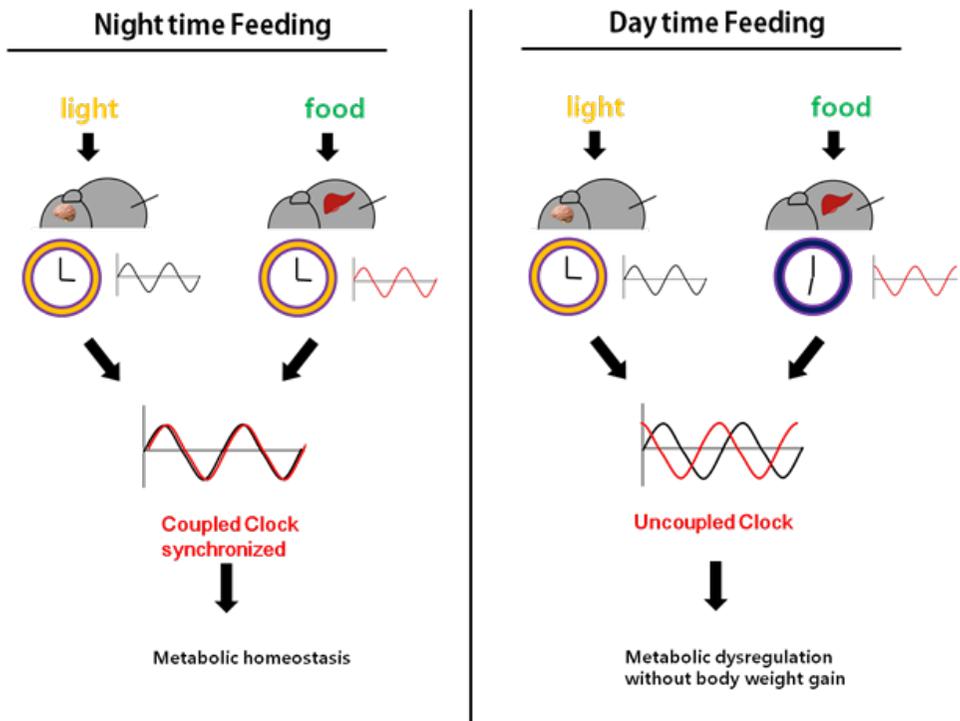
It has been reported that, HFD challenge disrupts behavioral and physiological circadian rhythms. For instance, HFD leads to alterations in the period of the locomotor activity and canonical circadian clock genes (Wang et al., 2008b). In this study, I observed that HFD fed mice exhibited distinct expression patterns of subset genes in liver, including circadian clock genes and metabolic genes, compared with NCD fed mice. In addition, ad libitum group exhibited different food consumption patterns depending upon nutrition source. HFD challenged mice consumed more food intake in day time than NCD challenged mice, implying that

food intake control would be dysregulated in HFD fed group. Since fasting period of HFD group might be shorter than that of NCD fed group under ad libitum (Kohsaka et al., 2007), it appears that NCD fed group might gain less body weight compared with HFD fed group through activation of fasting-induced signals with SIRT1 and AMPK, which mediated energy expenditure to burn extra energy (Canto et al., 2010; Kohsaka et al., 2007). Of course, I cannot rule out the possibility that physical activity or body temperature may contribute to compensate for body weight gain, which will be investigated in further study.

Disruption of circadian clock genes is exerted by various stimuli such as changes of light/dark cycle and food consumption. Interference of central circadian clock by light time modulation affects body weight gain (Karatsoreos et al., 2011). For instance, 10:10 light-dark (LD) cycle leads to body weight gain compared with 12:12 LD cycle, and 10:10 LD cycle regime increases several metabolic parameters such as plasma leptin, insulin, and glucose (Karatsoreos et al., 2011). Moreover, circadian clock defective mice such as clock gene mutant mice and HFD fed mice show irregular food intake pattern, accompanied with obesity due to increased food intake (Damiola et al., 2000; Turek et al., 2005). In contrast, *Bmal1* deficient mice show obese phenotype during 5 weeks of HFD, whereas they are no longer obese with 15 weeks of HFD (Hemmerlyckx et al., 2011). However, liver-specific *Bmal1* knockout mice also show more body weight gain compared with WT mice (Lamia et al., 2008).

Here, I revealed that feeding period restriction selectively alters expression patterns of peripheral circadian clock genes without body weight gain, concomitant with adjusted levels of metabolic genes and plasma profiles. Therefore, it is plausible to speculate that central circadian clock might play a key role to regulate body weight through regulation of energy intake and expenditure. Consistently, it appears the amounts of calorie intake would be a major factor to change body weight (Figure 13). On the contrary, peripheral circadian clock genes sensitively respond to changes of metabolic alterations upon nutrients and hormones. The exact molecular mechanism of central circadian clock to influence body weight gain remains to be elucidated. In conclusion, our data suggest that feeding period restriction would selectively modulate peripheral circadian clocks and metabolic regulation without a significant change in body weight.

Figure 13. Schematic diagram of the proposed model of chapter 1.



CHAPTER TWO:

**Hyperglycemia is exacerbated by dysregulation
of hepatic SREBP1c-CRY1 signaling pathway**

Abstract

SREBP1c is a key transcription factor of lipogenesis to store excess energy in postprandial state. Although SREBP1c appears to be involved in suppression of hepatic gluconeogenesis, the molecular mechanism(s) by which insulin-activated SREBP1c could repress hepatic gluconeogenesis is not thoroughly understood. Here, I demonstrate that CRY1 activation by insulin-induced SREBP1c led to decrease hepatic gluconeogenesis through FOXO1 degradation. In accordance with these, *SREBP1c*^{-/-} and *CRY1*^{-/-} mice showed higher blood glucose than wild type (WT) mice during pyruvate tolerance test, accompanied with enhanced expression of PEPCK and G6Pase genes. Moreover, CRY1 promoted degradation of nuclear FOXO1 by MDM2, one of the ubiquitin E3 ligases, by enhancing binding of FOXO1 and MDM2. Although hepatic SREBP1c failed to upregulate CRY1 expression in *db/db* mice, overexpression of CRY1 led to attenuate hyperglycemia through reduction of hepatic FOXO1 protein as well as gluconeogenic gene expression. Collectively, these data suggest that insulin-activated SREBP1c downregulates hepatic gluconeogenesis through CRY1-mediated FOXO1 degradation and thereby dysregulation of SREBP1c-CRY1 signaling cascade in liver could confer hyperglycemia in diabetic animals.

Introduction

Insulin, which is released from pancreatic β -cells, plays a key role in the maintenance of the whole-body energy homeostasis by actively regulating glucose and lipid metabolism. In the postprandial state, insulin lowers blood glucose by stimulating glucose uptake in adipose tissues and muscles as well as by inhibiting hepatic glucose production (Puigserver et al., 2003; Summers and Birnbaum, 1997). Moreover, in the liver, insulin stimulates the conversion of excess glucose into glycogen (glycogenesis) and triacylglyceride (lipogenesis) for the long term energy storage (Moller, 2001; Wang et al., 2013; Wong et al., 2009).

Suppression of hepatic gluconeogenesis by insulin is an important process to inhibit hyperglycemia. PEPCK and G6Pase are crucial enzymes that convert pyruvate to glucose, and their gene expression is regulated by several transcription factors such as Forkhead box O1 (FOXO1), cAMP Response Element-Binding protein (CREB), Hepatocyte Nuclear Factor 4 (HNF4), Glucocorticoid Receptor (GR), and Peroxisome proliferator-activated receptor Gamma Coactivator 1-Alpha (PGC1 α) (Mouchiroud et al., 2014; Sugden et al., 2010; Yoon et al., 2001). In the liver, FOXO1 is activated upon fasting and gets inactivated by feeding, which is one of the essential mechanisms by which insulin rapidly and efficiently represses hepatic glucose production during postprandial periods (Accili and Arden, 2004; Kops et al., 1999; Lu et al., 2012). After insulin treatment, FOXO1 protein is phosphorylated by AKT and then moves to the cytoplasm, resulting in the decrease of gluconeogenic

gene expression(Matsumoto and Accili, 2005). Although the translocation of hepatic FOXO1 from the nucleus to the cytoplasm is a well-defined mechanism mediating a quick decrease in glucose production by insulin, it is largely unknown how insulin endows a sustainable inhibition of hepatic gluconeogenesis during the postprandial state.

On the other hand, it has been proposed that SREBP1c might be involved in hepatic glucose metabolism. SREBP1c is a basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor that regulates de novo lipogenesis (Brown and Goldstein, 1997; Fajas et al., 1999; Moon et al., 2012; Tontonoz et al., 1993; Yokoyama et al., 1993). Activation of SREBP1c is mediated by AKT and mTORC1 upon insulin signaling (Laplante and Sabatini, 2010; Peterson et al., 2011). SREBP1c regulates lipogenic pathways by stimulating the expression of target genes such as those encoding fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) and acetyl-coenzyme A carboxylase (ACC) (Horton et al., 2003; Kim et al., 1998a; Liang et al., 2002). In addition, SREBP1c appears to be involved in hepatic carbohydrate metabolism. For example, SREBP1c affects the mRNA levels of PEPCK, G6Pase, and IRS-2 genes and inhibits the interaction between HNF4 and PGC1 α to suppress gluconeogenic genes (BeCARD et al., 2001; Ide et al., 2004; Lee et al., 2007; Ponugoti et al., 2007; Yamamoto et al., 2004). Although it has been reported that hepatic SREBP1c is upregulated in obese animals, it is unknown how increased SREBP1c fails to repress hepatic gluconeogenesis. Thus, it remains crucial to understand the

molecular mechanisms by which SREBP1c could modulate gluconeogenesis under physiological and pathological conditions.

CRY1 is a member of the mammalian clock transcription-translation feedback loop that also includes CLOCK, BMAL1, PER1, PER2, and CRY2, to modulate rhythmic oscillations. CLOCK and BMAL1 form a heterodimer to activate PER and CRY genes and then elevated PER and CRY proteins act as transcriptional repressors that decrease the transcriptional activity of CLOCK and BMAL1 (Miyamoto and Sancar, 1998; Thresher et al., 1998; Vitaterna et al., 1999; Ye et al., 2014). The hepatic circadian clock is affected by food intake as well by the expression of hormones such as insulin and glucagon, whereas the suprachiasmatic nucleus circadian clock is controlled by the light-dark cycle (Green et al., 2008). Recently, it has been shown that hepatic circadian clock genes also contribute to glucose homeostasis. For example, hepatic CRY proteins modulate glucose production by inhibiting the glucagon receptor signaling pathway and binding to GR (Lamia et al., 2011; Zhang et al., 2010). In addition, an agonist of CRY proteins has been reported to repress the expression of hepatic gluconeogenic genes, such as PEPCK and G6Pase (Hirota et al., 2012). Furthermore, *BMAL1*^{-/-} mice exhibited disrupted hepatic glucose homeostasis (Rudic et al., 2004). However, the molecular mechanisms by which CRY1 could repress hepatic glucose production during the postprandial state remain to be elucidated.

The fact that SREBP1c downregulates hepatic gluconeogenesis prompted

us to investigate novel target genes of SREBP1c by comparing *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice. Here, I demonstrate that SREBP1c attenuates hepatic glucose production via activation of CRY1, eventually leading to degradation of the FOXO1 protein upon insulin signaling. While hepatic FOXO1 is rapidly translocated from the nucleus into the cytoplasm by AKT-mediated phosphorylation triggered by insulin, the SREBP1c-CRY1 signaling pathway durably represses the execution of gluconeogenic genes by decreasing nuclear FOXO1 protein for long term insulin action. Additionally, in the liver of diabetic *db/db* mice, overexpression of CRY1 lowers blood glucose, accompanied with attenuated gluconeogenic genes and FOXO1 protein. Together, our data suggest that insulin activates the SREBP1c-CRY1 signaling pathway, resulting in FOXO1 degradation mediated by MDM2, which is one of the crucial mechanisms to maintain hepatic glucose homeostasis.

Methods

Animals

C57BL/6 mice were purchased from SAMTACO (Seoul, South Korea) and housed in colony cages. *db/+* and *db/db* mice were obtained from Central Lab (Seoul, Korea). *SREBP1c*^{-/-} mice were generously provided from Dr. J. Horton at the University of Texas Southwestern Medical Center and bred in isolated cages. All animals were maintained under 12 h light/ 12 h dark cycle in a pathogen-free animal facility. Following dissection, mouse tissue specimens were immediately stored at -80 °C until further analysis. All experiments with mice were approved by the Institute of Laboratory Animal Resources at Seoul National University and the Institutional Animal Care and Use Committee at the University of North Carolina.

***In vivo* imaging system**

C57BL/6 mice were injected with adenoviruses encoding GFP (Ad-Mock), SREBP1c (Ad-SREBP1c) and G6Pase luciferase (Ad-G6Pase-luc) through the tail vein. After 5 days, adenovirus-infected mice were injected intraperitoneally with 100 mg/kg sterile firefly D-luciferin. After 5 min, mice were anesthetized and imaged using an IVIS 100 imaging system (Xenogen, Alameda, CA, USA) as described(Lee et al., 2014b).

Pyruvate tolerance tests

For the pyruvate tolerance test, mice were fasted for 16 h and then injected intraperitoneally with pyruvate (2 g/kg body weight for mice). Blood glucose levels were measured in tail vein blood samples at the indicated time points by using a Free-Style blood glucose meter (Therasense, Sweden).

Antibodies, chemicals and plasmids

MG132 was purchased from Calbiochem (San Diego, CA, USA). I used antibodies to the following proteins in our study: MYC, HA, FOXO1, phosphor-FOXO1(Ser256), AKT, and phosphor-AKT(Ser473) (all purchased from Cell Signaling Technology, Beverly, MA, USA), FLAG, ACTIN (Sigma-Aldrich, St. Louis, MO, USA), G6Pase, POLII, GFP (Santa Cruz Biotechnology Santa Cruz, CA, USA), GAPDH (LabFrontier Co., Ltd, Seoul, Korea), SREBP1 (BD Bioscience, San Jose, CA, USA), MDM2 (Abcam, Cambridge, MA, USA), and CRY1 (Alpha Diagnostic International Inc., San Antonio, Texas, USA). GFP-CRY1 was cloned into the pEGFP-N1 vector and FLAG-MDM2 was cloned into pCMV-3 FLAG. Mouse CRY1 promoter was cloned into the pGL3-basic vector.

Cell-based ubiquitination assays

COS-1 cells were transfected with plasmids encoding FOXO1 WT-MYC, nFOXO1 (ADA)-MYC, GFP-CRY1 (or FLAG-CRY1), FLAG-MDM2 and Ubiquitin-HA in the presence of 20 μ M MG132 for 4 h. Total cell lysates were prepared using the TGN buffer. FOXO1 WT-MYC and nFOXO1 (ADA)-MYC were

immunoprecipitated with an anti-MYC antibody (Cell Signaling Technology, USA), and after washing in the TGN buffer, proteins were separated by SDS-PAGE followed by western blotting analyses with an anti-HA antibody.

ChIP assays

Cross-linking and chromatin immunoprecipitation assays with H4IIE cells were performed as described previously (Sakai et al., 2003). Extracted proteins from total cell lysates were immunoprecipitated with anti-SREBP1 (BD Bioscience) or IgG (Santa Cruz) for 2 h. Precipitated DNA fragments were analyzed by PCR using primer sets that encompassed the proximal (-100 to +100 base pairs) region of the rat *CRY1* promoter and negative control (+9670 to +9890 base pairs) region. The sequences of ChIP assay primers were as follows: sense, 5'-GTCCGAGCCAGCGTAGTAAA, antisense, 5'- GGATAGCGCGGGCTAGAG; negative control primer sense, 5'-CCAGCCACTTTGCTGAAGTT and antisense, 5'-CTAGACAAGGCTGCCCACTC.

Preparation of recombinant adenovirus

The adenovirus plasmid was constructed as previously described (Lee et al., 2014a). rat *SREBP1c* and mouse *CRY1* cDNAs were incorporated into the AdTrack-CMV shuttle vector and a recombinant vector was generated using the Ad-Easy adenoviral vector system. Adenoviruses were amplified in HEK293A cells and isolated by cesium chloride density centrifugation. The GFP was co-expressed from an

independent promoter with inserted cDNA. For *in vivo* experiments, mice were injected with 5×10^9 PFU of adenovirus (*db/db* mice, 2×10^{10} PFU) in 200 μ l PBS through the tail vein. Empty virus expressing only the gene for GFP served as the control (Mock).

Mouse primary hepatocytes cultures

Mouse primary hepatocytes were isolated as previously described (Jo et al., 2013). For adenoviral infection, isolated hepatocytes were incubated for 12 h with adenovirus at 50 PFU/cell with the serum-free medium, which was subsequently replaced by the 10% FBS-containing M199 medium.

Cell lysis and immunoprecipitation

After washing in cold PBS, cells were treated either with the TGN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20 and 0.3% NP-40) or the SDS lysis buffer (200 mM Tris-HCl, pH 6.8, 10% glycerol and 4% SDS) supplemented with 0.1% protease inhibitor cocktail (Roche, Basel, Switzerland). Total cell lysates were obtained by centrifugation at 12,000 rpm for 15 min at 4 °C, and 1–1.5 mg of lysates was used for immunoprecipitation. The lysates were incubated with primary antibodies for 2 h at 4 °C, followed by 1 h of further incubation with 50% slurry of protein A sepharose presaturated with the lysis buffer. After washing three times with the lysis buffer, the immunoprecipitated proteins were recovered from the beads by boiling for 10 min in the sample buffer and analyzed by SDS-PAGE and

immunoblotting.

Transient transfection and luciferase assays

HEK293T cells were transiently transfected with various DNA plasmids using the calcium-phosphate method described previously (Seo et al., 2004). After incubation for 36 h, transfected cells were harvested with the lysis buffer (25 mM Tris-phosphate pH 7.8, 10% glycerol, 2 mM EDTA, 2 mM DTT and 1% Triton X-100) and the activities of luciferase and β -galactosidase were measured according to the manufacturer's protocol (Promega, Madison, WI, USA). The relative luciferase activity was normalized to β -galactosidase activity in each sample.

RNA preparation and q-RT-PCR analyses

RNA was prepared as previously described (Kim et al., 2010). Briefly, total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, equal amounts of RNA were subjected to cDNA synthesis using the RevertAid M-MuLV reverse transcriptase (Fermentas, Canada). The relative amount of mRNA was evaluated by using a CFX real-time quantitative PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) and calculated following normalization to the level of TBP or cyclophilin mRNA. The primer sequences used for the real-time quantitative PCR analyses are described in the Supplementary Table 2.

siRNA transfection

Table 2.q-RT PCR primer sequence

Primer	Sequences
mrSREBP1c	Sense 5'-GGAGCCATGGATTGCACATT Antisense 5'-CAGGAAGGCTTCCAGAGAGG
mrCRY1	Sense 5'-CGTTTGGAAAGGCATTTGG Antisense 5'-CTTCCATTTGTCAAAGCGTG
mrPEPCK	Sense 5'-AGCCTTTGGTCAACAACCTGG Antisense 5'-TGCCTTCGGGGTTAGTTATG
mrG6Pase	Sense 5'-ACACCGACTACTACAGCAACAG Antisense 5'-CCTCGAAAGATAGCAAGAGTAG
hmrFOXO1	Sense 5'-CCAAGGCCATCGAGAGC Antisense 5'-GATTGAGCATCCACCAAGAACT
hmFASN	Sense 5'-GCTGCGGAAACTTCAGGAAAT Antisense 5'-AGAGACGTGTCACTCTGGACTT
mbmal1	Sense 5'-AACCTTCCCGCAGCTAACAG Antisense 5'-AGTCTCTTTGGGCCACCTT
mCLOCK	Sense 5'-TTGCGTCTGTGGGTGTTG Antisense 5'-TGCTTTGTCCTTGTCATCTCT
mPER2	Sense 5'-TGTGCGATGATGATTTCGTGA Antisense 5'-GGTGAAGGTACGTTTGGTTTGC
hmrCRY2	Sense 5'-GGAGCTGCCCAAGAAGC Antisense 5'-AGTAGAAGAGGCGGCAGGA
mSCD1	Sense 5'-CCGGAGACCCCTTAGATCGA Antisense 5'-TAGCCTGTAAAGATTTCTGCAAACC
mELOVL6	Sense 5'-TGCCATGTTTCATCACCTTGT Antisense 5'-TACTCAGCCTTCGTGGCTTT
mTBP	Sense 5'-GGGAGAATCATGGACCAGAA Antisense 5'-CCGTAAGGCATCATTGGACT

siRNA duplexes for *CRY1*, *MDM2*, and *FOXO1* were purchased from the Bioneer Inc. (Daejeon, South Korea). Primary hepatocytes were transiently transfected with the Lipofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequence information for siRNA is described in the Supplementary Table 3.

Glucose production assays

Glucose production by mouse primary hepatocytes was measured according to the manufacturer's protocol using a glucose oxidase assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the cells were incubated for 6 h at 37 °C and 5% CO₂ in the Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 10 mM lactate and 2 mM pyruvate, pH 7.4). The glucose production assays were performed in triplicate.

Statistical analysis

Data were compared using a paired Student's *t* test or analysis of variance as appropriate and are represented as the mean ± standard deviation (SD). Values of *P* < 0.05 were considered to indicate statistically significant differences.

Table 3. siRNA sequence

Primer	Sequences
Rat FOXO1	5'-GAAUGAAGGAACUGGGAAA
Mouse CRY1	5'-CCUCGCAACUGAAGUUGGU
Mouse MDM2	5'-CAGAGAAUGAUGGUAAAGA
Negative Control	5'-CCUACGCCACCAAUUUCGU

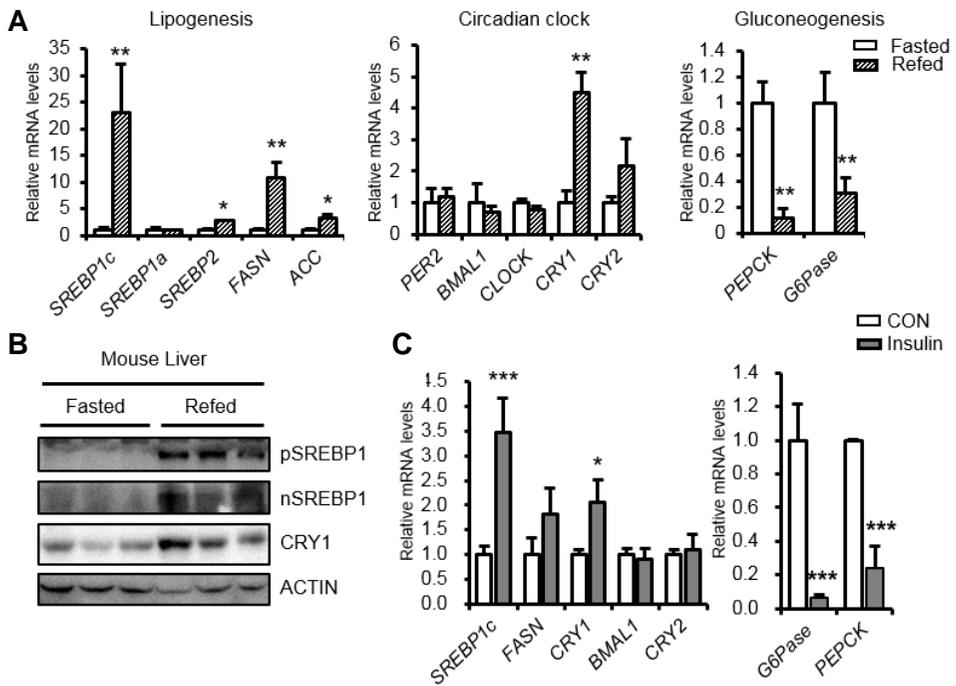
Results

CRY1 is promoted by feeding and insulin

In the liver, peripheral circadian clock genes are regulated by various nutritional and hormonal changes (Jang et al., 2012). Given that circadian clock genes are closely associated with energy homeostasis, I sought to investigate which circadian clock genes could affect feeding-dependent hepatic lipid and glucose metabolism. Since the expression of most circadian clock genes is oscillated in a time dependent manner, the end points of the fasting and/or refeeding experiment were fixed at ZT 3. Upon refeeding, the expression of most lipogenic genes including SREBP1c, FASN, and ACC was upregulated in the liver. In accordance with previous reports (McNeill et al., 1982; Oh et al., 2013; Pilgis et al., 1988), the expression of gluconeogenic genes such as *PEPCK* and *G6Pase* was downregulated in the postprandial state (Figure 14A). Interestingly, the level of hepatic CRY1 mRNA was selectively elevated in refeed mice, while the expression of other circadian clock genes such as PER2, CRY2, CLOCK, and BMAL1 was not significantly altered after nutritional changes (Figure 14A). Moreover, the expression of the CRY1 protein was markedly increased in the liver of refeed mice (Figure 14B). These findings prompted us to test whether insulin might elevate hepatic CRY1 gene expression. In primary hepatocytes, the level of CRY1 mRNA was increased by insulin, similar to SREBP1c mRNA (Figure 14C). These data indicate that hepatic CRY1 expression is upregulated by feeding and insulin, therefore this protein may participate in insulin-

Figure 14. CRY1 is stimulated by feeding and exposure to insulin

(A and B) *C57BL/6* mice were fasted for 24 h and then refed for 12 h. Both fasted and refed mice were sacrificed at ZT3. In the liver, levels of the CRY1 mRNA (A) and CRY1 protein (B) were determined using qRT-PCR with normalization to TBP mRNA levels and western blotting, respectively. Data are represented as mean \pm SD, $N=4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (C) CRY1 gene expression was measured in mouse primary hepatocytes following 12 h of insulin exposure and in control conditions using qRT-PCR. The level of the TBP mRNA was used for the qRT-PCR normalization. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test)



dependent energy metabolism.

SREBP1c regulates CRY1 gene expression

To investigate which transcription factors regulate insulin-dependent CRY1 gene expression, I analyzed *CRY1 promoters* in several species including monkey, cow, sheep, human, rat and mouse (Figure 16A). In the proximal CRY1 promoter, there are several sterol regulatory element (SRE) motifs as well as an E-BOX (CANNTG) motif, which is also a target motif for BMAL1 and CLOCK, the core circadian clock proteins (Figure 16A). Both SRE and E-BOX motifs are well known binding targets of SREBP1c with its dual DNA binding specificity (Griffin et al., 2007; Kim et al., 1995). To examine whether SREBP1c could regulate CRY1 gene expression, SREBP1c was overexpressed in mouse primary hepatocytes. As shown in Figure 15A and 15B, the levels of hepatic CRY1 mRNA and CRY1 protein were increased by SREBP1c overexpression (Figure 15A, 15B), implying that SREBP1c may be a key transcription factor that upregulates hepatic CRY1 gene expression in the postprandial state. Next, the effect of ectopic expression of SREBP1c on the CRY1 promoter activity was examined. Expression of luciferase from a wild-type CRY1 promoter was compared with expression from a promoter with mutated SRE motifs (3XSRE) in HEK293T cells (Figure 16B). I observed substantial loss of promoter activity with loss of the 3XSRE sequences but not E-BOX motif sequences

Figure 15. SREBP1c directly activates CRY1 gene expression

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-SREBP1c, as indicated. The levels of the CRY1 mRNA (A) and CRY1 protein (B) were determined using qRT-PCR with normalization to TBP mRNA levels and western blotting, respectively. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

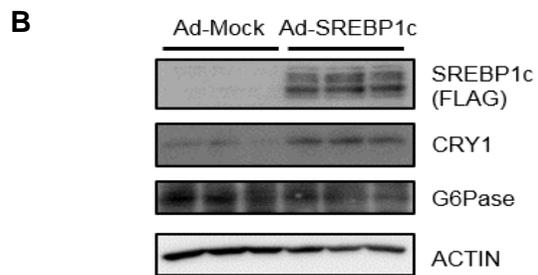
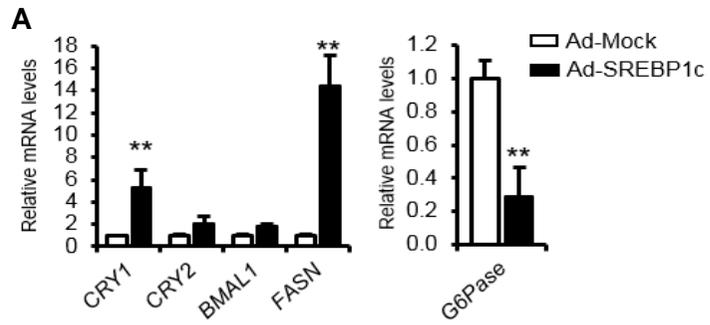


Figure 16. SREBP1c binds to SRE sequence in CRY1 promoter.

(A) SRE motifs and E-BOX sequences in the CRY1 promoter from various species.

(B) Luciferase activity of the WT CRY1 promoter and 3XSRE mutant promoter were measured following co-transfection with expression plasmids encoding either SREBP1c or Mock in HEK293T cells. Luciferase activity was normalized by β -gal activity. Data are represented as mean \pm SD, $N=5$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's t -test).

(C) HEK293T cells were co-transfected with a reporter plasmid containing the WT or E-BOX mutant mouse CRY1 promoter along with expression plasmids encoding either Mock or SREBP1c. The values represent the mean \pm SD ($N=4$ for each group). * $P < 0.05$, ** $P < 0.01$ (Student's t -test).

(Figure 16B, 16C). In addition, SREBP1c binding to the CRY1 promoter was confirmed by a ChIP assay (Figure 17). Meanwhile, consistent with previous reports (Becard et al., 2001; Ide et al., 2004; Lee et al., 2007), hepatic SREBP1c reduced G6Pase expression (Figure 15A, 15B).

To verify whether SREBP1c could modulate hepatic CRY1 gene expression *in vivo*, SREBP1c adenovirus was injected into mice via the tail vein and hepatic gene expression was investigated. As expected, hepatic SREBP1c overexpression increased the expression of lipogenic genes such as FASN, SCD1 and ELOVL6 (Figure 18A). As in the case with primary hepatocytes, the level of hepatic CRY1 mRNA was enhanced by SREBP1c *in vivo* (Figure 18A). Moreover, in contrast to *SREBP1c^{+/+}* mice, refeeding failed to increase hepatic CRY1 gene expression in *SREBP1c^{-/-}* mice (Figure 18B). This observation indicates that SREBP1c is an essential factor for the upregulation of hepatic CRY1 gene expression in the postprandial state.

SREBP1c-CRY1 pathway inhibits hepatic gluconeogenesis

Consistent with previous reports (Becard et al., 2001; Lamia et al., 2011; Zhang et al., 2010), SREBP1c overexpression decreased glucose production in mouse primary hepatocytes (Figure 19A). In addition, the optical *in vivo* imaging analysis revealed that hepatic SREBP1c overexpression remarkably repressed the promoter

Figure 17. SREBP1c directly binds to CRY1 promoter.

ChIP assay, performed as described in Methods, showing CRY1 promoter occupancy by SREBP1 in H4IIE cells.

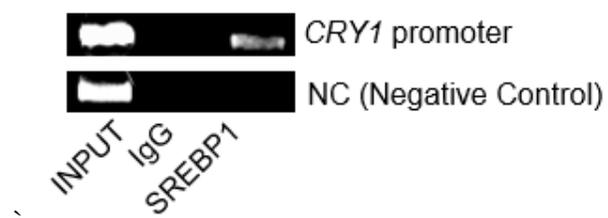


Figure 18. CRY1 regulation in SREBP1c modulated mice.

(A) *C57BL/6* mice were infected with adenoviruses encoding either a Mock or SREBP1c (adenoviral dose of 5×10^9 viral particles per mouse) through the tail vein. Mice were sacrificed 5 days following the injection of adenoviruses and relative mRNA levels were determined by qRT-PCR and normalized to the TBP mRNA signal. Data are represented as mean \pm SD, $N=3-4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (B) *SREBP1c*^{-/-} and *SREBP1c*^{+/+} mice were fasted for 24 h and then refed for 12 h. Both fasted and refed mice were sacrificed at ZT3. The levels of SREBP1c and CRY1 mRNAs were determined by qRT-PCR and normalized to TBP mRNA levels. Data are represented as mean \pm SD, $N=3-4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).

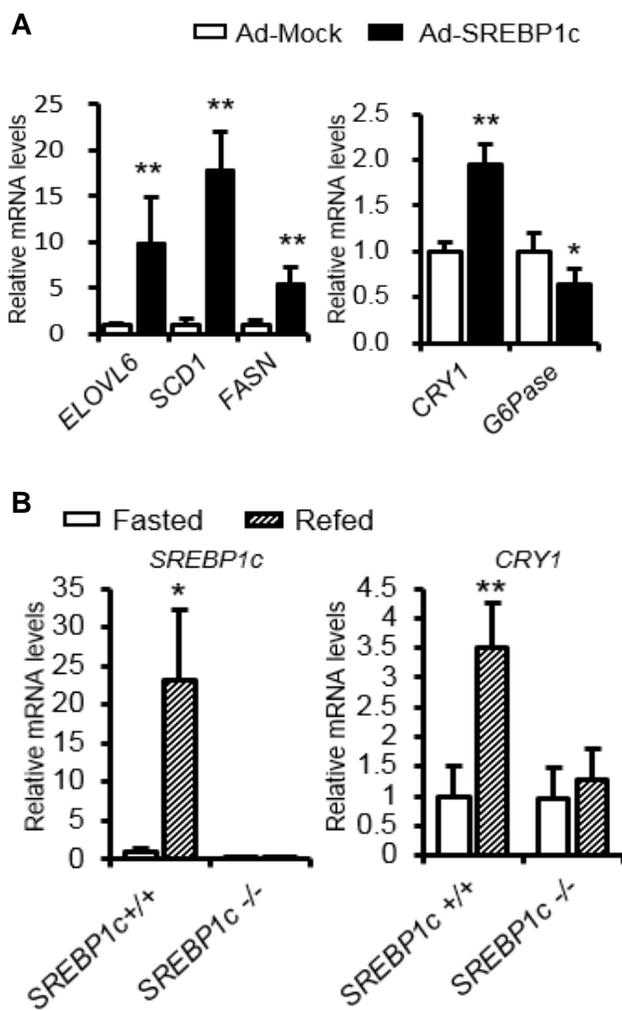
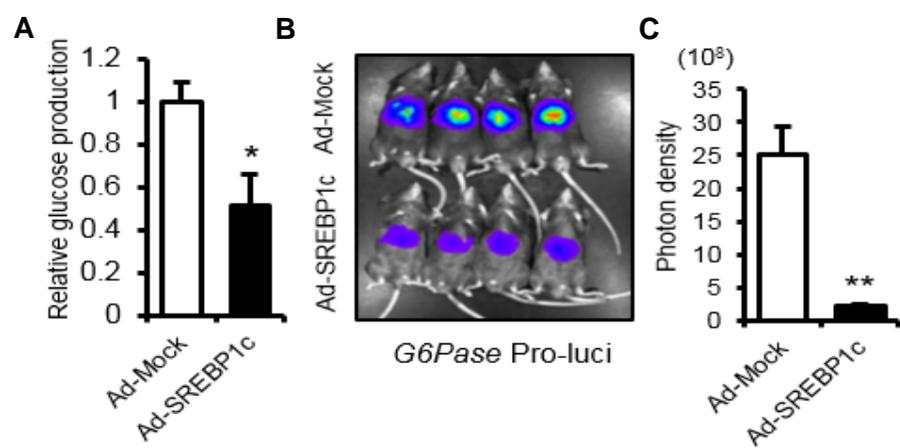


Figure 19. SREBP1c suppresses hepatic gluconeogenesis

(A) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-SREBP1c. Relative glucose secretion was measured using a glucose oxidase (GO) kit as described in Methods. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$ (Student's *t*-test). (B) *C57BL/6* mice were infected with Ad-G6Pase-luc and either Ad-Mock or Ad-SREBP1c. (C) The effect of hepatic SREBP1c overexpression on G6Pase promoter activity was measured by optical *in vivo* imaging analysis and converted to photon density.



activity of the *G6Pase* gene *in vivo* (Figure 19B, 19C). These findings led us to investigate the effect of SREBP1c on blood glucose level *in vivo*. Pyruvate tolerance test demonstrated that the adenoviral overexpression of SREBP1c significantly decreased blood glucose level following pyruvate injection (Figure 20A, 20B). Accordingly, *SREBP1c*^{-/-} mice showed higher blood glucose than *SREBP1c*^{+/+} mice (Figure 20C, 20D). Thus, these results suggest that hepatic SREBP1c would suppress gluconeogenesis, potentially by modulating gluconeogenic gene expression.

To examine whether CRY1, a novel target gene of SREBP1c, might modulate hepatic gluconeogenic gene expression, I suppressed CRY1 expression via siRNA in rat hepatoma H4IIE cells. Downregulation of CRY1 increased the expression of G6Pase and PEPCK genes (Figure 21A), which are crucial for hepatic gluconeogenesis. On the contrary, hepatic CRY1 overexpression decreased the expression of G6Pase and PEPCK genes in mouse primary hepatocytes (Figure 21B). To confirm these observations, I measured pyruvate-induced blood glucose level from *CRY1*^{+/+} and *CRY1*^{-/-} mice. As shown in Figure 22A and 22B, *CRY1*^{-/-} mice shows higher blood glucose level than *CRY1*^{+/+} mice. To test whether CRY1 could be a key mediator of the inhibition of hepatic gluconeogenesis by SREBP1c, I performed glucose production assays in primary hepatocytes. As indicated in Figure 23, SREBP1c overexpression significantly suppressed glucose production, while suppression of CRY1 with siRNA in SREBP1c-overexpressing hepatocytes rescued the ability to produce glucose. To establish whether the SREBP1c-CRY1 signaling

Figure 20. SREBP1c regulates pyruvate induced blood glucose level.

(A and B) *C57BL/6* mice were infected with Ad-Mock or Ad-SREBP1c and subjected to the PTT (A), as described in Methods. All mice were fasted at ZT10 and performed PTT at ZT3. The result was converted by the area-under-the curve (AUC) analysis (B). Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (C and D) Pyruvate tolerance test (C) was performed in *SREBP1c*^{-/-} and *SREBP1c*^{+/+} mice. All mice were fasted at ZT10 and performed PTT at ZT3. Results were converted to AUC values (D). Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).

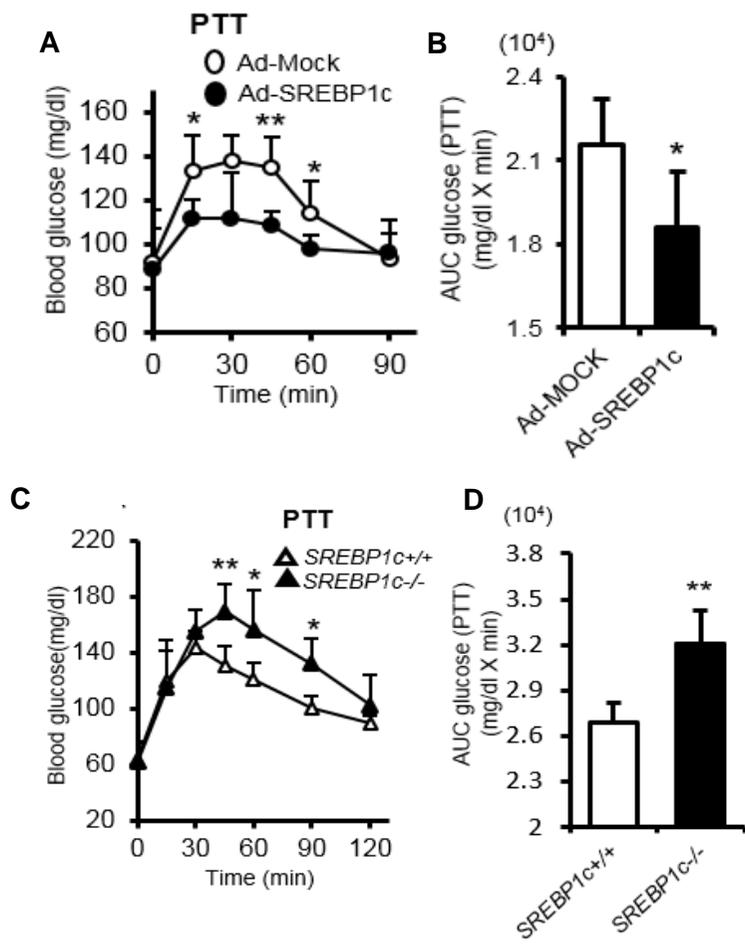


Figure 21. CRY1 regulates gluconeogenic gene expression.

(A) H4IIE cells were transfected with siCON or siCRY1. Relative PEPCK and G6Pase mRNA levels were determined by qRT-PCR and normalized to cyclophilin mRNA levels. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test). (B) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1. Relative PEPCK and G6Pase mRNA levels were determined by qRT-PCR and normalized to TBP mRNA levels. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$ (Student's *t*-test).

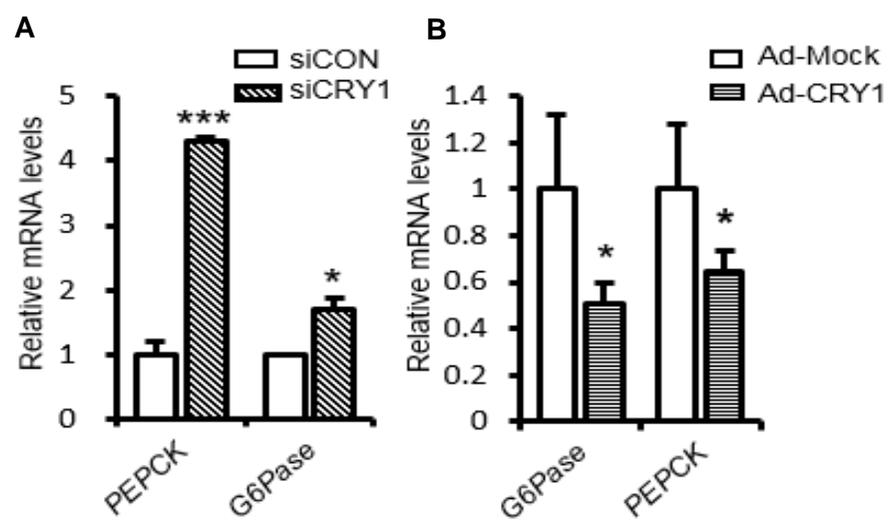


Figure 22. *CRY1*^{-/-} mice showed low level of glucose upon pyruvate challenge.

(A and B) Pyruvate tolerance test (A) was performed in *CRY1*^{-/-} and *CRY1*^{+/+} mice.

All mice were fasted at ZT10 and performed PTT at ZT3. Results were converted to

AUC values (B). Data are represented as mean \pm SD, $N=7$ for each group. * $P < 0.05$,

** $P < 0.01$ (Student's *t*-test).

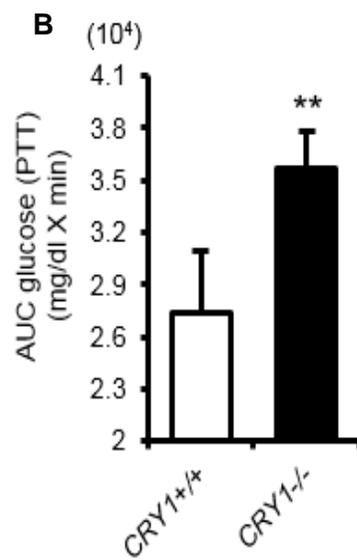
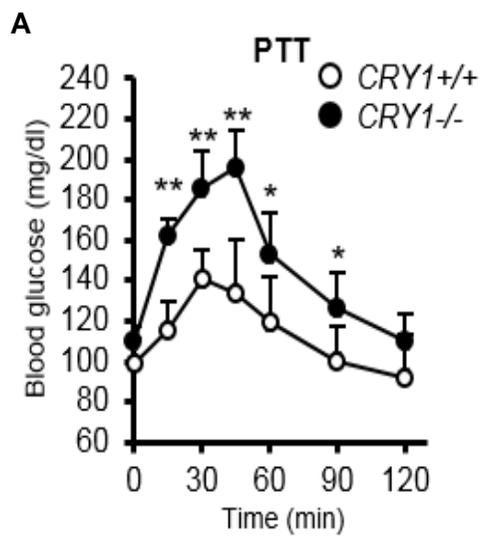
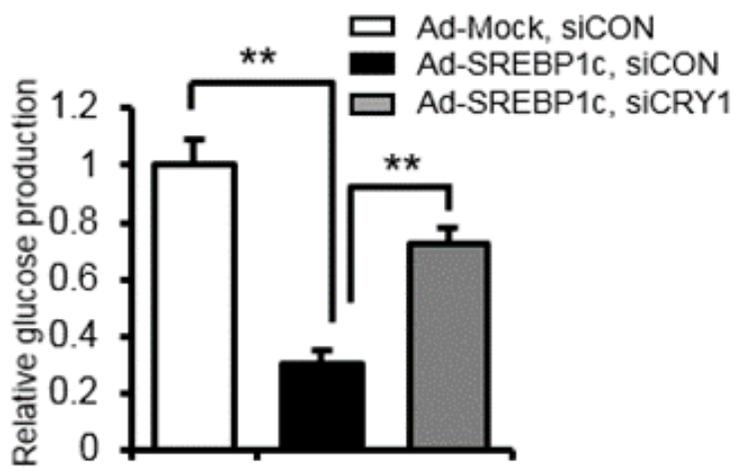


Figure 23. The SREBP1c-CRY1 signaling pathway regulates gluconeogenesis *in vitro*.

Mouse primary hepatocytes were infected with Ad-Mock or Ad-SREBP1c and transfected with either siCON or siCRY1. Relative glucose production was measured using a glucose oxidase (GO) kit. Data are represented as mean \pm SD, $N=4$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).



pathway could indeed repress hepatic glucose production *in vivo*, CRY1 was adenovirally overexpressed in the liver of *SREBP1c^{-/-}* mice. While *SREBP1c^{-/-}* mice showed higher blood glucose level than *SREBP1c^{+/+}* mice during the pyruvate tolerance test, *SREBP1c^{-/-}* mice with CRY1 overexpression exhibited an attenuated level of blood glucose, comparable to that of WT mice (Figure 24A, 24B). These data strongly indicate that the SREBP1c-CRY1 signaling pathway could inhibit hepatic gluconeogenesis *in vivo*.

CRY1 regulates FOXO1 protein

To decipher the underlying mechanism(s) by which insulin-induced CRY1 could repress hepatic gluconeogenesis, I focused on FOXO1, as its regulatory effects on insulin signaling and gluconeogenesis are well established. In mouse primary hepatocytes, the level of the FOXO1 protein was decreased by CRY1 overexpression (Figure 25A) while FOXO1 mRNA levels were not altered (Figure 25B). These data indicated that CRY1 might modulate the level of the FOXO1 protein, probably, independent of the FOXO1 mRNA. Furthermore, the level of the FOXO1 protein was enhanced in CRY1-suppressed hepatocytes (Figure 25C, 25D). In accordance with these *in vitro* data, the FOXO1 protein level was higher in the liver of *CRY1^{-/-}* mice than in the liver of the *CRY1^{+/+}* mice, whereas levels of the FOXO1 mRNA were not different between *CRY1^{+/+}* and *CRY1^{-/-}* mice (Figure 26A, 26B).

Figure 24. The SREBP1c-CRY1 signaling pathway regulates gluconeogenesis *in vivo*.

(A and B) Pyruvate tolerance test (A) in *SREBP1c^{+/+}* mice injected with Ad-Mock and in *SREBP1c^{-/-}* mice injected with either Ad-Mock or Ad-CRY1. Results were converted to AUC values (B) to assess the effect of *CRY1* overexpression in *SREBP1c^{-/-}* mice. All mice were fasted at ZT 10 and performed PTT at ZT 3. Data are represented as mean \pm SD, $N=4-5$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

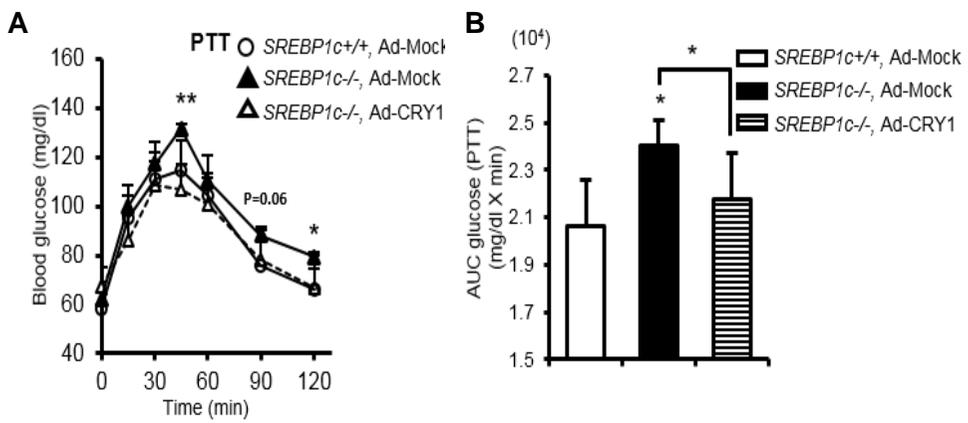


Figure 25. CRY1 regulates FOXO1 protein level *in vitro*.

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The expression profiles of FOXO1 were analyzed at the protein level (A) using western blotting and at the mRNA level (B) using qRT-PCR. Data are represented as mean \pm SD, $N=4$ for each group. $*P < 0.05$ (Student's t -test). (C and D) H4IIE cells were transfected with siCON or siCRY1. Immunocytochemical analysis (C) of endogenous FOXO1. DAPI, 4', 6-diamidino-2-phenylindole. Endogenous FOXO1 and CRY1 protein levels were analyzed using western blotting (D).

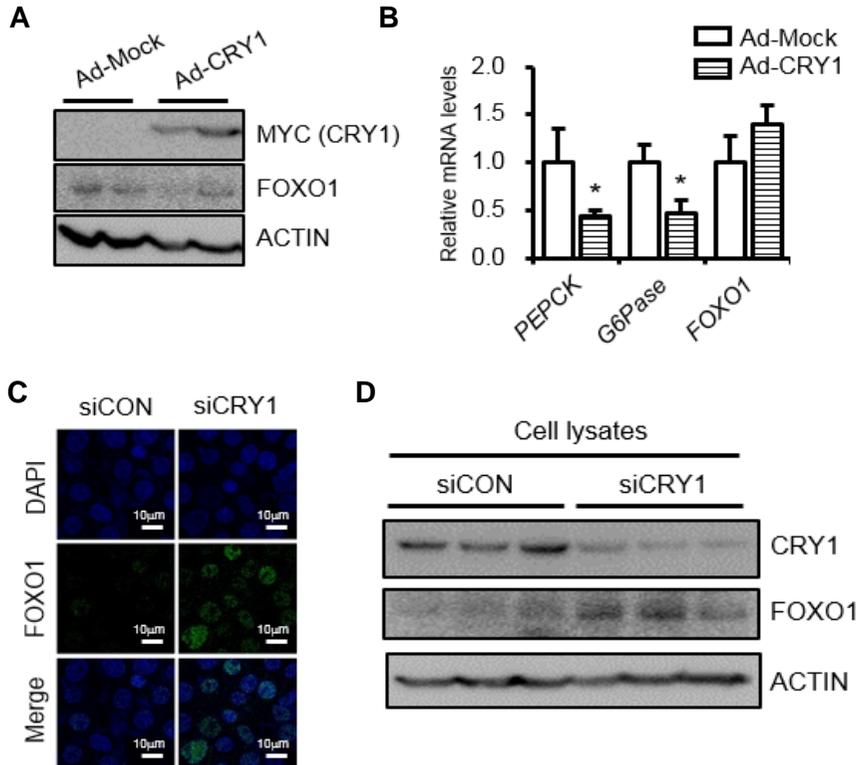
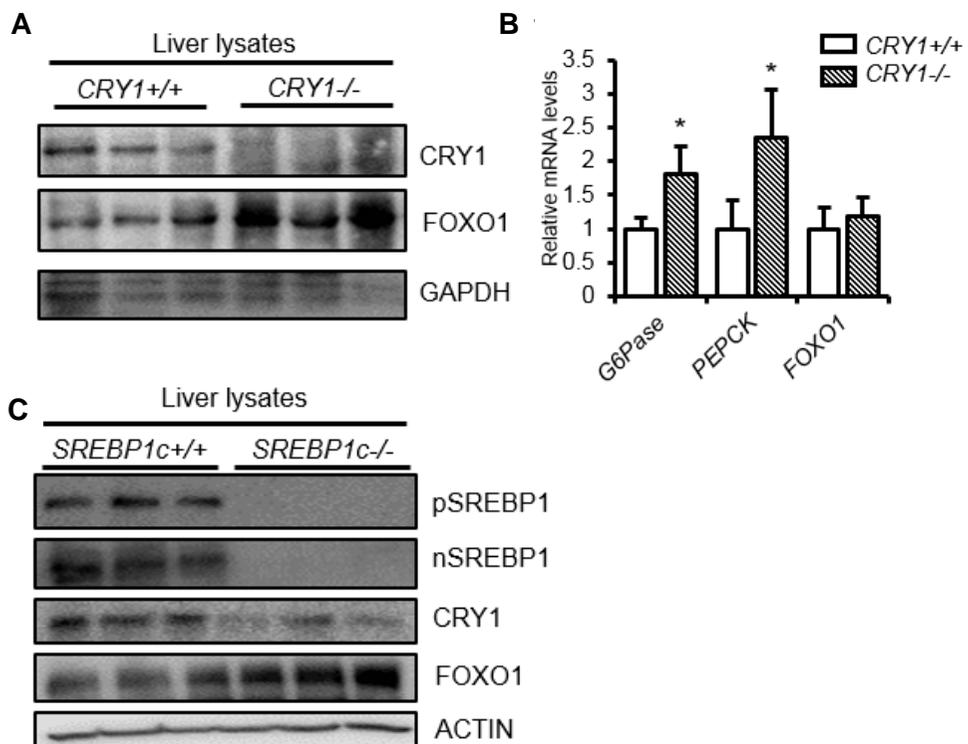


Figure 26. FOXO1 protein level is elevated in *SREBP1*^{-/-} and *CRY1*^{-/-}.

(A and B) The expression patterns of FOXO1 protein in the liver of *CRY1*^{+/+} and *CRY1*^{-/-} mice were analyzed by western blotting (A) and qRT-PCR (B). Relative mRNA levels were determined using qRT-PCR and normalized to the levels of the TBP mRNA. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test). (C) Expression of CRY1 and FOXO1 proteins in the liver of *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice was analyzed by western blotting.



Moreover, compared to the levels of proteins observed in the livers of the *SREBP1c*^{+/+} mice, the amount of the FOXO1 protein was higher and the level of the CRY1 protein was lower in the liver of *SREBP1c*^{-/-} mice (Figure 26C). To verify that CRY1 could inhibit hepatic gluconeogenesis via FOXO1 modulation, the effects of CRY1 and/or FOXO1 suppression on gluconeogenic gene expression were examined. Increased expression of G6Pase and PEPCK genes by CRY1 suppression was abolished when the FOXO1 gene was downregulated by siRNA (Figure 27), indicating that CRY1 could alleviate hepatic gluconeogenesis via suppression of the FOXO1 protein. Therefore, these *in vivo* and *in vitro* data suggest that hepatic FOXO1 protein could be regulated by CRY1.

FOXO1 protein is decreased by insulin-activated CRY1

FOXO1 translocation from the nucleus to the cytoplasm by AKT is a well-known mechanism by which insulin acutely inhibits hepatic glucose production (Rena et al., 1999). As insulin upregulates CRY1 that, in turn, downregulates the FOXO1 protein (Figure 14C and 25A), I investigated the time course of these events by examining the expression profiles of the FOXO1 and CRY1 proteins in insulin-treated primary hepatocytes. As shown in Figure 28A and 28B, AKT phosphorylation was clearly induced in cells treated with insulin for a relatively short period (0.5~4 h). Concomitantly, FOXO1 phosphorylation was also increased by

Figure 27. CRY1 inhibits gluconeogenesis via FOXO1 signaling pathway.

H4IIE cells were co-transfected with siCRY1 and/or siFOXO1. Relative mRNA levels were normalized to the cyclophilin mRNA level. Data are represented as mean \pm SD, $N=3$ for each group. # $P < 0.05$, ## $P < 0.01$ versus siCRY1, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus siCON (Student's t -test).

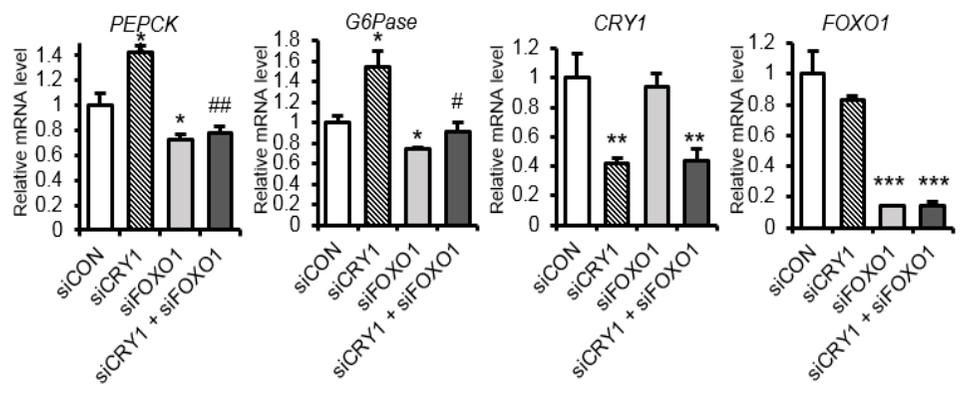
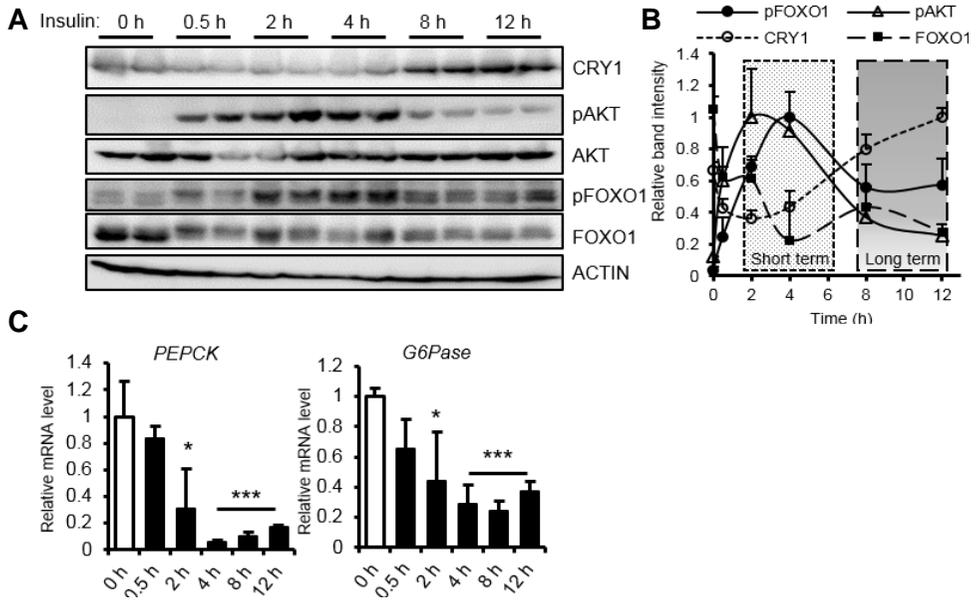


Figure 28. CRY1 is induced in long term insulin action with inhibition of gluconeogenesis.

(A, B, and C) Mouse primary hepatocytes were treated with insulin for different periods. Protein levels (A) were determined with western blotting and the results were converted to the band intensity graph (B). mRNA levels (C) were analyzed by qRT-PCR. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t -test).



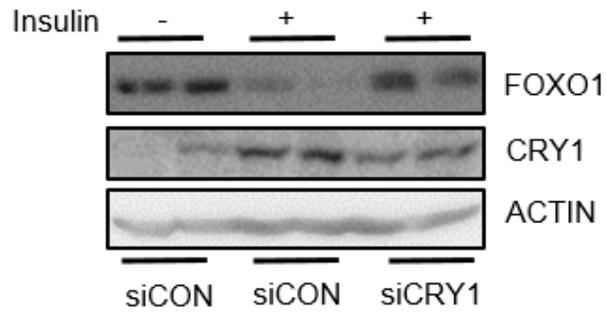
insulin. However, phosphorylation levels of AKT and FOXO1 were gradually and substantially decreased by a long-term (8~12 h) incubation with insulin, implying that FOXO1 translocation from the nucleus to the cytoplasm by AKT might be more pronounced after a short exposure to insulin rather than following a long-term insulin treatment. Intriguingly, in hepatocytes treated with insulin for long periods, the level of the CRY1 protein was markedly increased, while that of the total FOXO1 protein was decreased, indicating that the amount of the CRY1 protein appears to be inversely related to the total quantity of the FOXO1 protein. Moreover, the expression of PEPCK and G6Pase genes was repressed after either a long-term or a short-term insulin treatment (Figure 28C). These data suggest that reduction in the FOXO1 protein level might be involved in the suppression of hepatic gluconeogenesis as a result of a delayed effect of insulin treatment.

Next, I explored whether CRY1 could modulate the decrease in FOXO1 protein in insulin-treated hepatocytes. To address this, the expression of CRY1 was suppressed by siRNA with or without an insulin treatment. As shown in Figure 29A, long-term insulin challenge led to a decrease in FOXO1 protein, while suppression of CRY1 protein restored the level of the FOXO1 protein. In the presence of insulin, decreased levels of PEPCK and G6Pase mRNA were also substantially restored by CRY1 knockdown in hepatocytes (Figure 29B). Together, these data indicate that CRY1 could repress the expression of hepatic gluconeogenic genes via reduction of the FOXO1 protein level during the long-term insulin action.

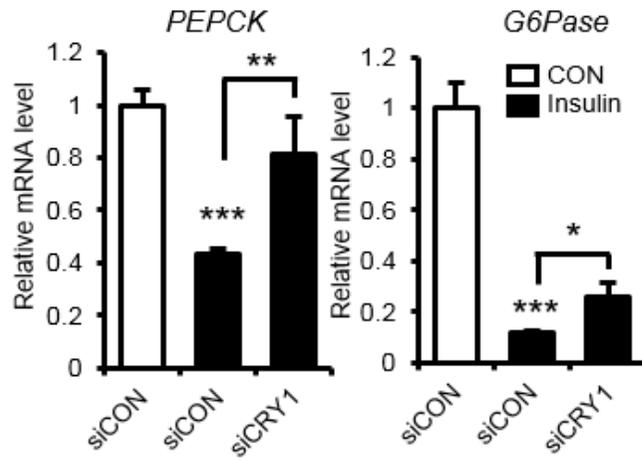
Figure 29. Insulin sustainably suppresses gluconeogenesis via CRY1.

(A and B) H4IIE cells were transfected with siCON or siCRY1 and treated with 10 nM insulin for 12 h. Protein levels (A) were analyzed by western blotting and relative mRNA levels (B) were determined by qRT-PCR and normalized to the cyclophilin mRNA level. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t -test).

A



B



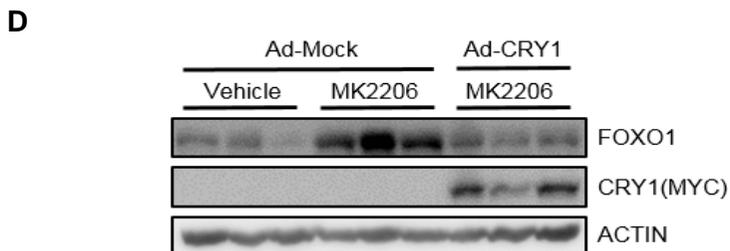
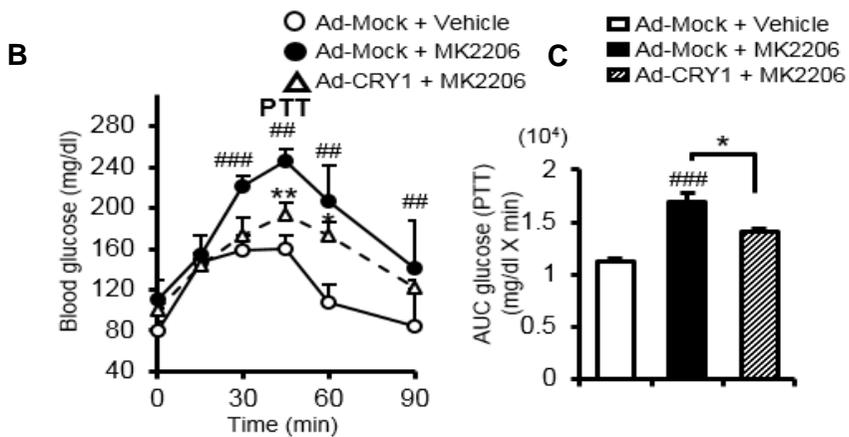
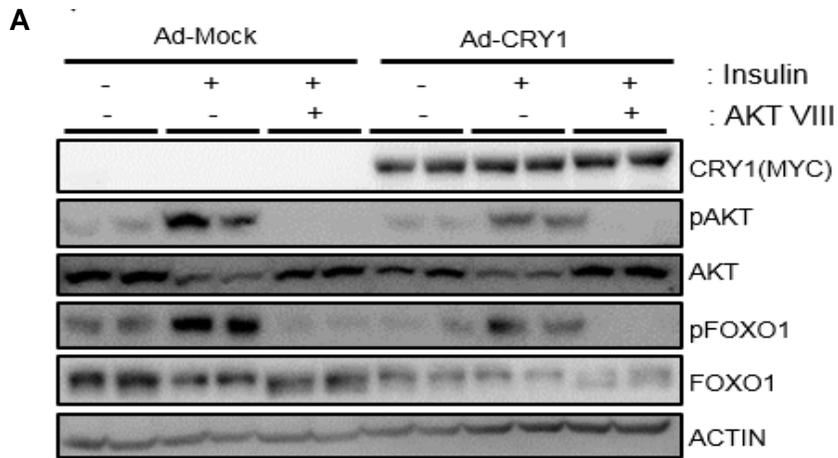
To test whether enhanced CRY1 could suppress hepatic gluconeogenesis even in the absence of a short-term insulin action, I employed AKT inhibitors. In primary hepatocytes, insulin increased phosphorylation levels of both AKT and FOXO1, while a co-treatment with the AKT inhibitor AKTVIII blocked phosphorylation of both proteins, as expected (Figure 30A). However, in Ad-CRY1 overexpressing hepatocytes, the total FOXO1 level was decreased in insulin and/or the AKT inhibitor treated cells, implying that CRY1 could downregulate FOXO1 protein independent of FOXO1 phosphorylation (Figure 30A). In order to confirm this observation *in vivo*, I tested another AKT inhibitor, MK2206, in mice. As expected, administration of MK2206 significantly increased blood glucose level upon pyruvate challenge; however, adenoviral CRY1 overexpression in mice significantly attenuated blood glucose level even in the presence of MK2206 (Figure 30B, 30C). It is noteworthy that the level of the FOXO1 protein was greatly augmented by MK2206, whereas CRY1 elevation suppressed FOXO1 protein expression *in vivo* (Figure 30D). Taken together, these data clearly indicate that CRY1-dependent FOXO1 reduction may contribute to the suppression of hepatic gluconeogenesis independent of AKT activation.

CRY1 stimulates proteasomal degradation of FOXO1

Since CRY1 overexpression appeared to decrease the level of the FOXO1

Figure 30. CRY1 suppresses FOXO1 protein level independent of AKT activity.

(A) Mouse primary hepatocytes were infected with Ad-Mock and Ad-CRY1, and then treated with insulin (10 nM) or insulin (10 nM) and AKTVIII (5 μ M) for 12 h. Protein levels were determined with western blotting. (B, C and D) *C57BL/6* mice were infected with Ad-Mock or Ad-CRY1 and subjected to the pyruvate tolerance test (B) with or without the AKT inhibitor MK2206. MK2206 (30 mg/kg) was given by oral gavage 10 min before the pyruvate tolerance test. All mice were fasted at ZT 10 and performed PTT at ZT 3. Results were converted to AUC values (C). After the pyruvate tolerance test, hepatic protein levels (D) were analyzed by western blotting. Data are represented as mean \pm SD, $N=5-7$ for each group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus Ad-Mock + vehicle control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Ad-Mock + MK2206 control (Student's t -test).

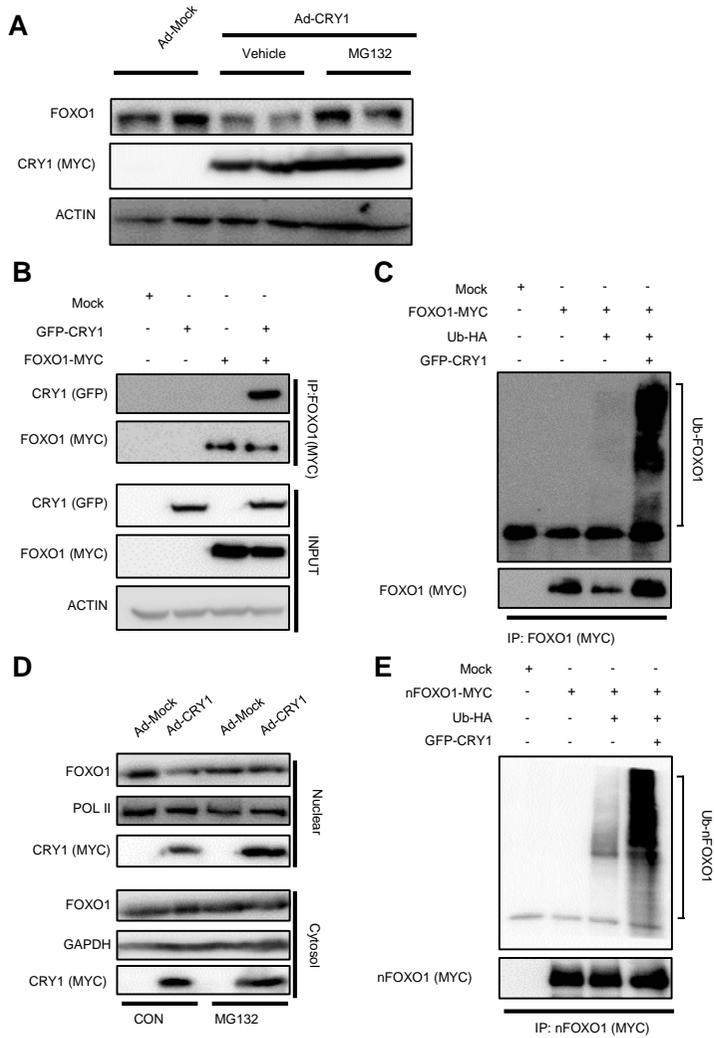


protein, but not the FOXO1 mRNA, I investigated whether the downregulation of the FOXO1 protein proceeds via proteasomal degradation. As shown in Figure 31A, the reduction in the FOXO1 protein by CRY1 overexpression was alleviated by MG132 treatment, indicating that the regulation of FOXO1 protein by CRY1 may be, at least in part, dependent on proteasomal degradation. When I tested physical interaction between FOXO1 and CRY1 proteins, co-immunoprecipitation assays revealed that CRY1 could associate with FOXO1 protein (Figure 31B). Then, I examined whether CRY1 might induce FOXO1 degradation via the ubiquitination-proteasome pathway. As shown in Figure 31C, CRY1 overexpression dramatically promoted FOXO1 poly-ubiquitination, implying that CRY1 could potentiate FOXO1 degradation, probably, through protein-protein interactions.

To explore the subcellular location of FOXO1 degradation induced by CRY1, levels of the nuclear and cytosolic FOXO1 protein were investigated. As shown in Figure 31D, the nuclear fraction of the FOXO1 protein was decreased by CRY1 overexpression, whereas incubation with MG132 blocked this decrease. At the same time, levels of cytosolic FOXO1 were not altered upon CRY1 overexpression irrespective of the presence of MG132. Consistent with these results, poly-ubiquitination of the nuclear form of the FOXO1 mutant protein (nFOXO1-MYC) was greatly augmented by CRY1 (Figure 31E). Therefore, it is plausible that the degradation of the FOXO1 protein via poly-ubiquitination is stimulated by CRY1 in the nucleus

Figure 31. CRY1 accelerates ubiquitin-mediated FOXO1 degradation

(A) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The cells were treated with 20 μ M MG132 or vehicle for 4 h. Total cell lysates were analyzed by western blotting with indicated antibodies. (B) HEK293T cells were transfected with GFP-CRY1 and/or FOXO1-MYC expression vectors. Co-immunoprecipitation with an anti-MYC antibody and western blotting were performed with the indicated antibodies. IP, immunoprecipitation. (C) COS-1 cells were co-transfected with plasmids encoding FOXO1-MYC, GFP-CRY1, and Ubiquitin-HA. After transfection, the cells were treated with MG132 (20 μ M) for 6 h and then the cell lysates were subjected to immunoprecipitation with an anti-MYC antibody followed by western blotting with indicated antibodies. IP, immunoprecipitation. (D) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1. After infection, the cells were treated with MG132 (20 μ M) for 4 h. Nuclear and cytosolic fractions were isolated and analyzed by western blotting with indicated antibodies. (E) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, GFP-CRY1, and Ubiquitin-HA. After transfection, the cells were challenged with MG132 (20 μ M) for 6 h. The cell lysates were subjected to immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation.



CRY1 is involved in MDM2-mediated FOXO1 degradation

Among several ubiquitin E3 ligases of the FOXO1 protein (Fu et al., 2009; Kato et al., 2008), I found that the MDM2 ubiquitin E3 ligase was involved in the CRY1-mediated FOXO1 degradation. As shown in Figure 32A, MDM2 suppression markedly rescued the level of the FOXO1 protein in CRY1-overexpressing cells, implying that MDM2 may participate in the CRY1-dependent FOXO1 reduction. To study the role of CRY1 in MDM2-mediated FOXO1 degradation, I tested whether CRY1 might regulate the subcellular localization of MDM2. Wild type CRY1 and cytosolic CRY1 (Δ NLS-CRY1) did not change the subcellular location of the nuclear MDM2 (Figure 32B). However, I revealed that CRY1 potentiates the association between FOXO1 and MDM2 (Figure 32C).

In another experiment, I explored if CRY1 could modulate MDM2-mediated FOXO1 degradation. As shown in Figure 33A and 33B, CRY1 overexpression promoted MDM2-mediated poly-ubiquitination of the nuclear form of FOXO1 protein (Figure 33A), whereas CRY1 suppression attenuated FOXO1 poly-ubiquitination by MDM2 (Figure 33B). These data indicate that CRY1 would participate in MDM2-induced FOXO1 degradation and repress FOXO1-mediated hepatic glucose production.

Figure 32. CRY1 represses FOXO1 protein through intensifying MDM2 and FOXO1 binding.

(A) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1 and/or siCON or siMDM2. Total cell lysates were analyzed by western blotting with indicated antibodies. (B) HEK293T cells were transfected with FLAG-MDM2 with GFP-WT CRY1 or GFP-cytosolic CRY1 (Δ NLS CRY1) and immunocytochemical analysis of FLAG-MDM2 and GFP was carried out. DAPI, 4',6-diamidino-2-phenylindole. (C) HEK293T cells were transfected with FLAG-MDM2, nFOXO1-MYC, and GFP-CRY1 expression vectors. Total cell lysates were subjected to co-immunoprecipitation with an anti-MYC antibody followed by western blotting with indicated antibodies. IP, immunoprecipitation.

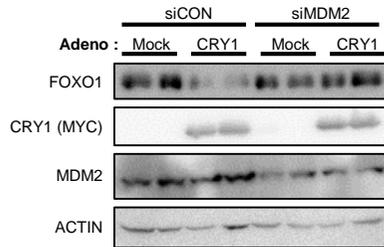
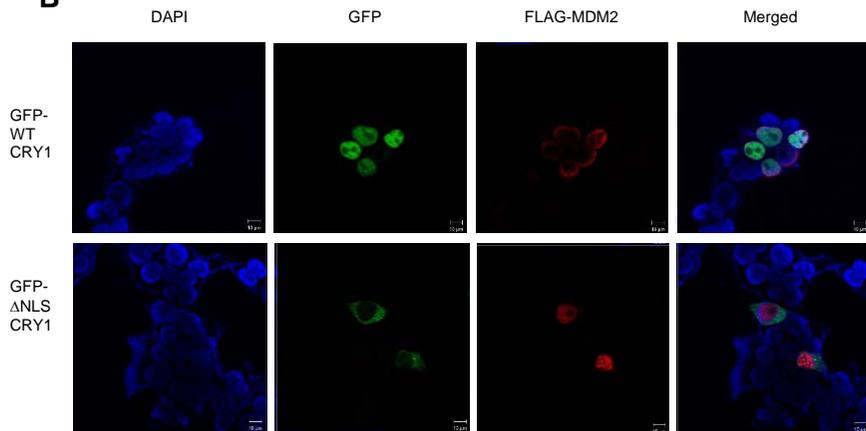
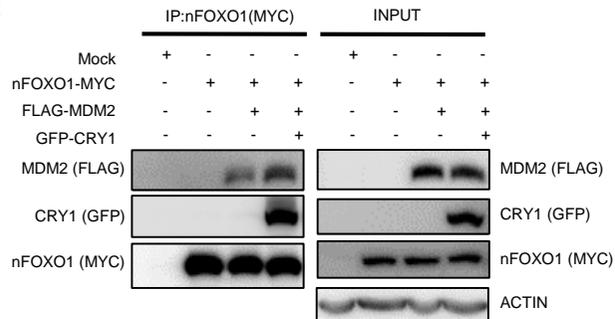
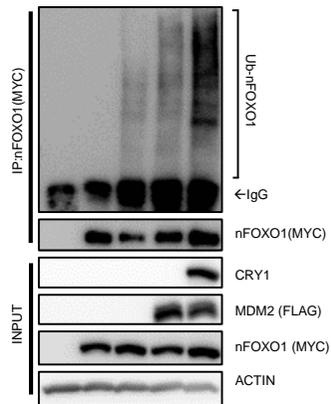
A**B****C**

Figure 33. CRY1 is involved in MDM2-mediated FOXO1 ubiquitination

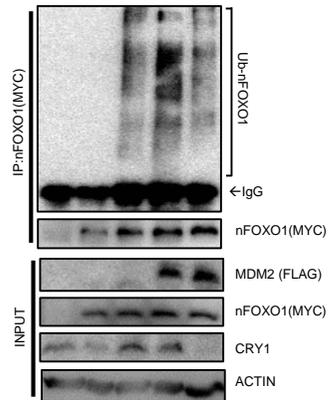
(A) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, FLAG-MDM2, FLAG-CRY1, and Ubiquitin-HA. After transfection, the cells were challenged with MG132 (20 μ M) for 6 h. Cell lysates underwent immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation (B) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, FLAG-MDM2, Ubiquitin-HA, and siCRY1. Cells were treated with MG132 (20 μ M) for 6 h. Cell lysates were subjected to immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation.

A

Mock	+	-	-	-	-
nFOXO1-MYC	-	+	+	+	+
Ub-HA	-	-	+	+	+
FLAG-MDM2	-	-	-	+	+
FLAG-CRY1	-	-	-	-	+

**B**

Mock	+	-	-	-	-
nFOXO1-MYC	-	+	+	+	+
Ub-HA	-	-	+	+	+
FLAG-MDM2	-	-	-	+	+
siCRY1	-	-	-	-	+



CRY1 mitigates hyperglycemia in *db/db* mice

In the liver of obese animals such as *db/db* and DIO (diet-induced-obesity) mice, SREBP1c level is elevated while hepatic gluconeogenesis is not repressed (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000; Yoon et al., 2010). To explore which process(es) might be dysregulated in the regulation of hepatic gluconeogenesis, I have examined mRNA and protein levels for SREBP1c-CRY1 axis and gluconeogenic genes in diabetic animals. Similar to previous reports (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000; Yoon et al., 2010), the mRNA levels of SREBP1c and gluconeogenic genes were elevated in *db/db* mice (Figure 34A, 34B). However, hepatic CRY1 protein was greatly decreased in *db/db* mice (Figure 34B). Similarly, DIO mice exhibited elevated SREBP1c and gluconeogenic genes whereas CRY1 was not activated (Figure 34C, 34D). To test the idea that dysregulated CRY1 protein might mediate hyperglycemia with enhanced FOXO1 protein in diabetic animals, CRY1 was adenovirally overexpressed in the liver of *db/db* mice. As shown in Figure 35A, the level of blood glucose was decreased by CRY1 overexpression. Moreover, ectopic CRY1 expression reduced the levels of FOXO1 protein as well as gluconeogenic gene expression in *db/db* mice (Figure 35B, 35C). These results propose that CRY1 could ameliorate hyperglycemia by repressing the level of FOXO1 protein in *db/db* mice.

Figure 34. SREBP1c is activated while CRY1 is not elevated in *db/db* and DIO mice.

(A, and B) Ten-week-old male *db/+* and *db/db* mice were sacrificed in fed states at ZT3. The relative mRNA levels of various hepatic genes (A) were determined by qRT-PCR analyses and normalized to the TBP mRNA level. Data are represented as mean \pm SD, $N=4$ for each group. * $P < 0.05$ versus *db/+* group. (Student's *t*-test). Protein levels (B) were determined with western blotting. (C and D) Eight-week-old *C57BL/6* mice were fed a NCD or HFD for 8 weeks. Hepatic gene expression levels (D) were determined by qRT-PCR and normalized by the level of the TBP mRNA. $N=5$ in each group. Hepatic protein levels (E) were analyzed by western blotting

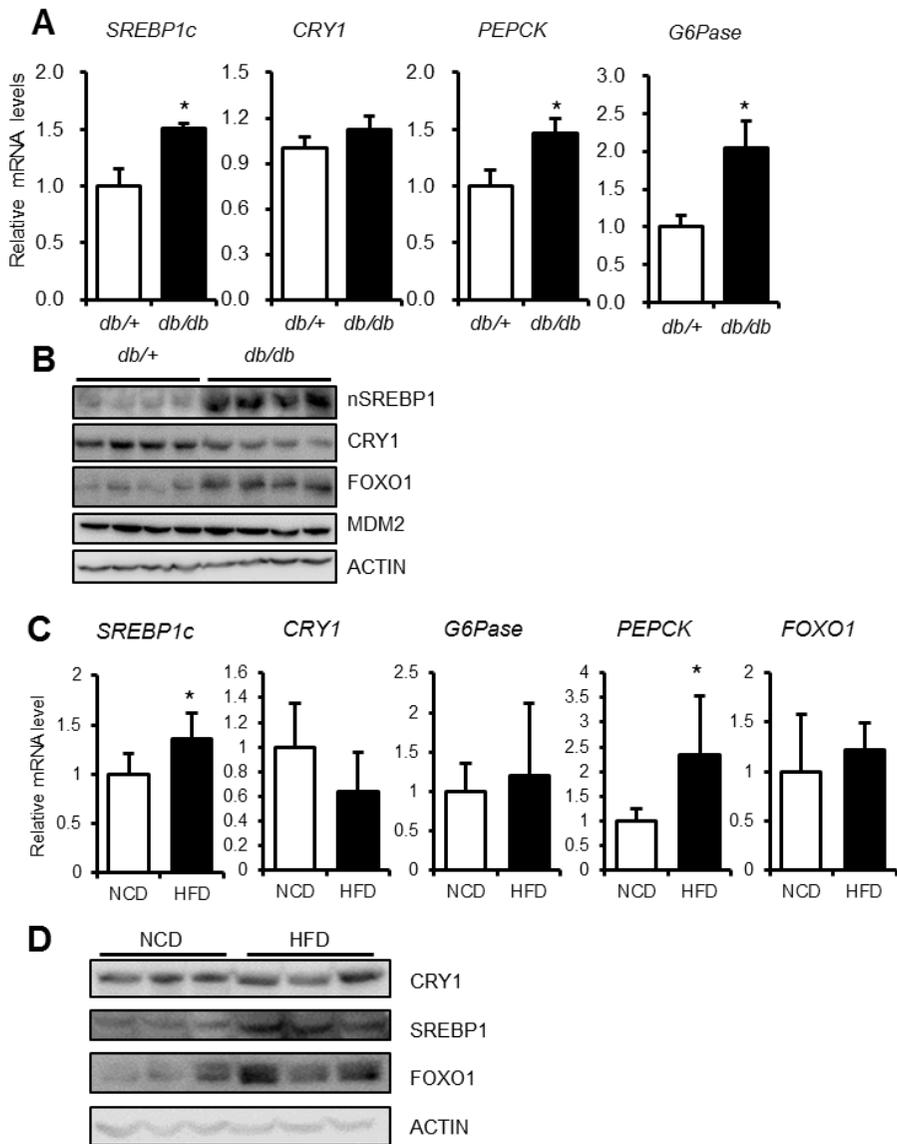
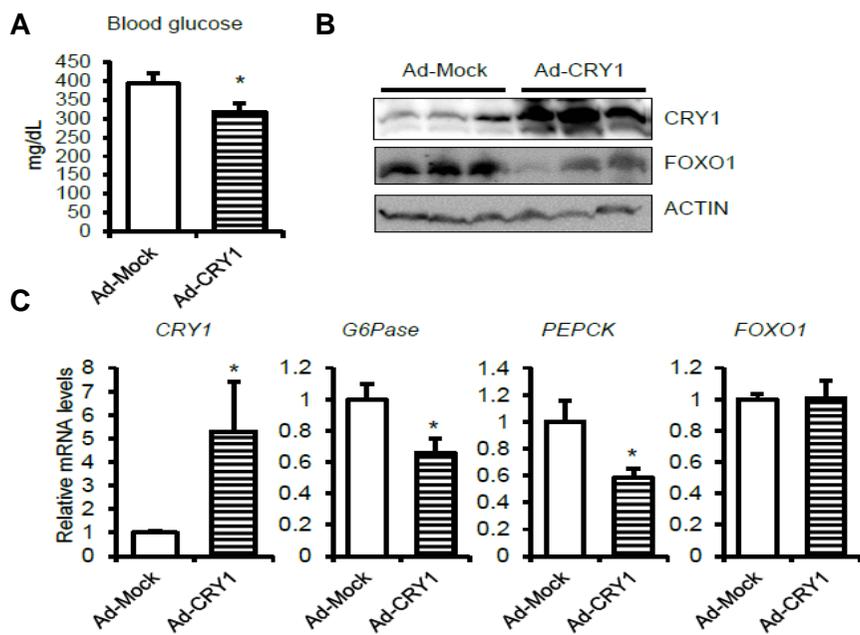


Figure 35. CRY1 alleviates gluconeogenesis in *db/db* mice

(A, B, and C) Ten-week-old male *db/db* mice were infected through the tail vein with adenovirus encoding GFP or CRY1 (adenoviral dose of 2×10^{10} viral particles per mouse). The blood glucose levels (A) were measured in ad libitum at ZT3. After all of the mice were sacrificed at ZT3, hepatic protein levels (B) were analyzed by western blotting, and the relative mRNA levels (C) were determined by qRT-PCR analyses and normalized to the TBP mRNA level. Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$ versus Ad-Mock group. (Student's *t*-test).



Discussion

As a major anabolic hormone, insulin stimulates lipogenesis and represses gluconeogenesis in the liver. Following insulin exposure, lipogenesis is upregulated by SREBP1c, and the expression of SREBP1c target genes such as FASN, SCD and ELOVL6 is thus induced (Chu et al., 2013; Kim et al., 1998a; Ponugoti et al., 2010). In contrast, insulin blocks hepatic gluconeogenesis through AKT-mediated phosphorylation of FOXO1 and PGC1 α (Gross et al., 2008; Li et al., 2007), both are major regulators of gluconeogenic genes including PEPCK and G6Pase. Here, I propose that the SREBP1c-CRY1 signaling pathway plays an important role to inhibit hepatic gluconeogenesis under anabolic state. Accumulating evidences from hepatic gluconeogenic gene expression, *in vitro* glucose output assays, time kinetics of insulin signaling cascades, and pyruvate tolerance test, which reflects both hepatic glucose output and peripheral glucose disposal, have consistently suggested the idea that maintenance of SREBP1c-induced CRY1 is crucial to prevent unnecessary hepatic gluconeogenesis during insulin action. In this regard, it has been reported that single nucleotide polymorphisms (SNPs) of SREBP1c and CRY1 genes are associated with hyperglycemia in human (Harding et al., 2006; Kelly et al., 2012).

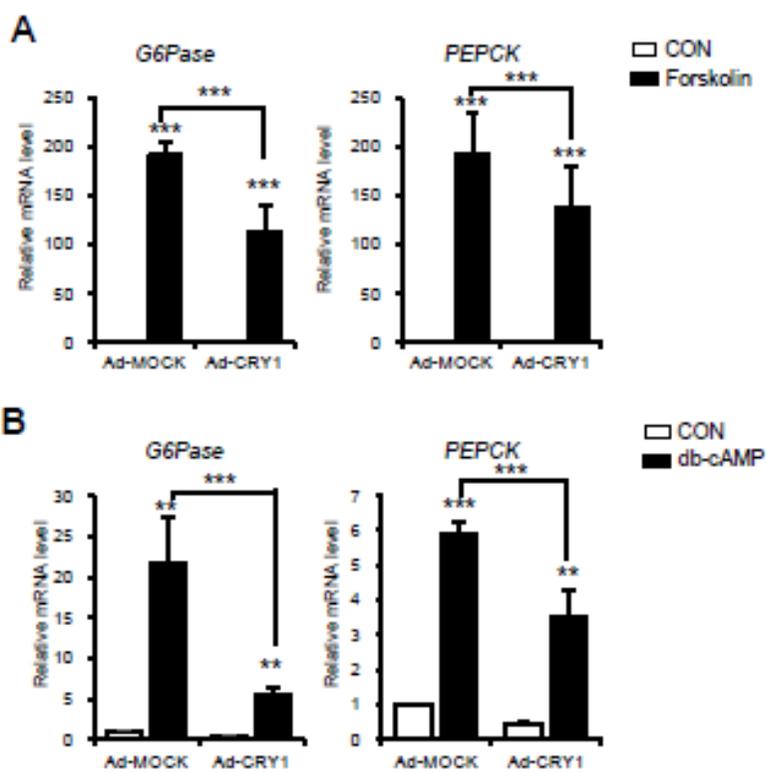
Similar to previous reports (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000), the expression of SREBP1c and gluconeogenic genes was increased in the liver of diabetic *db/db* mice (Figure 34A, 34B, 34C). Unexpectedly, the level of hepatic CRY1 protein was reduced in *db/db* mice. In this work, I have demonstrated

that ectopic overexpression of CRY1 in *db/db* mice alleviated hepatic gluconeogenesis by reducing FOXO1 protein (Figure 35A, 35B, 35C). Moreover, I have shown that hepatic CRY1 could attenuate blood glucose level by decreasing FOXO1 protein, independent of AKT activity (Figure 30A, 30B, 30C, 30D). Although it remains to be elucidated how elevated SREBP1c fails to increase CRY1 in the liver of *db/db* mice, it is very likely that increased FOXO1 protein might be resulted from reduced hepatic CRY1 protein in *db/db* mice.

It has been reported that CRY1 seems to suppress hepatic glucose production through interfering glucagon signaling (Hirota et al., 2012; Zhang et al., 2010). CRY1 interacts with GR (Lamia et al., 2011) and the α subunit of the glucagon receptor (Zhang et al., 2010), which are involved in the regulation of gluconeogenesis. Nonetheless, specific roles of CRY1 during the nutrient-rich state after exposure to insulin have not been clearly elucidated. To explore whether CRY1 might repress gluconeogenesis by inhibiting glucagon signaling, CRY1-overexpressing primary hepatocytes were treated with forskolin or db-cAMP to mimic the stimulation of glucagon signaling pathways. In primary hepatocytes, CRY1 partially repressed gluconeogenic gene expression in the presence of forskolin or db-cAMP (Figure 36A, 36B), implying that in addition to the glucagon signaling pathway there may be another signaling cascade, regulated by CRY1, which suppresses hepatic glucose production. Thus, CRY1 is appears to be involved in multiple regulatory pathways that control hepatic gluconeogenesis in response to insulin and glucagon.

Figure 36. Gluconeogenic gene expression is partially attenuated by CRY1 overexpression upon incubation with forskolin and db-cAMP

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The cells were treated with 10 μ M forskolin (A), 5 μ M db-cAMP (B), or vehicle (CON) for 4 h. PEPCK and G6Pase mRNA levels were determined using qRT-PCR and normalized to the level of the TBP mRNA. The values represent the mean \pm SD (N=3 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 (Student's t-test).



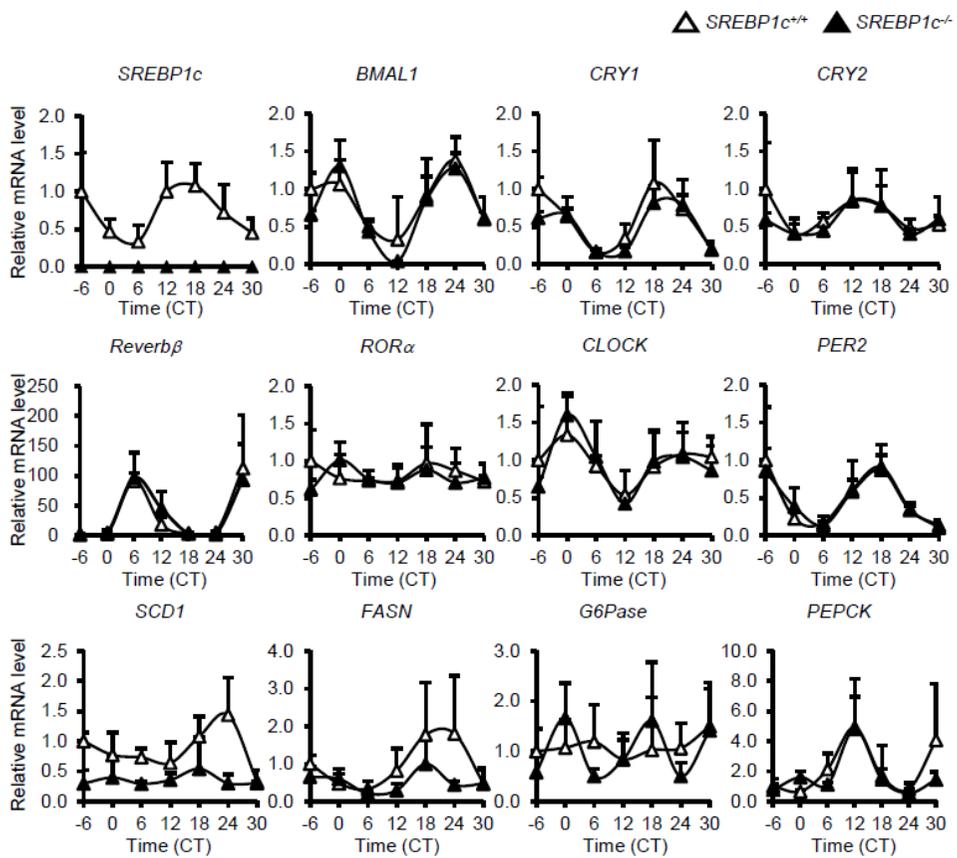
Activation of FOXO1-mediated gluconeogenesis is inhibited by insulin. AKT, a key downstream molecule of the insulin-activated signaling, phosphorylates FOXO1, which then is translocated from the nucleus to the cytoplasm through its association with the 14-3-3 protein (Zhao et al., 2004). In primary hepatocytes, FOXO1 phosphorylation was rapidly increased by insulin. However, hepatic gluconeogenic programming is persistently and efficiently suppressed regardless of the decreased FOXO1 phosphorylation at the late stage of insulin action. Intriguingly, hepatic CRY1 expression was enhanced at relatively late periods of insulin action (Figure 28A, 28B). Furthermore, in primary hepatocytes, a long-term insulin treatment downregulated FOXO1 expression, while suppression of CRY1 rescued FOXO1 protein levels as well as gluconeogenic gene expression (Figure 29A, 29B). It is noteworthy that CRY1 overexpressing mice showed a decrease of blood glucose level as well as of FOXO1 protein when AKT activity was pharmacologically repressed with AKT inhibitor MK2206 (Figure 30A, 30B, 30C, 30D). Collectively, our *in vitro* and *in vivo* data suggest that the CRY1-dependent FOXO1 degradation would be one of crucial mechanisms to attenuate hepatic gluconeogenesis for the long-term insulin action. Therefore, these observations prompted us to propose that the AKT-mediated FOXO1 phosphorylation provides an acute response during early insulin response, whereas SREBP1c-mediated CRY1 regulation would be a more durable process leading to the repression of futile hepatic gluconeogenesis throughout the anabolic state.

CRY1 is one of the key proteins in the circadian negative feedback loop. I observed that CRY1 levels were regulated by insulin and SREBP1c *in vivo* and *in vitro*. Given that hepatic circadian clock gene expression is altered in the STZ-injected insulin-deficient rats (Yamajuku et al., 2012), I investigated circadian clock gene oscillations in the liver of *SREBP1c^{+/+}* and *SREBP1c^{-/-}*. As shown in Figure 37, I did not observe any significant differences in hepatic circadian clock gene oscillations between *SREBP1c^{+/+}* and *SREBP1c^{-/-}* mice, implying that insulin-activated SREBP1c could stimulate *CRY1* gene expression in liver, probably, independent of circadian clock gene oscillations. Also, I cannot exclude the possibility that SREBP1c-induced CRY1 might contribute to minor roles for hepatic circadian oscillation in *SREBP1c^{-/-}* mice because it has been reported that *CRY1^{-/-}* mice exhibit fewer changes in circadian oscillations compared to *CRY1^{-/-}CRY2^{-/-}* double mutant mice (van der Horst et al., 1999). Furthermore, it is possible that remaining SREBP1a and/or SREBP2 activity in *SREBP1c^{-/-}* mice might maintain intact circadian clock gene oscillations and this homeostatic regulation needs to be addressed in future studies (Im et al., 2009; Liang et al., 2002). Nonetheless, hepatic CRY1 gene expression is clearly upregulated by SREBP1c in the postprandial condition.

As SREBP1c could simultaneously regulate both gluconeogenesis and lipogenesis, it is plausible to suggest that hepatic SREBP1c would effectively coordinate the anabolic pathways by upregulating fatty acid metabolism and

Figure 37. Expression profiles of various genes in the liver of *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice

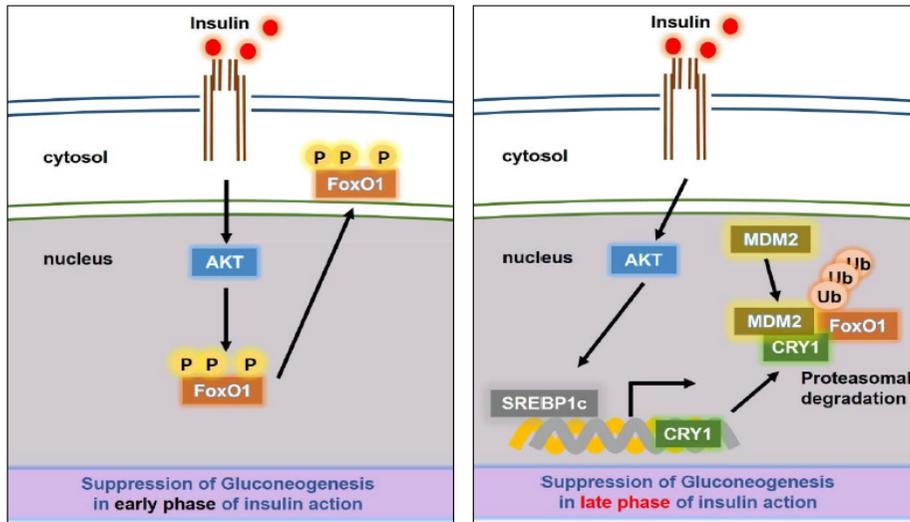
Livers were isolated every 6 h from *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice fed with normal chow diet and kept under 12 h: dark, 12 h: dark cycle for 1 week. Relative mRNA levels were determined by qRT-PCR and normalized by the level of the TBP mRNA. N=3-4 at each time point.



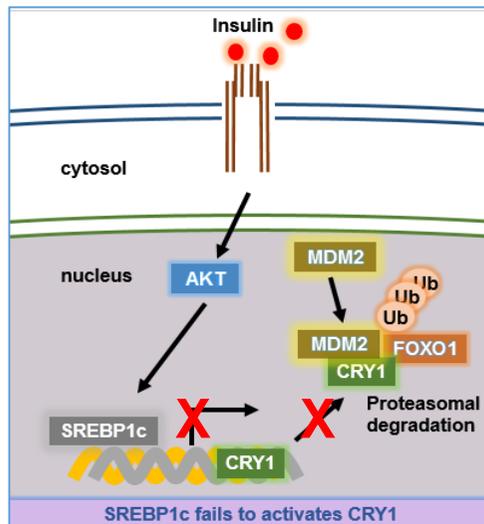
downregulating glucose metabolism upon insulin signaling with different target genes. Our study is the first report to reveal the role of SREBP1c in *CRY1* activation, which appears to be crucial in the regulation of hepatic glucose metabolism in the anabolic state. Based on the circadian oscillatory gene expression profile in *SREBP1c*^{-/-} mice, the SREBP1c protein may not actively govern hepatic circadian clock. However, increased expression of hepatic *CRY1* during the postprandial state is primarily regulated by the insulin-activated SREBP1c, which eventually leads to the suppression of glucose production via FOXO1 degradation (Figure 38). Although the roles of hepatic *CRY1* in energy metabolism need to be investigated further, our data provide an important clue to understand the molecular mechanisms that link hepatic SREBP1c and glucose homeostasis in physiological and pathological conditions.

Figure 38. Schematic diagram of the proposed model of chapter 2.

Normal Condition



Diabetic Condition



Conclusion and perspectives

1. Altering the hepatic circadian clock gene by feeding period restriction is associated with hepatic lipid and glucose metabolism, but not with body weight change

The harmony of the peripheral circadian clock and the central circadian clock is involved in diverse metabolic homeostasis. Therefore, it is likely that dysregulated circadian clocks are closely linked with metabolic disorders such as diabetes, obesity and cardiovascular disease. The central circadian clock is regulated by light signal whereas the peripheral circadian clock is controlled by food intake and diverse hormones. Interestingly, it has been reported that shift workers are prone to have high body mass index and cardiovascular events, indicating that unsynchronized circadian clock might participate in metabolic dysregulation (Aronoff et al., 2001; Colles et al., 2007; Tholin et al., 2009). In this aspects, it has been proposed that circadian clock regulation might be a potential therapeutic approach to cure for obesity or metabolic disorders.

As the prevalence of obesity continues to rise, its contribution to mortality increases. In particular, obesity-induced insulin resistance is one of the key factors for the development of metabolic diseases such as hypertension, atherosclerosis, and type 2 diabetes. The primary causes of obesity is simply expressed by higher energy intake compared to energy expenditure. To solve the relationship with

unsynchronized circadian clock and obesity, I analyzed two experimental groups that have equal amounts of energy intake with different circadian clock regulation by feeding period restriction.

In chapter 1, I have demonstrated that an unsynchronized circadian clock with the same amounts of energy intake had no effects on body weight change. Because rodents, including experimental mice, are nocturnal animals who consume more food in night time than day time, I have provided the night time feeding group with the equal amounts of food as the day time feeding group. Compared to the ad libitum group, the day time feeding and night time feeding group are offered restricted calorie intake with both NCD and HFD. Intriguingly, obtained data reveal that the same amounts of food intake with different feeding periods do not affect the body weight change regardless of NCD or HFD.

I also found out that lipid and glucose metabolisms were altered in liver upon feeding period restriction. The expression patterns of certain genes belonging to hepatic lipogenesis, gluconeogenesis, and lipid oxidation were influenced by feeding period alteration. Moreover, the levels of serum triacylglyceride, cholesterol, and glucose were changed, indicating that alteration of peripheral circadian clock genes is important for metabolic regulation. Although the mechanisms of how peripheral circadian clocks could regulate metabolic change are still to be elucidated, the results from chapter 1 is important for understanding the relationship between circadian clock and metabolic regulation.

2. Hepatic SREBP1c is a mediator of feeding dependent CRY1 gene alteration

Accumulating evidences have suggested that key metabolic regulators are involved in the modulation of circadian clock gene expression. For example, PPAR γ and its coactivator PGC1 α activate BMAL1 transcription and regulate circadian oscillation (Lamia et al., 2009; Schmutz et al., 2010; Wang et al., 2008a). Moreover, ROR α and Rev-erb α directly and inversely regulates BMAL1 gene expression to maintain circadian oscillation (Raspe et al., 2002; Tini et al., 1995). However, it has not been fully understood whether and how feeding or nutrient rich states might control circadian clock core genes in peripheral tissues.

In chapter two, I have shown that SREBP1c activates CRY1 gene expression. SREBP1c is expressed in a precursor form, which is bound to the ER membrane. Under insulin signaling, SREBP1c precursor is processed by site 1 protease and site 2 protease, and the truncated SREBP1c is located to the nucleus to activate its target gene such as FASN, SCD1 and itself. Further, SREBP1c is post-translationally activated with feeding signals accompanied with hormonal changes. CRY1 promoter contains both SRE motif and E-BOX motif which are binding motifs of SREBP1c. Also, E-box motif is a target element of BMAL1 and CLOCK heterodimer, which are the core regulators of circadian clock oscillation (Ramsey et al., 2007). I have demonstrated that SREBP1c stimulates CRY1 gene expression through SRE

sequence(s), not E-BOX motif. Luciferase assays performed with WT, 3XSRE mutants, and E-BOX have suggested that SRE sequences in the CRY1 promoter are target sites of SREBP1c. Because SREBP1c may not compete with BMAL1 and CLOCK at the E-box motif of CRY1 promoter, it appears that SREBP1c might have a little effects on circadian clock gene oscillation (Figure 37).

3. SREBP1c-CRY1 axis participates in inhibition of gluconeogenesis in postprandial state

SREBP1c is a well-known transcription activator that regulates lipid biosynthetic pathway in postprandial states. Recent data have also suggested that SREBP1c might participate in the suppression of hepatic gluconeogenesis (Lee et al., 2007; Yamamoto et al., 2004). As a transcriptional activator, SREBP1c directly regulates lipogenesis by binding to target gene promoters. However, the molecular mechanism by which transcriptional activator SREBP1c may suppress gluconeogenic genes are not fully elucidated.

In chapter two, I have revealed a novel signaling cascades of SREBP1c to mediate inhibition of hepatic gluconeogenesis. In liver, feeding or insulin mediated CRY1 activation by SREBP1c leads to degradation of FOXO1 protein, which is a crucial activator of hepatic gluconeogenesis. In primary hepatocytes, overexpression of SREBP1c downregulated glucose output and lowers PEPCK and G6Pase gene

expression. Overexpression of CRY1, as a novel target gene of SREBP1c, also repressed gluconeogenesis, whereas knock down of CRY1 led to increase in gluconeogenic gene expression. Among various signaling cascades that regulate gluconeogenesis, FOXO1 was regulated by CRY1. I have shown that CRY1 would act as a scaffold protein by binding with FOXO1 and MDM2 Ubiquitin E3 ligase MDM2 mediated FOXO1 ubiquitination, accompanied with FOXO1 protein degradation in liver. In addition, SREBP1c KO mice showed higher levels of blood glucose, and FOXO1 protein, compared to those of WT mice. Furthermore, CRY1 overexpression in SREBP1c KO mice alleviated pyruvate induced blood glucose level. CRY1 KO mice also exhibited increased FOXO1 protein and expression of gluconeogenic genes, concomitant with higher blood glucose level.

FOXO1 is a well-known transcription factor regulated by posttranslational modification. Under insulin signaling, insulin activated AKT phosphorylates FOXO1 protein, which accelerates the translocation of FOXO1 into cytoplasm (Nielsen et al., 2008; Tzivion et al., 2011; Wang et al., 2006). The key regulatory mechanism of insulin mediated suppression of gluconeogenesis has been reported that AKT mediated FOXO1 translocation. In this study, I have revealed a novel pathway that insulin sustainably suppresses hepatic gluconeogenesis by SREBP1c-CRY1. Insulin mediated FOXO1 phosphorylation occurs at an early time point whereas CRY1 is induced at the late stage of insulin action. I have discovered that CRY1 plays a key role to decrease FOXO1 protein upon insulin. To exclude the possibility that the effect

of AKT on FOXO1 phosphorylation, I have used AKT inhibitors. In both *in vitro* and *in vivo* experiments, AKT inhibitors intensified the stability of FOXO1 protein. In contrast, CRY1 overexpression dramatically lowers FOXO1 protein level with or without AKT inhibitor, implying that CRY1 would act as an inhibitor of FOXO1, independent of AKT activity.

Although AKT mediated FOXO1 phosphorylation is one of the major inhibitory mechanisms of hepatic gluconeogenesis, here, I proposed that SREBP1c mediated CRY1 induction would be another crucial signaling pathways that participates in the suppression of gluconeogenesis in the postprandial state.

4. Hyperglycemia is exacerbated by dysregulation of hepatic SREBP1c-CRY1 signaling pathway

Hepatic insulin signaling activates *de novo* lipogenesis for storage excess energy, and inhibits hepatic gluconeogenesis for maintaining blood glucose level. SREBP1c, one of key molecule in insulin signaling pathway, participates in both activation of *de novo* lipogenesis and inhibition of gluconeogenesis. Although hepatic SREBP1c activity is increased in obese animals, the inhibitory effect of SREBP1c on gluconeogenesis was not observed.

Here, I found out that SREBP1c was increased in HFD fed mice and *db/db* mice, while CRY1 was not elevated in both obese mice models. To address the

question which limited CRY1 might be a cause of hyperglycemia in obesity, I have investigated the effects of CRY1 overexpression on glucose metabolism. In *db/db* mice CRY1 overexpression lowered blood glucose level as well as hepatic gluconeogenic gene expression, implying that insufficient CRY1 may not be enough to block FOXO1 activity for normalizing blood glucose level in obese animals. Of course, it is necessary to further investigate why activated SREBP1c fails to increase CRY1 gene expression in obese animals. Moreover, it is likely that various approaches with CRY1 modulation, including stability control, gene expression regulation, chromatin structure modulation, and epigenetic management, will be possible as a therapeutic target.

Hyperglycemia is one of the many characters of obesity and diabetes. Lowering the blood glucose level is an important therapeutic approach for diabetes. Here, I have suggested that the peripheral circadian clock is important for metabolic regulation but not for body weight gain. Although obesity is associated with various metabolic complications, it appears that the primary cause of obesity is correlated with the amounts of food intake, but not circadian clock mediated metabolic changes. I have also elucidated a novel pathway that circadian clock is involved in the regulation of hepatic glucose metabolism. Under normal physiological condition, SREBP1c-CRY1 mediated FOXO1 degradation contributes to suppress gluconeogenesis. During this regulation, SREBP1c stimulated CRY1 expression and CRY1 acts as a scaffold protein by binding with MDM2 and FOXO1, leading to

FOXO1 degradation. Under pathophysiological conditions such as obesity, SREBP1c fails to activate CRY1 gene expression in liver, and as a consequence, gluconeogenesis is still highly activated. It is noteworthy that various approaches to modulate SREBP1c-CRY1 signaling pathway will give the opportunity for overcoming high blood glucose levels. Taken together, the characterization of the interaction between peripheral circadian clock and hepatic metabolism would be crucial for maintaining homeostasis of energy balance.

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국문 초록

최근 연구결과에 따르면 밤과 낮의 변화에 따라 발생하는 일주기성은 개체의 행동 뿐 아니라 분자신호전달에도 중추적인 역할을 담당한다. 또한, 정교하게 조절되는 일주기성은 몸의 에너지 항상성 유지에도 깊이 연관되어 있음이 보고되었다. 이러한 측면에서 일주기성이 유지되지 못하는 경우 포유동물은 비만 및 다양한 대사질환을 유발할 수 있다. 대표적인 에너지 대사 조직인 지방조직, 근육조직, 간조직의 일주기성은 지방생합성, 포도당생합성 및 지방 산화 작용 등을 조절한다. 또한, 중추조직과 말초조직의 일주기성이 일치하지 않는 야간 근로자의 경우 대사질환이 자주 발생하는 경향이 보고되고 있다. 그러나 일주기성의 생리적 및 병리적 역할과 체내 에너지 대사조절과의 상호 관계에 대해서는 분자수준의 연구가 부족한 상황이다.

본 연구를 통해 중추조직과 말초조직의 일주기성이 일치하지 않을 경우 간조직의 포도당 및 지방대사는 변화하는 반면 체중에는 영향을 미치지 않음을 발견하였다. 같은 양의 음식물을 다른 시간대에 섭취하는 조건에서 말초조직의 일주기성은 섭취 시간에 따라 변화하는 것을 관찰한 반면, 중추조직의 일주기성은 변하지 않는다는 것을 관찰할 수 있었다. 간조직에서 대표적인 일주기성 유전자인 *BMAL1*, *CLOCK*, *PER2*는

음식 섭취시간대에 따라 그 발현이 변함을 관찰하였다. 간조직의 지방대사, 포도당대사, 지방 산화에 연관된 유전자들 또한 음식섭취 시간대의 변화에 따라 발현양상이 다르게 조절되었다. 이와 같은 현상은 저칼로리 음식물이나 고칼로리 음식물을 섭취한 야생형 생쥐 모두에서 관찰되었다. 본 연구를 통하여 체중증가에 있어 음식물 섭취 시간대의 변화는 보다는 음식물의 섭취량이 핵심적임을 확인할 수 있었다.

본 학위 논문연구 동안 본인은 음식섭취와 인슐린에 의해 증가되는 CRY1 유전자가 포도당 생합성을 억제함을 발견하였다. 전사인자인 SREBP1c는 음식섭취에 의해 활성화되어 간조직에서 지방생합성을 관장하는데, 간세포의 SREBP1c가 음식섭취에 의해 CRY1의 발현을 증가 시킴을 발견하였다. 간세포주에 CRY1을 과발현 시켰을 경우 포도당생합성 유전자인 PEPCK, G6Pase의 발현이 억제되었고, FOXO1의 단백질량이 감소하였다. 간세포주에서 CRY1은 인슐린을 8시간 이상 처리했을 경우 증가되었고 이상의 연구를 통하여 CRY1은 인슐린이 지속적으로 포도당 생합성을 억제하는 과정 중 중요한 매개자의 역할을 수행하는 것으로 추정된다. 흥미롭게도, SREBP1c에 의해 증가된 CRY1은 FOXO1의 단백질 분해 기전을 촉진시켰으며, FOXO1의 E3 ubiquitin ligase인 MDM2와의 결합을 강화시키는 Scaffold protein의 역할을 담당할 수 있음을 발견하였다. 이와 관련하여, 간에서 SREBP1c가 증가되어 있음이 보고된

db/db 생쥐와 고지방성식이 유도 비만 생쥐 모델에서 CRY1의 발현이 동반하여 증가하지 않았으며, 그 결과 포도당생합성이 증가될 수 있음을 발견하였다. 그러나 *db/db* 생쥐에 CRY1을 과발현 시킨 경우 혈당을 낮추는 동시에 간에서 포도당생합성 관련 유전자들의 발현이 억제됨을 관찰하였다.

본 연구의 결과들을 종합하여 살펴볼 때, 일주기성 유전자는 간조직의 에너지대사를 능동적으로 조절하는 것으로 생각된다. 다양한 일주기성 유전자 가운데 SREBP1c에 의해 증가하는 CRY1은 FOXO1의 단백질 분해 과정 유도를 통해 간세포 내 포도당생합성 유전자의 발현을 억제할 수 있다. 또한, 체중 증가는 말초기관의 일주기성 유전자의 발현 변화에 의한 것이 아니라 섭취한 음식물의 에너지 양에 의해 결정되는 것으로 추정된다. 그러므로 적절한 SREBP1c-CRY1 신호전달 경로의 조절은 체내 에너지대사 항상성 유지에 핵심적인 신호전달 경로 중 하나임을 제안한다.

주요어: 비만, 당뇨, 일주기성, SREBP1c, CRY1, FOXO1, MDM2, PEPCK, G6Pase, 포도당생합성, 인슐린 신호전달경로

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말초조직 일주기성이 에너지 대사 조절에
미치는 역할 규명

Roles of peripheral circadian clock
in the regulation of energy metabolism

2016년 2월

서울대학교 대학원

생명과학부

장 하 군

ABSTRACT

Roles of peripheral circadian clock in the regulation of hepatic energy homeostasis

Hagoon Jang

Emerging evidence has suggested that the circadian clock is a control tower in the regulation of behavioral and molecular processes under day/night cycle. Also, precise regulation of the circadian clock is crucial for maintaining whole-body energy homeostasis. Thus, dysregulation of the circadian clock is closely associated with obesity and metabolic complications. In metabolic organs, such as adipose tissue, muscle and liver, the circadian clocks affect various metabolic processes including lipogenesis, gluconeogenesis and lipid oxidation. Moreover, unsynchronized cooperation between the hypothalamic central clock and the peripheral clock, in shift workers, is prone to occur metabolic disorders. However, the pathophysiological role of the circadian clock and its metabolic regulatory processes have not been thoroughly elucidated.

In this study, I have demonstrated that unsynchronized circadian clock influences hepatic glucose and lipid metabolisms without altering body weight. With the same amounts of caloric intake during different time periods, the expression patterns of peripheral circadian clock genes showed different by day time feeding and

night time feeding, while that of light-regulated hypothalamic circadian clock genes was not affected. For instance, the expression pattern of core circadian clock genes, such as BMAL1, CLOCK and PER2 in peripheral tissues, was altered by different feeding periods. In addition, hepatic expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation genes was also changed by feeding periods. In conclusion, it is likely that the amounts of food consumed might be a crucial factor to induce obesity compared to feeding time because feeding period restriction has no effects on body weight gain either normal chow diet (NCD) or high fat diet (HFD).

In addition, I have revealed that feeding-induced CRY1 gene expression leads to suppression of hepatic gluconeogenesis. Given that SREBP1c is a well-known transcriptional activator in postprandial state for activating lipogenesis, I found out that SREBP1c stimulated CRY1 gene expression. CRY1 was induced by feeding and insulin challenge. In addition, I have shown that hepatic SREBP1c contributed to repress gluconeogenesis through CRY1 induction. Hepatocytes overexpressing CRY1 inhibited hepatic gluconeogenic genes, such as PEPCK and G6Pase, via lowering FOXO1 protein. Furthermore, CRY1 was elevated in long term insulin action for sustainable suppression of hepatic gluconeogenesis. Intriguingly, SREBP1c-induced CRY1 accelerated FOXO1 degradation via ubiquitination. I discovered that CRY1 would act as a scaffold protein by binding with MDM2, an E3 ubiquitin ligase, and FOXO1. Although SREBP1c is increased in obese diabetic animals, such as *db/db* mice and HFD fed mice, SREBP1c failed to stimulate CRY1

and thereby hepatic gluconeogenesis was not suppressed in obese animals. When I overexpressed CRY1 in the liver of *db/db* mice to test the CRY1 effects on gluconeogenesis suppression. CRY1 overexpression reduced blood glucose level as well as downregulated hepatic gluconeogenic gene expression. Taken together, these data suggest that circadian clock genes actively and dynamically regulate energy metabolism in the liver. Among many circadian clock genes, SREBP1c-induced CRY1 especially contributes to the suppression of hepatic glucose production through FOXO1 degradation in liver. However, body weight gain is mainly determined by the amounts of calorie intake rather than alteration of peripheral circadian clock gene. Therefore, it is likely that appropriate regulation of SREBP1c-CRY1 signaling pathway would be crucial for maintaining whole-body energy homeostasis.

Key words: Obesity, Diabetes, Circadian clock, SREBP1c, CRY1, FOXO1, MDM2, PEPCCK, G6Pase, Hepatic gluconeogenesis, Insulin signaling

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BACKGROUND

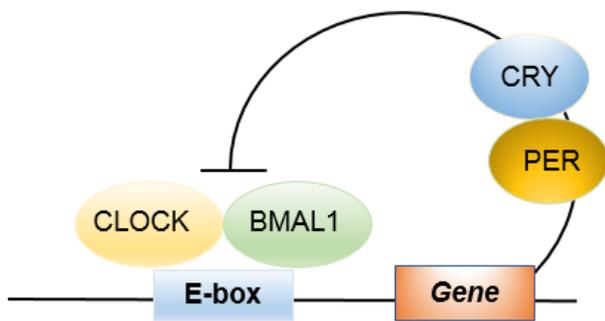
1. Circadian clock and metabolic regulation

(1) Circadian clock

Circadian clocks control behavioral and molecular processes with the day and night cycle. The molecular circadian clock in mammals exists within pacemaker neurons of the suprachiasmatic nucleus (SCN) and peripheral tissues. The central SCN clock regulates standard time for peripheral tissue clock (Ramsey et al., 2007). Various physiological changes, such as sleep-wake cycles, body temperature, blood pressure, and hormone secretion, are associated with the circadian clock. Molecular circadian oscillation is composed of transcription-translational auto-regulatory negative feedback loops; BMAL1, CLOCK, PER, and CRY are key circadian molecules that produce rhythmic oscillations in a cell-autonomous manner (Thresher et al., 1998; Vitaterna et al., 1999). BMAL1 and CLOCK play key roles in inducing PER and CRY. Induced PER and CRY then form a transcriptional repressor complex to suppress BMAL1 and CLOCK, which eventually leads to negative feedback regulation (Figure 1). In addition, BMAL1 and CLOCK also increase the mRNA levels of Rev-erb α and ROR α , which then compete to bind to the retinoic acid-related orphan receptor response elements (ROREs) in BMAL1 promoter and repress or activate the expression of BMAL1, respectively (Raspe et al., 2002; Tini et al., 1995). This alternating promoter occupancy is due to the rhythmic expression of Rev-erb α

Figure 1. Molecular clock: circadian negative feedback loop.

CLOCK and BMAL1, heterodimer initiates transcription of target genes such 'period' genes (PER1, PER2, and PER3) and two cryptochrome genes (CRY1 and CRY2). Negative feedback is achieved by PER/CRY complexes which translocate into the nucleus to suppress their own transcription by blocking the activity of the CLOCK/BMAL1 heterodimer.



and ROR α .

Moreover, these auto-regulatory loops are modulated by various post-translational modifications such as phosphorylation, sumoylation, acetylation, and ubiquitination. For instance, casein kinase phosphorylates the PER proteins for degradation via the Skp1, cullin1, F-box protein (SCF)/ B-TrCP ubiquitin ligase complex. Adenosine monophosphate kinase (AMPK) phosphorylates CRYs and leads to ubiquitin-mediated proteasomal degradation via the SCF/FBXL3 ubiquitin ligase complex (Lamia et al., 2009; Zheng et al., 2014). In addition, cellular NAD⁺ level regulates SIRT1 activity which participates in CLOCK protein deacetylation (Peek et al., 2013; Ramsey et al., 2009).

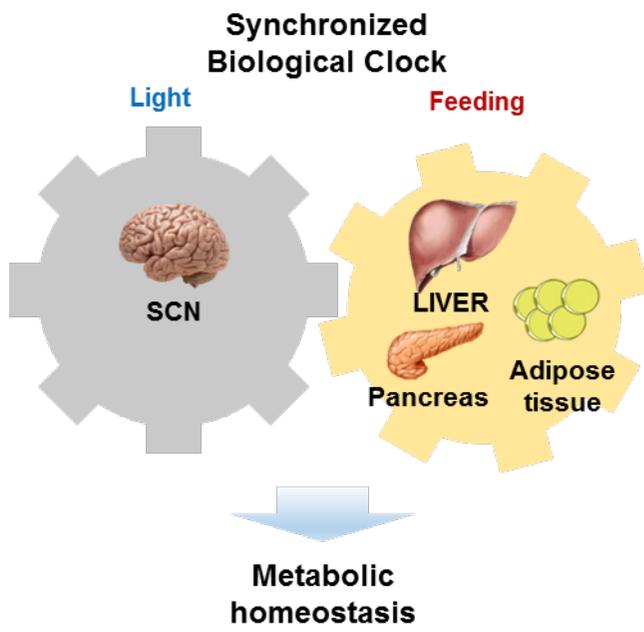
Central SCN circadian oscillation is primarily regulated by light, whereas peripheral circadian oscillation is affected by food intake along with hormones such as insulin and glucagon (Gamble et al., 2014; Hoyle and O'Neill, 2013). These clocks regulate biological processes to maintain whole-body homeostasis with the environmental changes of light and nutrients (Figure 2). Many recent studies have shown that numerous aspects of metabolic regulation, such as circulating hormones, intracellular metabolites, and feeding behaviors, exhibit daily rhythmicity (Brandenberger and Weibel, 2004).

(2) Circadian clock and metabolic regulation

Emerging evidences suggest that the circadian clock is closely associated

Figure 2. Light-dependent central clock and feeding-mediated peripheral clock.

The master pacemaker within SCN, although clock genes are also expressed in other tissues such as liver, adipose tissue, and pancreas. Emerging evidence suggests that peripheral clock synchronized with central clock regulates whole-body energy homeostasis.



with whole-body energy homeostasis. For example, the finding that participation of the orphan nuclear hormone receptors, Rev-erb α and the opposing ROR α , in a short feedback loop by controlling BMAL1 transcription provides a direct evidence for metabolic input into the molecular clock (Raspe et al., 2002; Tini et al., 1995). In addition, peroxisome proliferator-activated receptor α (PPAR α) and peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1 α) also regulate BMAL1 transcription via circadian core feedback loop (Liu et al., 2007). Moreover, microarray analysis reveals that more than half of transcripts show rhythmic gene oscillation, with some variation among different tissues such as liver, skeletal muscle, and brown and white adipose tissue (Yang et al., 2006). The numbers of transcripts rhythmic oscillation are up to 20%, indicating that a large proportion of the transcriptomes seem to be affected by circadian genes. These oscillating genes are involved in biosynthetic and metabolic processes such as cholesterol and lipid metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation, and detoxification pathways (Akhtar et al., 2002; McCarthy et al., 2007). Interestingly, most rate-limiting enzymes in these metabolic pathways are regulated by circadian clocks, implying that the clocks could influence on biosynthetic and metabolic processes whole-body metabolic homeostasis

(3) Circadian clock and metabolic disorder

In the aspect of clinical studies, several evidences suggest that circadian

disruption is associated with metabolic complications across large portions of the human population (Karlsson et al., 2001). Cross-sectional studies have revealed an increased prevalence of metabolic syndrome, such as high body mass index and cardiovascular events, in shift workers. These observations suggest the possibility that chronic disharmony between central and peripheral clocks, combined with light/dark and fasting/feeding, might contribute to body weight gain and metabolic complications (Kalra and Kalra, 2004). In addition, intriguing human behavioral studies propose that nocturnal feeding patterns might be related with metabolic diseases (Ayala et al., 2009). These findings have been recapitulated by recent rodent experimental studies, indicating that diet-induced obesity (DIO) with HFD may lead to increased energy intake only in the rest/light period and not in the active/dark period (Kohsaka et al., 2007). In addition, several rodent models have suggested that circadian clock regulation is associated with metabolic disease. For example, CLOCK-defective mice show increased body weight and hyperphagia with disrupted circadian oscillation (Turek et al., 2005). HFD-fed mice become obese and diabetic concomitantly with altered expression of circadian clock genes (Kohsaka et al., 2007). BMAL1 knockout mice show dysregulation of hepatic glucose homeostasis (Rudic et al., 2004). Furthermore, key metabolic genes, such as PPAR α , PPAR γ , and AMPK, are involved in the regulation of circadian genes, and the circadian clocks in turn modulate whole-body energy metabolism (Lamia et al., 2009; Schmutz et al., 2010; Wang et al., 2008a). In addition to rodent studies, the control of glucose metabolism

by circadian oscillation in humans is a well-known aspect of clinical diabetes management, and a variation of the normal cyclic pattern of glucose tolerance is an important indicator of type 2 diabetes (Allison et al., 2007; Tasali et al., 2008).

Unsynchronized central and peripheral clocks caused by different regulation of light/dark cycle and the feeding/fasting cycle have been implicated in metabolic disorder, along with higher calorie intake in the case of shift-workers (Ellingsen et al., 2007; Karlsson et al., 2001). Under physiological conditions, the central and peripheral clocks are synchronized by the light/dark cycle and the feeding/fasting cycle. However, it has been also demonstrated that disharmonious signaling by these two cues leads to the independent regulation of each circadian oscillation. Although it appears that there is a close relationship between the circadian clock and metabolic regulation, the effects of unsynchronized SCN and peripheral-tissue circadian clocks on metabolic regulation are largely unknown. In addition, it is still elusive whether feeding period alteration might be a key determinant of body weight change without change of total calorie intake.

2. Hepatic lipid and glucose metabolism

(1) Lipogenesis

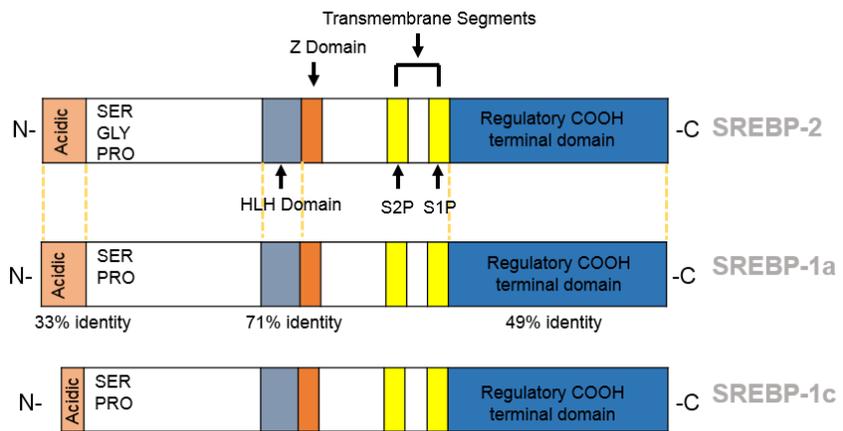
In liver, synthesis of triacylglycerides in liver is nutritionally and hormonally regulated. The ingestion of a high-carbohydrate diet causes a marked elevation of enzymes involved in key metabolic pathways, including glycolysis,

lipogenesis, fatty acid elongation and desaturation steps, and finally triacylglyceride synthesis. Especially, lipogenesis is the process that converts acetyl-CoA to fatty acids. Acetyl-CoA is an intermediate metabolite produced from glucose, a source of primary energy of living organisms. Through lipogenesis and subsequent triacylglyceride synthesis, the excess energy can be efficiently stored in the form of neutral lipid metabolites (Ferre and Foufelle, 2010). Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA, and increased malonyl-CoA level leads to produce long chain fatty acids. This reaction is the controlling step in fatty acid synthesis and takes place in the cell cytosol. The overall synthesis of fatty acids is catalyzed by the fatty acid synthase (FASN) complex, a single polypeptide containing seven distinct enzymatic activities (Griffin and Sul, 2004).

Sterol regulatory element binding proteins (SREBPs) including SREBP1a, SREBP1c, and SREBP2 belong to the basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family that regulates de novo lipogenesis and cholesterol biosynthetic pathway (Figure 3) (Brown and Goldstein, 1997). SREBP precursor consists of structurally three functional domains; an NH₂-terminal domain that contains the bHLH-LZ region for DNA binding, two hydrophobic transmembrane spanning domains interrupted by a short loop which are inserted into the lumen of ER, and COOH-terminal domain that recruits the gene regulatory machinery (Figure 3). Precursors of SREBPs are processed by several SREBPs processing apparatus.

Figure 3. Structure of SREBP isotypes.

Sterol regulatory element binding protein (SREBP) was identified as a protein that bound to the sterol regulatory element (SRE). SREBPs are a family of transcription factors that control lipid homeostasis by regulating the expression of enzymes required for cholesterol, fatty acid, triacylglycerol, and phospholipid synthesis. SREBPs are synthesized as precursor forms bound to the endoplasmic reticulum membranes. Upon activation, the precursor undergoes a sequential two step cleavage process to release the N-terminal active domain in the nucleus.



SREBP cleavage-activating protein (SCAP) is a sensor of cholesterol, and escorts the SREBP from the ER to the Golgi, where there are two proteases involved in the cleavage (DeBose-Boyd et al., 1999; Tomita et al., 1998). In the Golgi apparatus, SREBP is released from the Golgi membrane through cleavage by Site-1 protease (S1P) and Site-2 protease (S2P). The NH₂-terminal domain of SREBPs is translocated to the nucleus, where it stimulates target gene transcription by binding to sterol response elements (SREs) or E-BOX in the promoter/enhancer regions of various target genes (Kim et al., 1995).

SREBP1c is the master regulator of de novo lipogenesis in fat tissue and liver (Shimomura et al., 1999a). Insulin, which is released from pancreatic β -cells, stimulates, leading to fatty acid synthesis during nutrient rich status (Kim et al., 1998a). However, glucagon, which is released from pancreatic α -cells during fasting, suppresses SREBP1c mediated lipogenic action (Lee et al., 2014a). SREBP1c regulates lipogenic pathways by stimulating the expression of fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) and acetyl-coenzyme A carboxylase (ACC) (Kim et al., 1998a; Shimomura et al., 1999b). The level of SREBP1c falls in streptozotocin-treated animals by accelerating pancreatic β -cell apoptosis and increases after insulin injection (Shimomura et al., 1999b). Moreover, overexpression of nuclear SREBP1c in livers of transgenic mice prevents the reduction of lipogenic action in fasting (Takahashi et al., 2005). Taken together, accumulating evidences indicate that SREBP1c is a key player in insulin-mediated lipogenic activation in liver.

(2) Glucose metabolism

During fasting, most animals maintain energy balance by shifting from glucose utilization to fat burning. The levels of glycogenolysis and gluconeogenesis are increased to provide glucose as an energy source in many organs and tissues such as the brain and the red blood cell compartment, which are deficient in enzymes for burning free fatty acids (Altarejos and Montminy, 2011; Roach et al., 2012). Fasting also triggers elevation of circulating free amino acids, which are also major precursors for hepatic glucose production. When fasting is prolonged, hepatic gluconeogenesis is blocked to protect against excessive muscle wasting by over-converting protein to glucose, and liver derived ketone bodies become the primary energy source for the brain (Morris, 2005).

During fasting, elevated levels of circulating pancreatic glucagon stimulate the gluconeogenic program through the activation of the protein kinase A (PKA) pathway. Increased cAMP levels activate PKA, then gluconeogenic genes such as phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are upregulated (Montminy et al., 2004). cAMP response element binding protein (CREB), one of major regulators of hepatic gluconeogenesis, binds to promoters for the PEPCK and G6Pase genes to directly stimulate the gluconeogenic program. The significance of CREB in the activation of hepatic gluconeogenesis has been revealed by a study utilizing albumin-ACREB TG mice overexpressing a dominant negative CREB in liver (Herzig et al., 2001). These mice show lower blood glucose levels with

downregulated mRNA levels for hepatic gluconeogenic genes, indicating that CREB is a physiological transcriptional regulator of gluconeogenesis *in vivo*.

In parallel, decrement of insulin signaling upon fasting also stimulates gluconeogenic gene expression through dephosphorylation and nuclear translocation of the forkhead box (FOXO) domain proteins (Matsumoto and Accili, 2005). FOXO1 belongs to a subclass of the forkhead family of transcription factors which have a forkhead box type DNA binding domain. FOXO1 recognizes insulin response element (IRE) located in the promoters of PEPCK and G6Pase. Regulation of subcellular localization of FOXO1 is one of the important regulatory pathways to control its activity, which is modulated by the phosphorylation status of Ser/Thr residues. Insulin and PI3K signaling pathway activates AKT-dependent phosphorylation of FOXO1 and phosphorylated FOXO1 binds with 14-3-3 shuttle protein to translocate to the cytoplasm (Nielsen et al., 2008; Tzivion et al., 2011; Wang et al., 2006). The cytoplasm-localized FOXO1 then undergoes subsequent degradation by an ubiquitin-proteasome pathway. Although the translocation of hepatic FOXO1 from the nucleus to the cytoplasm is a well-defined mechanism mediating a quick decrease in glucose production by insulin, it is largely unknown how insulin provides a sustainable inhibition of hepatic gluconeogenesis during the postprandial state.

Interestingly, SREBP1c appears to be involved in hepatic carbohydrate metabolism. For example, SREBP1c affects the mRNA levels of PEPCK, G6Pase,

and IRS-2 genes and inhibits the interaction between HNF4 and PGC1 α to suppress gluconeogenic genes (Lee et al., 2007; Yamamoto et al., 2004). Although it has been reported that hepatic SREBP1c suppresses hepatic glucose production, the molecular mechanism(s) by which SREBP1c could repress hepatic gluconeogenesis is unclear.

(3) Selective insulin resistance in liver

Under nutrient rich status, pancreatic β -cell secretes insulin, which is one of the major anabolic hormones in human body. Insulin accelerates glucose uptake in fat tissue and muscle as well as inhibits hepatic gluconeogenesis for lowering blood glucose level (Biddinger and Kahn, 2006). Moreover, insulin elevates lipogenic activity to store excess energy as triacylglyceride in liver and fat tissue (Kim et al., 1998a).

Type 2 diabetes are closely associated with hyperinsulinemia, hyperglycemia, and hypertriacylglyceridemia. Insulin resistance is one of the characteristics of type 2 diabetes. In insulin resistant animals, increased insulin is not enough to lower blood glucose, and consequently hyperglycemia reveals (Saltiel and Kahn, 2001). In diabetic subjects, liver, muscle, and adipose tissue are the major insulin resistant organs and the precise contributions of each organ to hyperglycemia and hypertriacylglyceridemia are unclear.

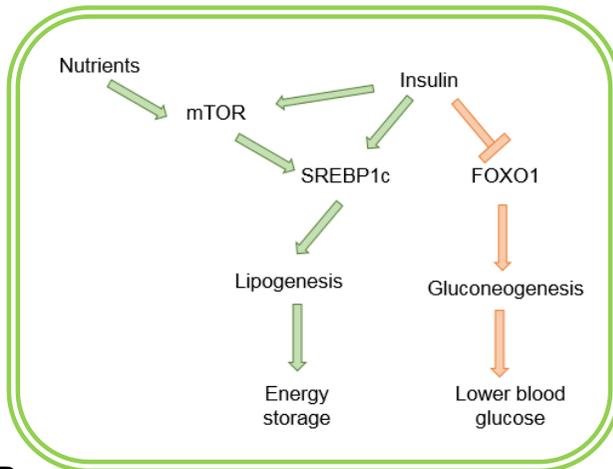
Upon feeding conditions, increased plasma insulin levels elicit two key actions at the level of gene expression, at least, in the liver. First, insulin activates

transcription factor SREBP1c which enhances transcription of genes required for fatty acid and triacylglyceride biosynthesis such as ACC and FASN (Kim et al., 1998a). In liver, the newly synthesized triacylglycerides are secreted in the form of very low density lipoproteins (VLDL), which are delivered to adipose tissues for storage and to muscles for fueling. The uptake of VLDL-derived fatty acids in adipose tissue is facilitated by insulin, which activates lipoprotein lipase on the surface of endothelial cells (Bourgeois et al., 1995; Lewis et al., 1994). Second, insulin stimulates the phosphorylation of FOXO1, a transcription factor that activates gluconeogenesis (Puigserver et al., 2003; Schilling et al., 2006; Xiong et al., 2013). Insulin-mediated FOXO1 phosphorylation prevents FOXO1 from entering the nucleus, which downregulates the expression of genes required for gluconeogenesis, including PEPCK and G6Pase. Blocking of hepatic glucose production by insulin is one of the crucial pathways to maintain blood glucose homeostasis. Interestingly, in diabetic liver, FOXO1 pathway becomes insulin resistant whereas SREBP1c-mediated lipogenesis shows insulin sensitive (Figure 4) (Brown and Goldstein, 2008). Despite extremely high insulin levels, the mRNAs of PEPCK and G6Pase remain high, and gluconeogenesis continues in diabetic liver. Moreover, since nuclear SREBP1c levels are high, fatty acid synthesis is accelerated and triacylglycerides are accumulated, leading to hepatic steatosis (Lee et al., 2014a). Further, elevated hepatic triacylglycerides are secreted via VLDL, raising plasma triacylglyceride levels in obese animals. Consequently, fatty acids derived from these triacylglycerides

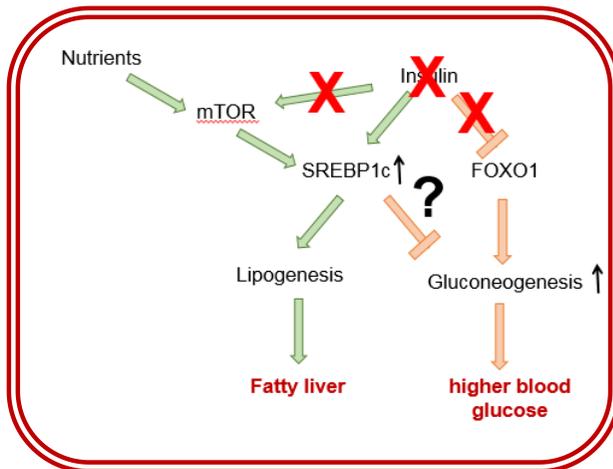
Figure 4. Selective insulin resistance in liver.

(A) Normal insulin response in liver. Insulin activates SREBP1c-mediated lipogenesis whereas inhibits gluconeogenesis. (B) Selective insulin resistance in liver of type 2 diabetes. Hyperinsulinemia still activates lipogenic action while is not able to suppress glucose production.

A Normal condition of insulin signaling



B Selective insulin resistance



aggravate insulin resistant state in muscle and adipose tissue, and the net results become hyperglycemia, hyperinsulinemia, and hypertriacylglyceridemia in type 2 diabetic animals (Figure 4).

An understanding the selective insulin resistance in diabetic liver is important for providing appropriate approaches to type 2 diabetes. Although it has been reported that hepatic SREBP1c is upregulated in obese animals, it is unknown why increased SREBP1c appears to fail to repress hepatic gluconeogenesis. Thus, it is crucial that understanding the molecular mechanisms by which SREBP1c could modulate gluconeogenesis under physiological and pathological conditions.

3. Purpose of this study

Circadian clock has a close correlation with metabolic regulation. Feeding is a major trigger that modulates the peripheral circadian clock while light activates the central circadian clock. These biological clocks are synchronized and synergically regulates various whole-body homeostasis including metabolism, body temperature, and sleep/wake cycle. Emerging evidence for the roles of circadian clock in metabolic tissues, such as adipose tissue, liver and muscle, proposes that the circadian clock actively interacts with metabolic signaling pathway to maintain the whole-body biological clock and energy homeostasis.

In this study, I have focused on body weight change according to feeding period restriction to investigate whether the restriction of feeding periods may affect body weight gain upon NCD and HFD. Whether the modulated peripheral circadian clock or the different amounts of food intake might be the primarily cause of obesity in shift workers is unclear. I have demonstrated that body weight gain in mice is not significantly changed by restricting feeding period to daytime or to nighttime if the animals take same calorie intake. On the contrary, the expression of peripheral circadian clock genes was altered by feeding period restriction, while the expression of light-regulated hypothalamic circadian clock genes was unaffected by both a NCD and HFD. The expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation related genes in the liver was also altered by feeding period restriction. Given that feeding period restriction does not affect body weight gain with both a NCD and HFD, it is likely that the amount of food consumed might be a crucial factor in determining body weight. In the second part, I have aimed to find out the new target gene of SREBP1c to find the signaling pathway that suppresses the gluconeogenesis. Although SREBP1c suppresses hepatic glucose production, the underlying molecular signaling pathway is unclear. To find out missing link between SREBP1c and inhibition of gluconeogenesis, I discovered CRY1 as a new target gene of SREBP1c. With fasting and refeeding experiment, I have shown that CRY1 is increased by feeding and insulin, which suppresses hepatic glucose production by FOXO1 degradation. Taken together, I would like to propose that the interpretation

of the relationship between circadian clock and hepatic glucose/lipid metabolism is important for understanding the pathophysiology of obesity and diabetes.

CHAPTER ONE:

**Feeding period restriction alters expression of
peripheral circadian rhythm genes without body
weight change in mice**

Abstract

Accumulating evidence suggests that circadian clock is closely associated with metabolic regulation. However, it has not been clearly understood whether impaired circadian clock is a direct cause of metabolic dysregulation including body weight gain. In this study, I demonstrate that mice body weight gain is not significantly changed by feeding period restriction; ad libitum, day time feeding and night time feeding. Expression of peripheral circadian clock genes was altered by feeding period restriction, while that of light-regulated hypothalamic circadian clock genes was not affected with either normal chow diet (NCD) or high fat diet (HFD). In liver, the expression pattern of circadian clock genes including BMAL1, CLOCK, and PER2 was changed by different restrictions of feeding period. Moreover, the expression of lipogenic genes, gluconeogenic genes, and fatty acid oxidation-related genes was also altered in liver by feeding period restriction. Taken together, these data suggest that restriction of feeding period would modulate expression of peripheral circadian clock genes, which is uncoupled from light-sensitive hypothalamic circadian clock genes, even though feeding period restriction might not be a crucial factor to affect body weight gain under NCD or HFD, implying that the amounts of energy intake seems to be a crucial factor to determine body weight.

Introduction

Various physiological and behavioral oscillations such as sleep-wake cycles, body temperature, blood pressure, and hormone secretion are associated with circadian clock (Bass and Takahashi, 2010). Circadian oscillation is composed of auto-regulatory negative feedback loops; BMAL1, CLOCK, PER, and CRY are key circadian transcription factors that determine rhythmic oscillation in a cell-autonomous manner. BMAL1 and CLOCK play a key role to induce PER and CRY. Then, elevated PER and CRY form a transcriptional repressor complex to suppress BMAL1 and CLOCK, which eventually leads to negative feedback regulation. In addition to the PER and CRY targets, BMAL1 and CLOCK also activate mRNA level of Rev-erb α and ROR α , which thereafter compete for binding to the retinoic acid related orphan receptor response elements (ROREs) as to repress or activate expression of Bmal1, respectively. This alternating promoter occupancy is due to rhythmic expression of Rev-erb α (Green et al., 2008). In addition, these auto-regulatory loops are modulated by various post-translational modifications such as phosphorylation, sumoylation, acetylation, and ubiquitination (Lamia et al., 2009; Nakahata et al., 2009; Shirogane et al., 2005).

Circadian clock exists in the hypothalamic suprachiasmatic nucleus (SCN) as well as peripheral tissues including liver and fat (Bass and Takahashi, 2010). SCN circadian oscillation is primarily regulated by light signal whereas peripheral circadian oscillation is affected by food intake accompanied by hormones such as

insulin and glucagon (Green et al., 2008). Emerging evidences have suggested that circadian clock is deeply associated with whole-body energy homeostasis. For instance, CLOCK defective mice exhibit obesity and hyperphagia with disrupted circadian oscillation (Turek et al., 2005). High fat diet (HFD) fed wild type mice become obese and show altered expression of circadian clock genes with metabolic dysregulation (Kohsaka et al., 2007). Further, liver specific Bmal1 knockout mice lose their hepatic glucose homeostasis (Canaple et al., 2006). Moreover, key metabolic genes such as PPAR α , PPAR γ , and AMPK are involved in the regulation of circadian genes, and circadian clocks in turn modulate whole-body energy metabolism (Canaple et al., 2006; Lamia et al., 2009; Wang et al., 2008b). Under physiological conditions, SCN clock and peripheral clock are synchronized by light-dark cycle and feeding-fasting cycle. Nonetheless, it has been also demonstrated that disharmonious signaling of these two cues, light-dark cycle and feeding-fasting cycle, independently regulates each circadian oscillation (Damiola et al., 2000). Although it appears that there is a close relationship between circadian clock and metabolic regulation, the effects of unsynchronized circadian clocks in SCN and peripheral tissues on metabolic regulation are largely unknown. In addition, it is unclear whether alteration of feeding periods would be a major determinant of body weight change even when there is no change of calorie intake.

In this study, I have investigated whether the restriction of feeding periods may affect body weight gain upon NCD and HFD. Also, I have analyzed gene

expression profiles of circadian clocks from SCN and peripheral tissue under different feeding periods. Our data suggest that feeding period restriction would not influence body weight gain whereas it would differently regulate circadian oscillations in peripheral tissues but not in SCN.

Methods

Animal care and experimental protocol

Four-week-old male C57BL/6N mice were obtained from SAMTAKO BIO KOREA Co., Ltd. Mice were maintained according to the guidelines of Seoul National University Animal Experiment Ethics Committee. They were housed in individual cages for pair feeding in 12 hr light/ 12 hr dark cycles. After a minimum 1-week stabilization period, ad libitum group mice (5 weeks old) were exposed to food freely, and the night time fed mice were pair fed to match the amount of food of day time group with NCD and 60% HFD (Research Diets, Inc.) for 4 weeks (Figure 5). Only those pair-fed mice (night time fed) with similar body weights as those of day time fed mice were subjected to the procedure from 5 weeks of age to 9 weeks of age. Body weight and food intake were measured daily at ZT0 (7 a.m.) and ZT12 (7 p.m.) during the experimental protocol. The average initial body weights in each group of mice were not different. All mice were euthanized, and dissected tissue specimens were immediately stored at -80C until analysis.

Quantitative real-time RT-PCR analysis

Total RNAs were isolated from liver and hypothalamus as described previously (Choe et al., 2007), and cDNA was synthesized using the M-MuLV reverse

transcriptase kit (Fermentas, Glen Burnie, MD). The primers used for the real-time PCR analyses were produced in Bioneer (Korea). The primer sequences used for real-time PCR analyses are provided in supplementary Table 1.

Biochemical analysis

The levels of plasma cholesterol and triacylglycerides were measured using Infinity reagents (Thermo, Melbourne, Australia). Plasma glucose levels were measured with a freestyle blood glucose meter (Therasense; Uppsala, Sweden).

Table 1. q-RT PCR primer sequence.

Gene	Primer Sequence
Clock	5'-TTGCGTCTGTGGGTGTTG-3' 5'-TGCTTTGCCTTGTCATCTTCT-3'
Bmal1	5'-AACCTTCCCGCAGCTAACAG-3' 5'-AGTCCTCTTTGGGCCACCTT-3'
Per2	5'-TGTGCGATGATGATTCGTGA-3' 5'-GGTGAAGGTACGTTTGGTTTGC-3'
TBP	5'-GGGAGAATCATGGACCAGAA-3' 5'-CCGTAAGGCATCATTGGACT-3'
Srebp1c	5'-GGAGCCATGGATTGCACATT-3' 5'-CAGGAAGGCTTCCAGAGAGG-3'
Fasn	5'-GCTGCGGAACTTCAGGAAAT-3' 5'-AGAGACGTGTCACCTCCTGGACTT-3'
G6pase	5'-ACACCGACTACTACAGCAACAG-3' 5'-CCTCGAAAGATAGCAAGAGTAG-3'
Pepck	5'-AAAAGCCTTTGGTCAACAAC-3' 5'-AAACTTCATCCAGGCAATGT-3'
Cpt1	5'-ACTCCTGGAAGAAGAAGTTCAT-3' 5'-AGTATCTTTGACAGCTGGGAC-3'
Ppara	5'-ATGCCAGTACTGCCGTTTTTC-3' 5'-GGCCTTGACCTTGTTTCATGT-3'

Result

Feeding period restriction does not change body weight gain

To address the question whether body weight gain is sensitively altered by feeding behavior, accompanied with changes of circadian clock genes, I have investigated the effects of feeding period restriction on body weight. Restriction of feeding period has been designed with the following three groups: 1) ad libitum in which mice were freely exposed to food, 2) restriction feeding to day time (RF Day), in which mice could access food only in day time, and 3) pair-feeding in night time (PF Night), in which mice were given the same amount of food as the RF Day group only in night time (Figure 5). Since mice are nocturnal animals that would take most food at night time than during day time, I measured the amount of food intake in day time, and the same amount of food was given to night time feeding (PF Night) group. I have designed the pair-feeding group to investigate the effect of feeding period variation on body weight with the same amount of calorie intake. Moreover, mice were fed with either normal chow diet (NCD) or high fat diet (HFD) in order to test the effects of different nutrition sources on body weight gain upon feeding period restriction.

Interestingly, I observed that RF Day and PF Night groups revealed a similar pattern of body weight gain, regardless of diet source (NCD or HFD) (Figure 6A, 6B, 6D, 6E). Compared to restricted feeding groups such as RF Day and PF Night,

Figure 5. Feeding period restriction scheme for the three feeding groups in this study.

The ad libitum (AL) group was freely exposed to food; the feeding restricted to day time (RF Day) group could access food only during daytime; and the pair-feeding at night (PF Night) group was given the same amount of food as the RF Day group but only at night. Mice were fed with a normal chow diet (NCD) or a high-fat diet (HFD). The light was turned on at ZT0 and turned off at ZT12. At ZT2 and ZT14, mice were sacrificed to prepare tissues and harvest blood samples.

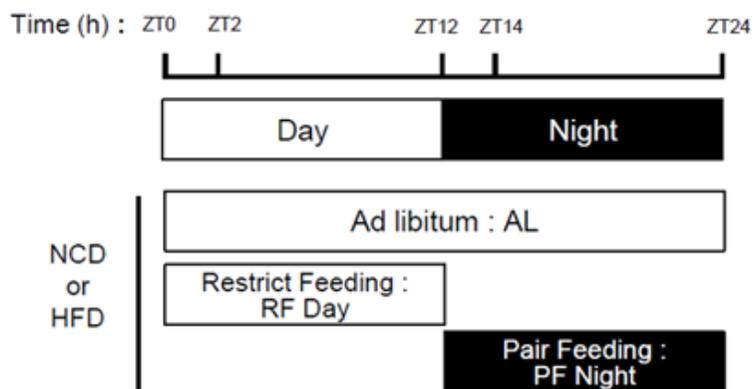
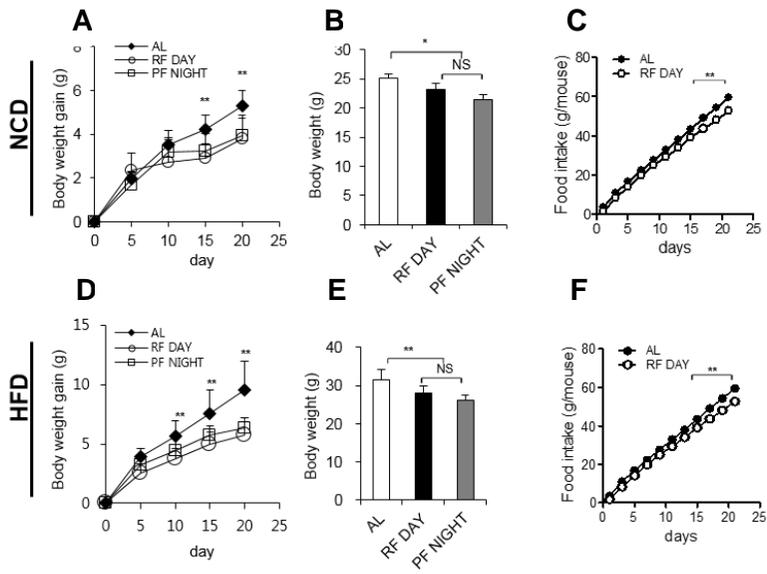


Figure 6. Feeding period restriction does not change body weight gain.

(A and B) Body weight gain or total body weight of AL, RF Day, and PF Night NCD-fed mice. (C) Total food intake in NCD-fed AL and RF Day mice. (D and E) Body weight gain or total body weight of AL, RF Day, and PF Night HFD-fed mice. (F) Total food intake in HFD-fed AL and RF Day mice. Each bar represents mean \pm SD of each group of mice (n=6), *P<0.05, **P<0.01.



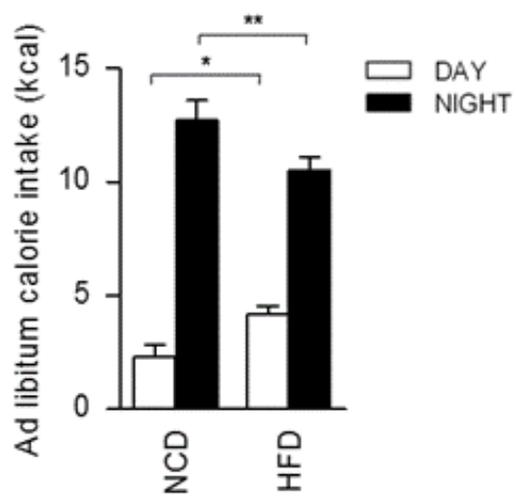
body weight gain of ad libitum group was significantly increased with either NCD or HFD (Figure 6A, 6B, 6D, 6E). When the amounts of cumulative food intakes were measured, ad libitum group gradually consumed more food than RF Day or PF Night group (Figure 6C, 6F). Recently, it has been reported that circadian clock is disrupted by HFD (Kohsaka et al., 2007). Thus, I examined food intake patterns during day time (ZT0-ZT12) and night time (ZT12-ZT24) time periods in ad libitum group. Expectedly, mice ate more food during night time period than day time periods (Figure 7). Interestingly, it seemed that HFD fed mice slightly, but substantially, consumed more food in day time period than NCD fed mice (Figure 7). These data suggest that the amount of food intake rather than feeding periods would be a major determinant of body weight gain, which is both applicable in different nutrition sources such as NCD and HFD.

Day time feeding changes the expression of circadian clock genes in liver but not in hypothalamus

I next investigated the expression of circadian clock genes upon feeding period restriction. In order to examine the expression profiles of circadian clock genes, mice were sacrificed at ZT2 and ZT14, which may reflect different expression pattern of circadian clock genes in 12:12 light-dark cycle. Total RNAs were isolated from liver and hypothalamus as representative peripheral clock and central clock tissues,

Figure 7. Ad libitum calorie intake during day and night with NCD and HFD feeding.

Each bar represents mean \pm SD of each group of mice (n=6), *P<0.05, **P<0.01.



respectively, and analyzed by qRT-PCR. As shown in Figure 8 and 9, the expression patterns of circadian clock genes such as BMAL11, PER2 and CLOCK were altered by feeding period restrictions in liver but not in hypothalamus with either NCD or HFD. Overall, ad libitum group and PF Night group showed the similar expression patterns of circadian clock genes in liver, which would be resulted from the behavior of nocturnal mice that took most food during night time. In contrast, in liver, RF Day group revealed distinct expression pattern of circadian genes from either ad libitum or PF Night group. Unlike liver, the expression patterns of hypothalamic circadian genes were not changed by feeding period restrictions with either NCD or HFD (Figure 8, 9). These data strongly indicate that feeding period restriction would influence expression of circadian clock genes in peripheral tissues, probably via modulating nutritional hormones such as insulin or glucagon, which may not affect expression of circadian clock genes in hypothalamus.

Feeding period restriction alters expression of metabolic genes and plasma metabolites

It is well known that feeding is one of important factors to regulate circadian clock genes as well as hepatic lipid and glucose metabolism (Kohsaka et al., 2007; Lamia et al., 2008). Given that RF Day changed expression patterns of hepatic circadian clock genes, I decided to examine whether RF Day might also influence expression of metabolic genes in liver. I analyzed hepatic gene expression via qRT-

Figure 8. Daytime feeding changes expression of circadian clock genes in the liver but not in the hypothalamus in NCD.

Hepatic and hypothalamic BMAL1, PER2, and CLOCK gene expression profiles in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<,0.05, **P<0.01.

NCD

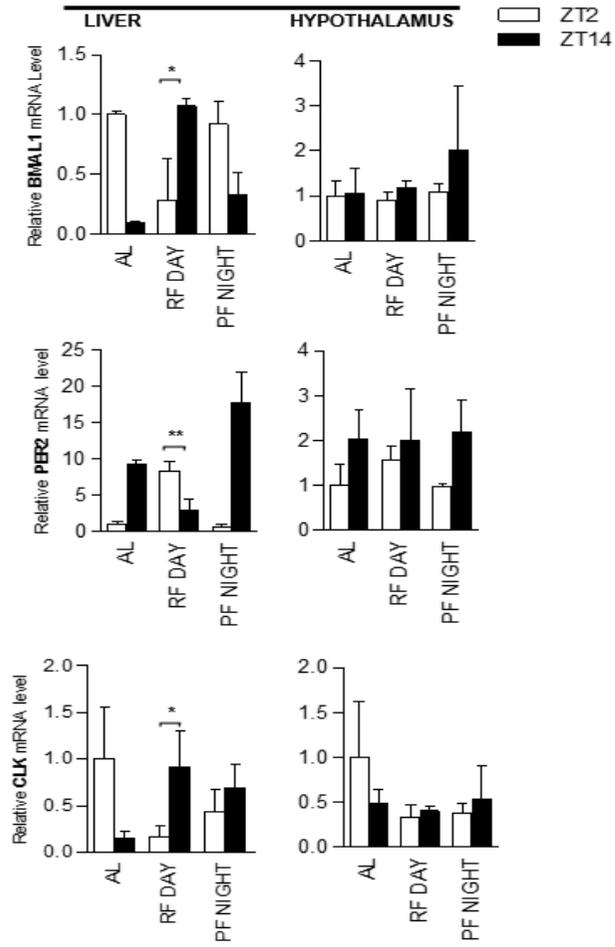
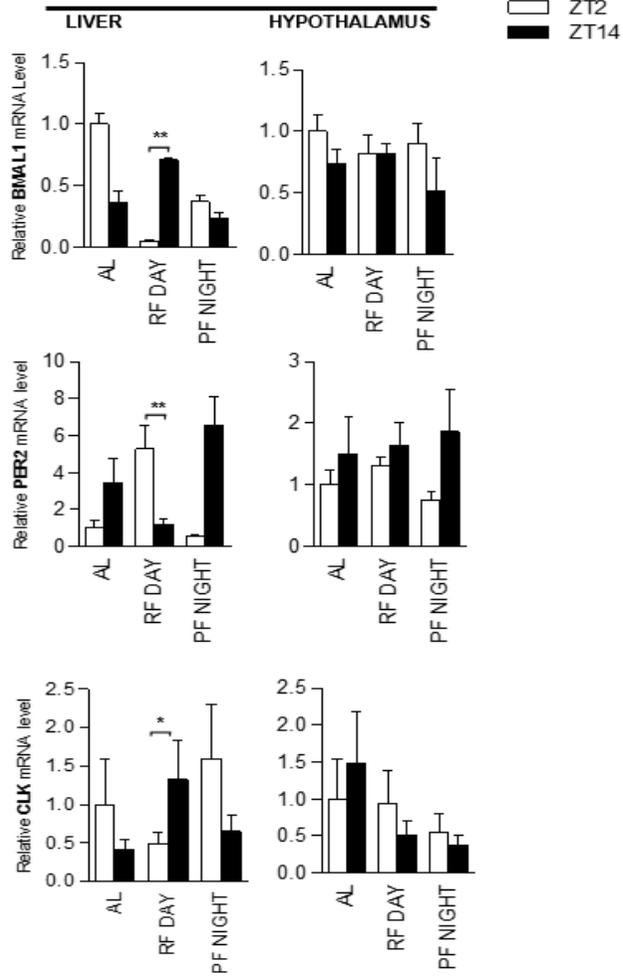


Figure 9. Daytime feeding changes expression of circadian clock genes in the liver but not in the hypothalamus in HFD.

Hepatic and hypothalamic BMAL1, PER2, and CLOCK gene expression profiles in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

HFD



PCR from NCD (Figure 10A, 10B, 10C) or HFD (Figure 11A, 11B, 11C) fed mice at ZT2 and 14. In order to conjecture key metabolic changes, I have investigated expression of several lipid and carbohydrate metabolism genes such as SREBP1c, FASN, PEPCK, G6PASE, PPAR α , and CPT1. Since SREBP1c is a master transcription factor for lipogenesis, it regulates the expression of *FASN* upon nutritional and hormonal changes (Kim et al., 1998a; Kim and Spiegelman, 1996; Kim et al., 1995; Kim et al., 1998b; Kim et al., 2004). Under NCD feeding, expression of SREBP1c mRNA was greatly suppressed in RF Day at ZT14, while it was up-regulated at ZT14 in PF Night (Figure 10A). Despite of this, FASN did not show significant change at ZT2 and 14 in RF Day and PF Night, implying that it may need several hours to reflect SREBP1c target gene expression *in vivo*. Upon HFD feeding, FASN was increased in RF Day at ZT2, while it was not altered in PF Night (Figure 11A). Conversely, the expression of gluconeogenic genes such as PEPCK and G6Pase, which are well known as fasting-induced genes, was not changed at ZT2 and 14 both in RF Day and PF Night in NCD (Figure 10B). When fatty acid oxidation genes such as PPAR α and CPT1 were analyzed, their mRNA levels were not significantly different in RF Day and PF Night groups (Figure 10C). These data propose that expression of several metabolic genes might to be partly altered by feeding period restriction but not as much as that of circadian clock genes in liver.

Since feeding period restriction altered expression of subset of metabolic genes in liver without body weight gain, I have examined serum metabolites,

Figure 10. Feeding period restriction changes the expression of metabolic genes in NCD fed mice.

(A) Expression profiles of lipogenic genes such as SREBP1c and FASN in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (B) Expression profiles of the gluconeogenic genes PEPCCK and G6Pase in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (C) Expression profiles of the fatty acid oxidation genes PPAR α and CPT1 in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

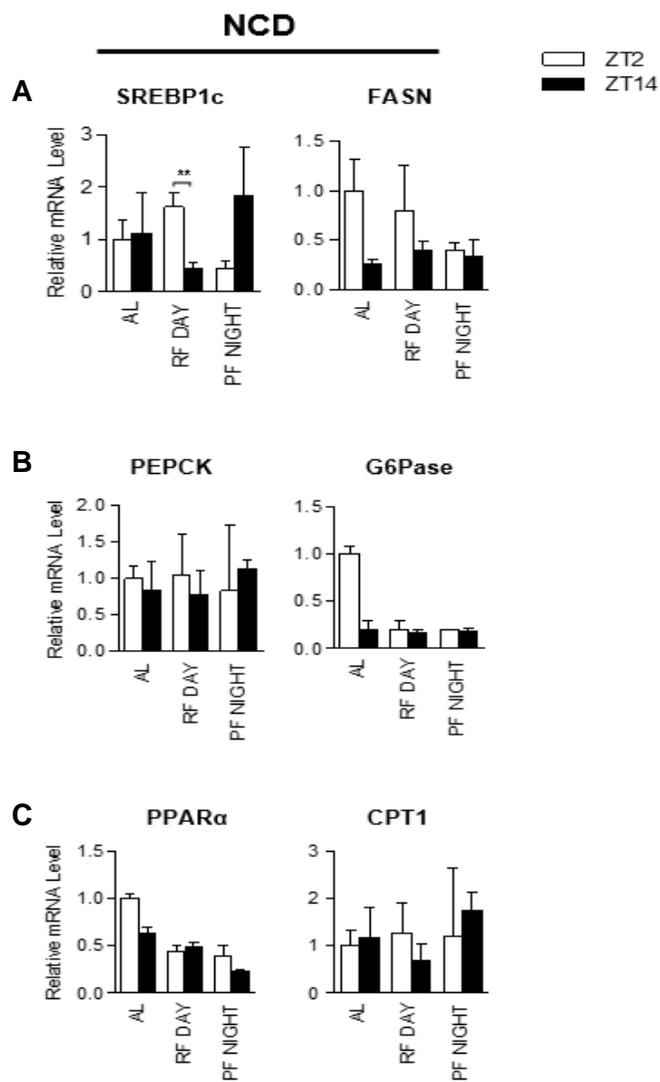
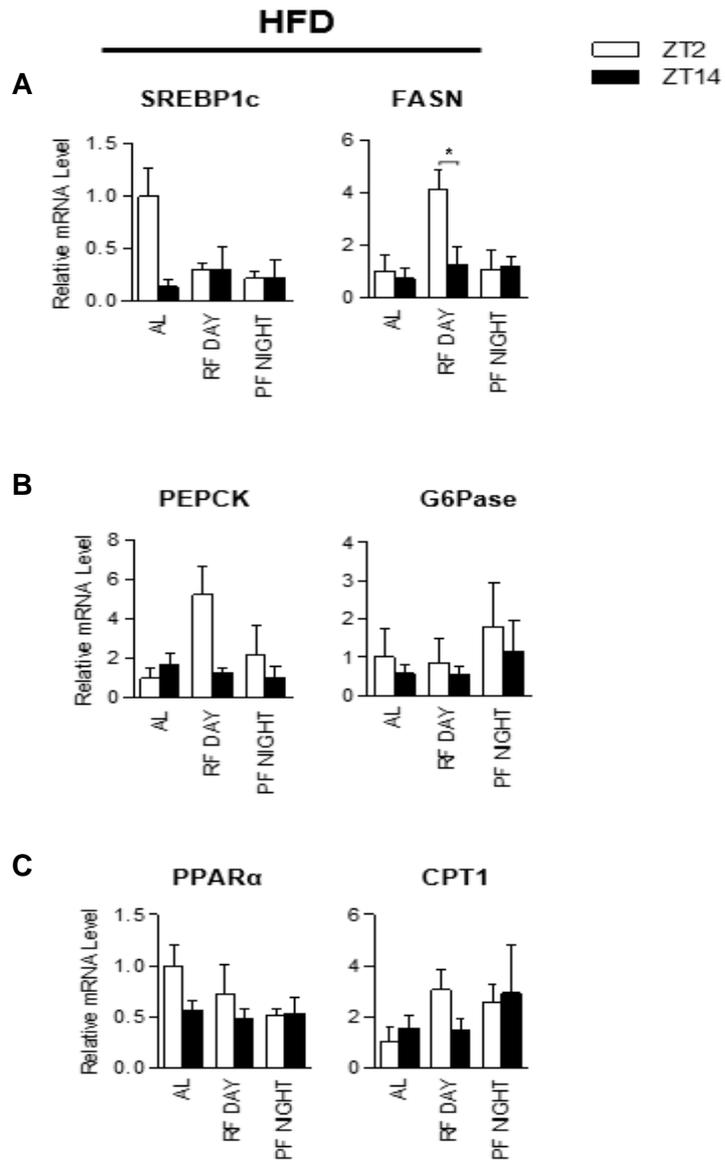


Figure 11. Feeding period restriction changes the expression of metabolic genes in HFD fed mice.

(A) Expression profiles of lipogenic genes such as SREBP1c and FASN in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. (B) Expression profiles of the gluconeogenic genes PEPCCK and G6Pase in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. (C) Expression profiles of the fatty acid oxidation genes PPARa and CPT1 in AL, RF Day, and PF Night HFD-fed mice at ZT2 and ZT14. All RNA samples are normalized to TBP mRNA. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

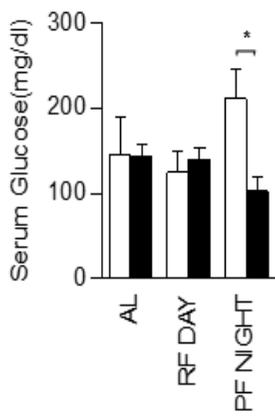


relevant to whole-body energy state, in different feeding period restriction groups. Although it has been reported that serum metabolite levels are varied throughout feeding-fasting cycle (Morris et al., 2012), our experimental windows limited at ZT2 and 14 did not sensitively reflect whole-body energy state. When I measured serum glucose, triacylglyceride (TG), and cholesterol levels at ZT2 and 14, I failed to observe distinct patterns of those metabolites. Nonetheless, in PF Night group, the levels of serum glucose, TG, and cholesterol were higher at ZT2 than at ZT14 under NCD, implying that there might be a correlation between peripheral circadian oscillation and serum metabolites (Figure 12A, 12B, 12C). On the contrary, HFD challenged animals exhibited disrupted serum metabolite profiles as previously reported (data not shown) (Hatori et al., 2012). These data indicate that feeding period restriction may modulate the profiles of several serum metabolites without changes in body weight gain, concomitant with alteration of circadian clock genes as well as metabolic genes.

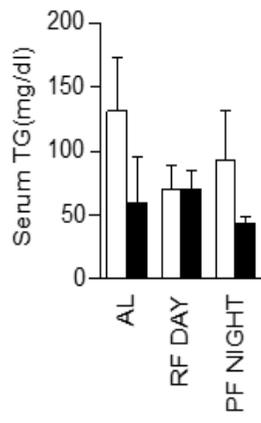
Figure 12. Feeding period restriction changes plasma metabolites.

(A) Serum glucose levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (B) Serum TG levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. (C) Serum cholesterol levels in AL, RF Day, and PF Night NCD-fed mice at ZT2 and ZT14. Each bar represents mean \pm SD of each group of mice (n=3), *P<0.05, **P<0.01.

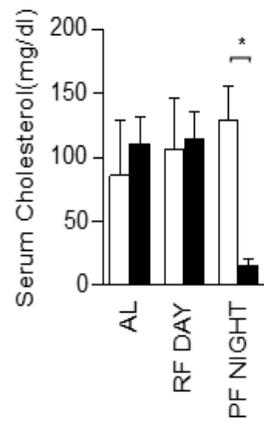
A



B



C



Discussion

Obesity is characterized by increase of adipose tissue and lipid metabolism, which is caused by chronic excess of energy intake than energy expenditure. Very recent reports have suggested that feeding period restriction would be an important factor to influence body weight gain, leading to obesity (Arble et al., 2009; Hatori et al., 2012). Since Arble et al. have reported that day time HFD-fed mice were more obese than night time HFD-fed mice with no differences in calorie intake and activity (Arble et al., 2009), it has been proposed that change of feeding behavior by shifting from night to day time would be an important factor to regulate energy metabolism contribute to. In accordance with this, it has been reported that night-eating syndrome in human might lead to weight gain as a result of excess calories consumption at night (Aronoff et al., 2001; Colles et al., 2007; Tholin et al., 2009). On the contrary, several studies have revealed that there is no correlation of night eating syndrome and body weight gain in human (Gluck et al., 2008; Striegel-Moore et al., 2008; Striegel-Moore et al., 2004). Nonetheless, it has not been clearly understood whether dysregulation of any circadian clock gene expression is an essential factor to alter body weight gain.

In the present work, I have investigated the relationship between body weight gain and feeding period restriction by providing the same amount of food at either night time or day time. I observed that feeding period restriction evidently altered expression pattern of peripheral circadian clock genes, provably via nutrition sensitive hormones. However, the expression of central circadian clock genes in

hypothalamus was not modulated by feeding restriction regardless of NCD or HFD. These data implied that central circadian clock and peripheral circadian clock would be independently regulated, at least, at transcriptional level, upon feeding period restriction (Damiola et al., 2000). Furthermore, hepatic expression of metabolic genes including lipogenesis, gluconeogenesis, and fatty acid oxidation were modulated upon feeding period restriction. Similarly, the levels of serum glucose, triacylglyceride, and cholesterol were also adjusted by feeding period restriction. Despite these changes of peripheral metabolic gene expression, there were no significant body weight differences between RF Day and PF Night groups with NCD or HFD, indicating that the change of peripheral circadian clock genes may not be a key factor to influence body weight. Instead of feeding behavior, it appears that the amount of energy intake would be a crucial factor to determine body weight, which might be closely linked with central circadian genes.

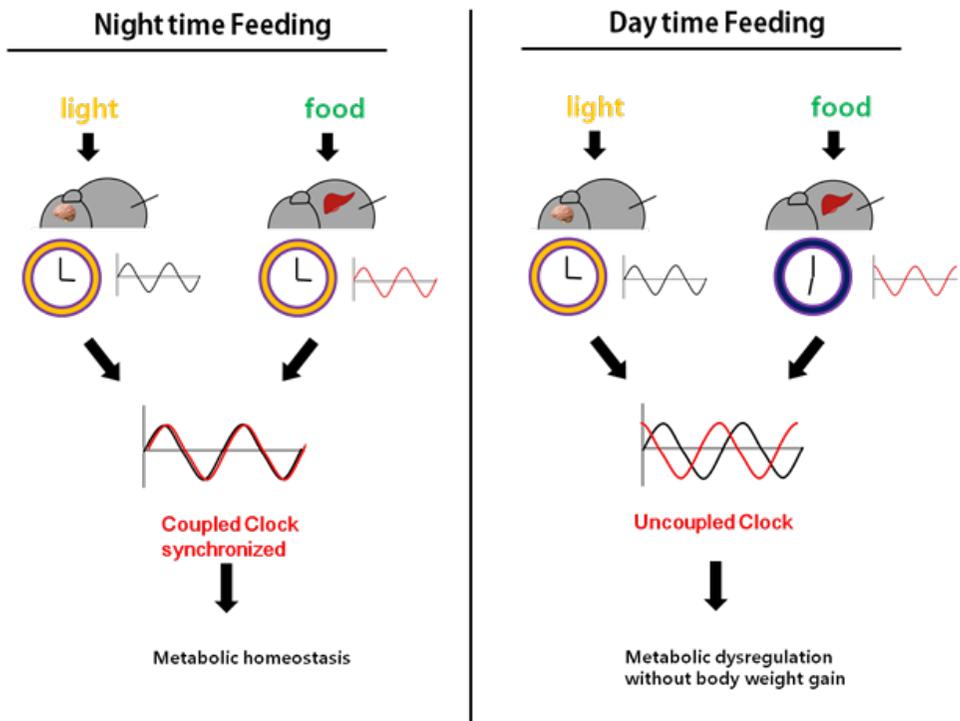
It has been reported that, HFD challenge disrupts behavioral and physiological circadian rhythms. For instance, HFD leads to alterations in the period of the locomotor activity and canonical circadian clock genes (Wang et al., 2008b). In this study, I observed that HFD fed mice exhibited distinct expression patterns of subset genes in liver, including circadian clock genes and metabolic genes, compared with NCD fed mice. In addition, ad libitum group exhibited different food consumption patterns depending upon nutrition source. HFD challenged mice consumed more food intake in day time than NCD challenged mice, implying that

food intake control would be dysregulated in HFD fed group. Since fasting period of HFD group might be shorter than that of NCD fed group under ad libitum (Kohsaka et al., 2007), it appears that NCD fed group might gain less body weight compared with HFD fed group through activation of fasting-induced signals with SIRT1 and AMPK, which mediated energy expenditure to burn extra energy (Canto et al., 2010; Kohsaka et al., 2007). Of course, I cannot rule out the possibility that physical activity or body temperature may contribute to compensate for body weight gain, which will be investigated in further study.

Disruption of circadian clock genes is exerted by various stimuli such as changes of light/dark cycle and food consumption. Interference of central circadian clock by light time modulation affects body weight gain (Karatsoreos et al., 2011). For instance, 10:10 light-dark (LD) cycle leads to body weight gain compared with 12:12 LD cycle, and 10:10 LD cycle regime increases several metabolic parameters such as plasma leptin, insulin, and glucose (Karatsoreos et al., 2011). Moreover, circadian clock defective mice such as clock gene mutant mice and HFD fed mice show irregular food intake pattern, accompanied with obesity due to increased food intake (Damiola et al., 2000; Turek et al., 2005). In contrast, *Bmal1* deficient mice show obese phenotype during 5 weeks of HFD, whereas they are no longer obese with 15 weeks of HFD (Hemmerlyckx et al., 2011). However, liver-specific *Bmal1* knockout mice also show more body weight gain compared with WT mice (Lamia et al., 2008).

Here, I revealed that feeding period restriction selectively alters expression patterns of peripheral circadian clock genes without body weight gain, concomitant with adjusted levels of metabolic genes and plasma profiles. Therefore, it is plausible to speculate that central circadian clock might play a key role to regulate body weight through regulation of energy intake and expenditure. Consistently, it appears the amounts of calorie intake would be a major factor to change body weight (Figure 13). On the contrary, peripheral circadian clock genes sensitively respond to changes of metabolic alterations upon nutrients and hormones. The exact molecular mechanism of central circadian clock to influence body weight gain remains to be elucidated. In conclusion, our data suggest that feeding period restriction would selectively modulate peripheral circadian clocks and metabolic regulation without a significant change in body weight.

Figure 13. Schematic diagram of the proposed model of chapter 1.



CHAPTER TWO:

**Hyperglycemia is exacerbated by dysregulation
of hepatic SREBP1c-CRY1 signaling pathway**

Abstract

SREBP1c is a key transcription factor of lipogenesis to store excess energy in postprandial state. Although SREBP1c appears to be involved in suppression of hepatic gluconeogenesis, the molecular mechanism(s) by which insulin-activated SREBP1c could repress hepatic gluconeogenesis is not thoroughly understood. Here, I demonstrate that CRY1 activation by insulin-induced SREBP1c led to decrease hepatic gluconeogenesis through FOXO1 degradation. In accordance with these, *SREBP1c*^{-/-} and *CRY1*^{-/-} mice showed higher blood glucose than wild type (WT) mice during pyruvate tolerance test, accompanied with enhanced expression of PEPCK and G6Pase genes. Moreover, CRY1 promoted degradation of nuclear FOXO1 by MDM2, one of the ubiquitin E3 ligases, by enhancing binding of FOXO1 and MDM2. Although hepatic SREBP1c failed to upregulate CRY1 expression in *db/db* mice, overexpression of CRY1 led to attenuate hyperglycemia through reduction of hepatic FOXO1 protein as well as gluconeogenic gene expression. Collectively, these data suggest that insulin-activated SREBP1c downregulates hepatic gluconeogenesis through CRY1-mediated FOXO1 degradation and thereby dysregulation of SREBP1c-CRY1 signaling cascade in liver could confer hyperglycemia in diabetic animals.

Introduction

Insulin, which is released from pancreatic β -cells, plays a key role in the maintenance of the whole-body energy homeostasis by actively regulating glucose and lipid metabolism. In the postprandial state, insulin lowers blood glucose by stimulating glucose uptake in adipose tissues and muscles as well as by inhibiting hepatic glucose production (Puigserver et al., 2003; Summers and Birnbaum, 1997). Moreover, in the liver, insulin stimulates the conversion of excess glucose into glycogen (glycogenesis) and triacylglyceride (lipogenesis) for the long term energy storage (Moller, 2001; Wang et al., 2013; Wong et al., 2009).

Suppression of hepatic gluconeogenesis by insulin is an important process to inhibit hyperglycemia. PEPCK and G6Pase are crucial enzymes that convert pyruvate to glucose, and their gene expression is regulated by several transcription factors such as Forkhead box O1 (FOXO1), cAMP Response Element-Binding protein (CREB), Hepatocyte Nuclear Factor 4 (HNF4), Glucocorticoid Receptor (GR), and Peroxisome proliferator-activated receptor Gamma Coactivator 1-Alpha (PGC1 α) (Mouchiroud et al., 2014; Sugden et al., 2010; Yoon et al., 2001). In the liver, FOXO1 is activated upon fasting and gets inactivated by feeding, which is one of the essential mechanisms by which insulin rapidly and efficiently represses hepatic glucose production during postprandial periods (Accili and Arden, 2004; Kops et al., 1999; Lu et al., 2012). After insulin treatment, FOXO1 protein is phosphorylated by AKT and then moves to the cytoplasm, resulting in the decrease of gluconeogenic

gene expression(Matsumoto and Accili, 2005). Although the translocation of hepatic FOXO1 from the nucleus to the cytoplasm is a well-defined mechanism mediating a quick decrease in glucose production by insulin, it is largely unknown how insulin endows a sustainable inhibition of hepatic gluconeogenesis during the postprandial state.

On the other hand, it has been proposed that SREBP1c might be involved in hepatic glucose metabolism. SREBP1c is a basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor that regulates de novo lipogenesis (Brown and Goldstein, 1997; Fajas et al., 1999; Moon et al., 2012; Tontonoz et al., 1993; Yokoyama et al., 1993). Activation of SREBP1c is mediated by AKT and mTORC1 upon insulin signaling (Laplante and Sabatini, 2010; Peterson et al., 2011). SREBP1c regulates lipogenic pathways by stimulating the expression of target genes such as those encoding fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1) and acetyl-coenzyme A carboxylase (ACC) (Horton et al., 2003; Kim et al., 1998a; Liang et al., 2002). In addition, SREBP1c appears to be involved in hepatic carbohydrate metabolism. For example, SREBP1c affects the mRNA levels of PEPCK, G6Pase, and IRS-2 genes and inhibits the interaction between HNF4 and PGC1 α to suppress gluconeogenic genes (BeCARD et al., 2001; Ide et al., 2004; Lee et al., 2007; Ponugoti et al., 2007; Yamamoto et al., 2004). Although it has been reported that hepatic SREBP1c is upregulated in obese animals, it is unknown how increased SREBP1c fails to repress hepatic gluconeogenesis. Thus, it remains crucial to understand the

molecular mechanisms by which SREBP1c could modulate gluconeogenesis under physiological and pathological conditions.

CRY1 is a member of the mammalian clock transcription-translation feedback loop that also includes CLOCK, BMAL1, PER1, PER2, and CRY2, to modulate rhythmic oscillations. CLOCK and BMAL1 form a heterodimer to activate PER and CRY genes and then elevated PER and CRY proteins act as transcriptional repressors that decrease the transcriptional activity of CLOCK and BMAL1 (Miyamoto and Sancar, 1998; Thresher et al., 1998; Vitaterna et al., 1999; Ye et al., 2014). The hepatic circadian clock is affected by food intake as well by the expression of hormones such as insulin and glucagon, whereas the suprachiasmatic nucleus circadian clock is controlled by the light-dark cycle (Green et al., 2008). Recently, it has been shown that hepatic circadian clock genes also contribute to glucose homeostasis. For example, hepatic CRY proteins modulate glucose production by inhibiting the glucagon receptor signaling pathway and binding to GR (Lamia et al., 2011; Zhang et al., 2010). In addition, an agonist of CRY proteins has been reported to repress the expression of hepatic gluconeogenic genes, such as PEPCK and G6Pase (Hirota et al., 2012). Furthermore, *BMAL1*^{-/-} mice exhibited disrupted hepatic glucose homeostasis (Rudic et al., 2004). However, the molecular mechanisms by which CRY1 could repress hepatic glucose production during the postprandial state remain to be elucidated.

The fact that SREBP1c downregulates hepatic gluconeogenesis prompted

us to investigate novel target genes of SREBP1c by comparing *SREBP1c^{+/+}* and *SREBP1c^{-/-}* mice. Here, I demonstrate that SREBP1c attenuates hepatic glucose production via activation of CRY1, eventually leading to degradation of the FOXO1 protein upon insulin signaling. While hepatic FOXO1 is rapidly translocated from the nucleus into the cytoplasm by AKT-mediated phosphorylation triggered by insulin, the SREBP1c-CRY1 signaling pathway durably represses the execution of gluconeogenic genes by decreasing nuclear FOXO1 protein for long term insulin action. Additionally, in the liver of diabetic *db/db* mice, overexpression of CRY1 lowers blood glucose, accompanied with attenuated gluconeogenic genes and FOXO1 protein. Together, our data suggest that insulin activates the SREBP1c-CRY1 signaling pathway, resulting in FOXO1 degradation mediated by MDM2, which is one of the crucial mechanisms to maintain hepatic glucose homeostasis.

Methods

Animals

C57BL/6 mice were purchased from SAMTACO (Seoul, South Korea) and housed in colony cages. *db/+* and *db/db* mice were obtained from Central Lab (Seoul, Korea). *SREBP1c*^{-/-} mice were generously provided from Dr. J. Horton at the University of Texas Southwestern Medical Center and bred in isolated cages. All animals were maintained under 12 h light/ 12 h dark cycle in a pathogen-free animal facility. Following dissection, mouse tissue specimens were immediately stored at -80 °C until further analysis. All experiments with mice were approved by the Institute of Laboratory Animal Resources at Seoul National University and the Institutional Animal Care and Use Committee at the University of North Carolina.

***In vivo* imaging system**

C57BL/6 mice were injected with adenoviruses encoding GFP (Ad-Mock), SREBP1c (Ad-SREBP1c) and G6Pase luciferase (Ad-G6Pase-luc) through the tail vein. After 5 days, adenovirus-infected mice were injected intraperitoneally with 100 mg/kg sterile firefly D-luciferin. After 5 min, mice were anesthetized and imaged using an IVIS 100 imaging system (Xenogen, Alameda, CA, USA) as described(Lee et al., 2014b).

Pyruvate tolerance tests

For the pyruvate tolerance test, mice were fasted for 16 h and then injected intraperitoneally with pyruvate (2 g/kg body weight for mice). Blood glucose levels were measured in tail vein blood samples at the indicated time points by using a Free-Style blood glucose meter (Therasense, Sweden).

Antibodies, chemicals and plasmids

MG132 was purchased from Calbiochem (San Diego, CA, USA). I used antibodies to the following proteins in our study: MYC, HA, FOXO1, phosphor-FOXO1(Ser256), AKT, and phosphor-AKT(Ser473) (all purchased from Cell Signaling Technology, Beverly, MA, USA), FLAG, ACTIN (Sigma-Aldrich, St. Louis, MO, USA), G6Pase, POLII, GFP (Santa Cruz Biotechnology Santa Cruz, CA, USA), GAPDH (LabFrontier Co., Ltd, Seoul, Korea), SREBP1 (BD Bioscience, San Jose, CA, USA), MDM2 (Abcam, Cambridge, MA, USA), and CRY1 (Alpha Diagnostic International Inc., San Antonio, Texas, USA). GFP-CRY1 was cloned into the pEGFP-N1 vector and FLAG-MDM2 was cloned into pCMV-3 FLAG. Mouse CRY1 promoter was cloned into the pGL3-basic vector.

Cell-based ubiquitination assays

COS-1 cells were transfected with plasmids encoding FOXO1 WT-MYC, nFOXO1 (ADA)-MYC, GFP-CRY1 (or FLAG-CRY1), FLAG-MDM2 and Ubiquitin-HA in the presence of 20 μ M MG132 for 4 h. Total cell lysates were prepared using the TGN buffer. FOXO1 WT-MYC and nFOXO1 (ADA)-MYC were

immunoprecipitated with an anti-MYC antibody (Cell Signaling Technology, USA), and after washing in the TGN buffer, proteins were separated by SDS-PAGE followed by western blotting analyses with an anti-HA antibody.

ChIP assays

Cross-linking and chromatin immunoprecipitation assays with H4IIE cells were performed as described previously (Sakai et al., 2003). Extracted proteins from total cell lysates were immunoprecipitated with anti-SREBP1 (BD Bioscience) or IgG (Santa Cruz) for 2 h. Precipitated DNA fragments were analyzed by PCR using primer sets that encompassed the proximal (-100 to +100 base pairs) region of the rat *CRY1* promoter and negative control (+9670 to +9890 base pairs) region. The sequences of ChIP assay primers were as follows: sense, 5'-GTCCGAGCCAGCGTAGTAAA, antisense, 5'- GGATAGCGCGGGCTAGAG; negative control primer sense, 5'-CCAGCCACTTTGCTGAAGTT and antisense, 5'-CTAGACAAGGCTGCCCACTC.

Preparation of recombinant adenovirus

The adenovirus plasmid was constructed as previously described (Lee et al., 2014a). rat *SREBP1c* and mouse *CRY1* cDNAs were incorporated into the AdTrack-CMV shuttle vector and a recombinant vector was generated using the Ad-Easy adenoviral vector system. Adenoviruses were amplified in HEK293A cells and isolated by cesium chloride density centrifugation. The GFP was co-expressed from an

independent promoter with inserted cDNA. For *in vivo* experiments, mice were injected with 5×10^9 PFU of adenovirus (*db/db* mice, 2×10^{10} PFU) in 200 μ l PBS through the tail vein. Empty virus expressing only the gene for GFP served as the control (Mock).

Mouse primary hepatocytes cultures

Mouse primary hepatocytes were isolated as previously described (Jo et al., 2013). For adenoviral infection, isolated hepatocytes were incubated for 12 h with adenovirus at 50 PFU/cell with the serum-free medium, which was subsequently replaced by the 10% FBS-containing M199 medium.

Cell lysis and immunoprecipitation

After washing in cold PBS, cells were treated either with the TGN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween-20 and 0.3% NP-40) or the SDS lysis buffer (200 mM Tris-HCl, pH 6.8, 10% glycerol and 4% SDS) supplemented with 0.1% protease inhibitor cocktail (Roche, Basel, Switzerland). Total cell lysates were obtained by centrifugation at 12,000 rpm for 15 min at 4 °C, and 1–1.5 mg of lysates was used for immunoprecipitation. The lysates were incubated with primary antibodies for 2 h at 4 °C, followed by 1 h of further incubation with 50% slurry of protein A sepharose presaturated with the lysis buffer. After washing three times with the lysis buffer, the immunoprecipitated proteins were recovered from the beads by boiling for 10 min in the sample buffer and analyzed by SDS-PAGE and

immunoblotting.

Transient transfection and luciferase assays

HEK293T cells were transiently transfected with various DNA plasmids using the calcium-phosphate method described previously (Seo et al., 2004). After incubation for 36 h, transfected cells were harvested with the lysis buffer (25 mM Tris-phosphate pH 7.8, 10% glycerol, 2 mM EDTA, 2 mM DTT and 1% Triton X-100) and the activities of luciferase and β -galactosidase were measured according to the manufacturer's protocol (Promega, Madison, WI, USA). The relative luciferase activity was normalized to β -galactosidase activity in each sample.

RNA preparation and q-RT-PCR analyses

RNA was prepared as previously described (Kim et al., 2010). Briefly, total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, equal amounts of RNA were subjected to cDNA synthesis using the RevertAid M-MuLV reverse transcriptase (Fermentas, Canada). The relative amount of mRNA was evaluated by using a CFX real-time quantitative PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) and calculated following normalization to the level of TBP or cyclophilin mRNA. The primer sequences used for the real-time quantitative PCR analyses are described in the Supplementary Table 2.

siRNA transfection

Table 2.q-RT PCR primer sequence

Primer	Sequences
mrSREBP1c	Sense 5'-GGAGCCATGGATTGCACATT Antisense 5'-CAGGAAGGCTTCCAGAGAGG
mrCRY1	Sense 5'-CGTTTGGAAAGGCATTTGG Antisense 5'-CTTCCATTTGTCAAAGCGTG
mrPEPCK	Sense 5'-AGCCTTTGGTCAACAACCTGG Antisense 5'-TGCCTTCGGGGTTAGTTATG
mrG6Pase	Sense 5'-ACACCGACTACTACAGCAACAG Antisense 5'-CCTCGAAAGATAGCAAGAGTAG
hmrFOXO1	Sense 5'-CCAAGGCCATCGAGAGC Antisense 5'-GATTGAGCATCCACCAAGAACT
hmFASN	Sense 5'-GCTGCGGAAACTTCAGGAAAT Antisense 5'-AGAGACGTGTCACTCTGGACTT
mbmal1	Sense 5'-AACCTTCCCGCAGCTAACAG Antisense 5'-AGTCTCTTTGGGCCACCTT
mCLOCK	Sense 5'-TTGCGTCTGTGGGTGTTG Antisense 5'-TGCTTTGTCCTTGTCATCTCT
mPER2	Sense 5'-TGTGCGATGATGATTTCGTGA Antisense 5'-GGTGAAGGTACGTTTGGTTTGC
hmrCRY2	Sense 5'-GGAGCTGCCCAAGAAGC Antisense 5'-AGTAGAAGAGGCGGCAGGA
mSCD1	Sense 5'-CCGGAGACCCCTTAGATCGA Antisense 5'-TAGCCTGTAAAGATTTCTGCAAACC
mELOVL6	Sense 5'-TGCCATGTTTCATCACCTTGT Antisense 5'-TACTCAGCCTTCGTGGCTTT
mTBP	Sense 5'-GGGAGAATCATGGACCAGAA Antisense 5'-CCGTAAGGCATCATTGGACT

siRNA duplexes for *CRY1*, *MDM2*, and *FOXO1* were purchased from the Bioneer Inc. (Daejeon, South Korea). Primary hepatocytes were transiently transfected with the Lipofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequence information for siRNA is described in the Supplementary Table 3.

Glucose production assays

Glucose production by mouse primary hepatocytes was measured according to the manufacturer's protocol using a glucose oxidase assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the cells were incubated for 6 h at 37 °C and 5% CO₂ in the Krebs-Ringer buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 10 mM lactate and 2 mM pyruvate, pH 7.4). The glucose production assays were performed in triplicate.

Statistical analysis

Data were compared using a paired Student's *t* test or analysis of variance as appropriate and are represented as the mean ± standard deviation (SD). Values of *P* < 0.05 were considered to indicate statistically significant differences.

Table 3. siRNA sequence

Primer	Sequences
Rat FOXO1	5'-GAAUGAAGGAACUGGGAAA
Mouse CRY1	5'-CCUCGCAACUGAAGUUGGU
Mouse MDM2	5'-CAGAGAAUGAUGGUAAAGA
Negative Control	5'-CCUACGCCACCAAUUUCGU

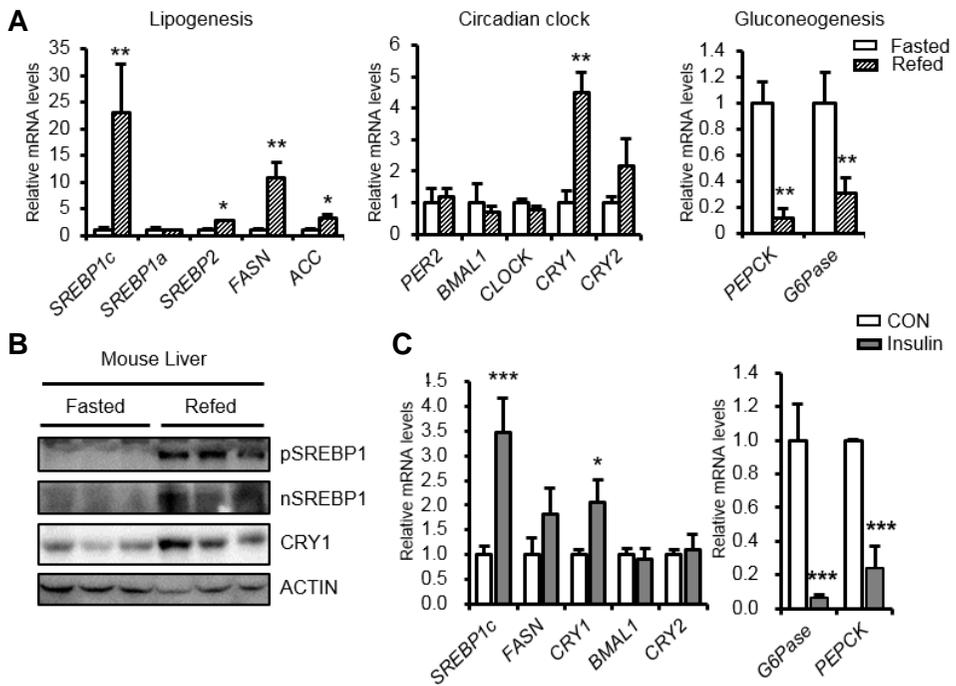
Results

CRY1 is promoted by feeding and insulin

In the liver, peripheral circadian clock genes are regulated by various nutritional and hormonal changes (Jang et al., 2012). Given that circadian clock genes are closely associated with energy homeostasis, I sought to investigate which circadian clock genes could affect feeding-dependent hepatic lipid and glucose metabolism. Since the expression of most circadian clock genes is oscillated in a time dependent manner, the end points of the fasting and/or refeeding experiment were fixed at ZT 3. Upon refeeding, the expression of most lipogenic genes including SREBP1c, FASN, and ACC was upregulated in the liver. In accordance with previous reports (McNeill et al., 1982; Oh et al., 2013; Pilgis et al., 1988), the expression of gluconeogenic genes such as *PEPCK* and *G6Pase* was downregulated in the postprandial state (Figure 14A). Interestingly, the level of hepatic CRY1 mRNA was selectively elevated in refeed mice, while the expression of other circadian clock genes such as PER2, CRY2, CLOCK, and BMAL1 was not significantly altered after nutritional changes (Figure 14A). Moreover, the expression of the CRY1 protein was markedly increased in the liver of refeed mice (Figure 14B). These findings prompted us to test whether insulin might elevate hepatic CRY1 gene expression. In primary hepatocytes, the level of CRY1 mRNA was increased by insulin, similar to SREBP1c mRNA (Figure 14C). These data indicate that hepatic CRY1 expression is upregulated by feeding and insulin, therefore this protein may participate in insulin-

Figure 14. CRY1 is stimulated by feeding and exposure to insulin

(A and B) *C57BL/6* mice were fasted for 24 h and then refed for 12 h. Both fasted and refed mice were sacrificed at ZT3. In the liver, levels of the CRY1 mRNA (A) and CRY1 protein (B) were determined using qRT-PCR with normalization to TBP mRNA levels and western blotting, respectively. Data are represented as mean \pm SD, $N=4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (C) CRY1 gene expression was measured in mouse primary hepatocytes following 12 h of insulin exposure and in control conditions using qRT-PCR. The level of the TBP mRNA was used for the qRT-PCR normalization. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test)



dependent energy metabolism.

SREBP1c regulates CRY1 gene expression

To investigate which transcription factors regulate insulin-dependent CRY1 gene expression, I analyzed *CRY1 promoters* in several species including monkey, cow, sheep, human, rat and mouse (Figure 16A). In the proximal CRY1 promoter, there are several sterol regulatory element (SRE) motifs as well as an E-BOX (CANNTG) motif, which is also a target motif for BMAL1 and CLOCK, the core circadian clock proteins (Figure 16A). Both SRE and E-BOX motifs are well known binding targets of SREBP1c with its dual DNA binding specificity (Griffin et al., 2007; Kim et al., 1995). To examine whether SREBP1c could regulate CRY1 gene expression, SREBP1c was overexpressed in mouse primary hepatocytes. As shown in Figure 15A and 15B, the levels of hepatic CRY1 mRNA and CRY1 protein were increased by SREBP1c overexpression (Figure 15A, 15B), implying that SREBP1c may be a key transcription factor that upregulates hepatic CRY1 gene expression in the postprandial state. Next, the effect of ectopic expression of SREBP1c on the CRY1 promoter activity was examined. Expression of luciferase from a wild-type CRY1 promoter was compared with expression from a promoter with mutated SRE motifs (3XSRE) in HEK293T cells (Figure 16B). I observed substantial loss of promoter activity with loss of the 3XSRE sequences but not E-BOX motif sequences

Figure 15. SREBP1c directly activates CRY1 gene expression

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-SREBP1c, as indicated. The levels of the CRY1 mRNA (A) and CRY1 protein (B) were determined using qRT-PCR with normalization to TBP mRNA levels and western blotting, respectively. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's t -test).

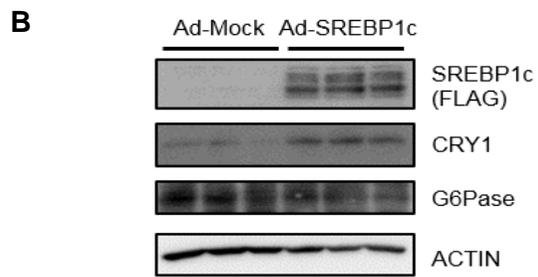
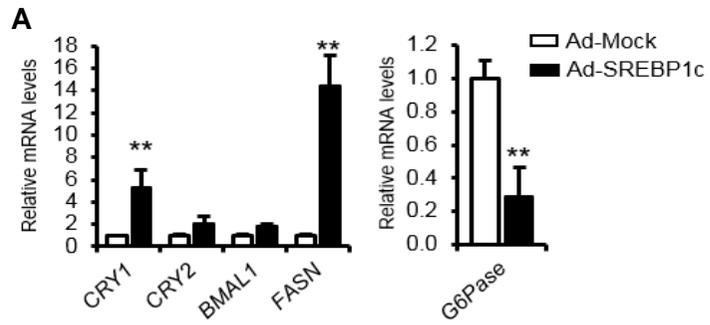


Figure 16. SREBP1c binds to SRE sequence in CRY1 promoter.

(A) SRE motifs and E-BOX sequences in the CRY1 promoter from various species.

(B) Luciferase activity of the WT CRY1 promoter and 3XSRE mutant promoter were measured following co-transfection with expression plasmids encoding either SREBP1c or Mock in HEK293T cells. Luciferase activity was normalized by β -gal activity. Data are represented as mean \pm SD, $N=5$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's t -test).

(C) HEK293T cells were co-transfected with a reporter plasmid containing the WT or E-BOX mutant mouse CRY1 promoter along with expression plasmids encoding either Mock or SREBP1c. The values represent the mean \pm SD ($N=4$ for each group). * $P < 0.05$, ** $P < 0.01$ (Student's t -test).

(Figure 16B, 16C). In addition, SREBP1c binding to the CRY1 promoter was confirmed by a ChIP assay (Figure 17). Meanwhile, consistent with previous reports (Becard et al., 2001; Ide et al., 2004; Lee et al., 2007), hepatic SREBP1c reduced G6Pase expression (Figure 15A, 15B).

To verify whether SREBP1c could modulate hepatic CRY1 gene expression *in vivo*, SREBP1c adenovirus was injected into mice via the tail vein and hepatic gene expression was investigated. As expected, hepatic SREBP1c overexpression increased the expression of lipogenic genes such as FASN, SCD1 and ELOVL6 (Figure 18A). As in the case with primary hepatocytes, the level of hepatic CRY1 mRNA was enhanced by SREBP1c *in vivo* (Figure 18A). Moreover, in contrast to *SREBP1c*^{+/+} mice, refeeding failed to increase hepatic CRY1 gene expression in *SREBP1c*^{-/-} mice (Figure 18B). This observation indicates that SREBP1c is an essential factor for the upregulation of hepatic CRY1 gene expression in the postprandial state.

SREBP1c-CRY1 pathway inhibits hepatic gluconeogenesis

Consistent with previous reports (Becard et al., 2001; Lamia et al., 2011; Zhang et al., 2010), SREBP1c overexpression decreased glucose production in mouse primary hepatocytes (Figure 19A). In addition, the optical *in vivo* imaging analysis revealed that hepatic SREBP1c overexpression remarkably repressed the promoter

Figure 17. SREBP1c directly binds to CRY1 promoter.

ChIP assay, performed as described in Methods, showing CRY1 promoter occupancy by SREBP1 in H4IIE cells.

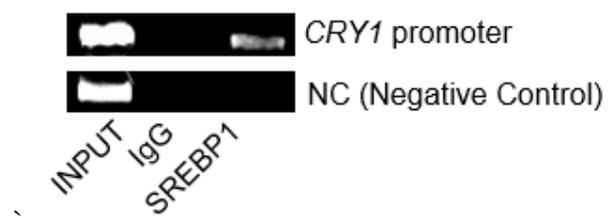


Figure 18. CRY1 regulation in SREBP1c modulated mice.

(A) *C57BL/6* mice were infected with adenoviruses encoding either a Mock or SREBP1c (adenoviral dose of 5×10^9 viral particles per mouse) through the tail vein. Mice were sacrificed 5 days following the injection of adenoviruses and relative mRNA levels were determined by qRT-PCR and normalized to the TBP mRNA signal. Data are represented as mean \pm SD, $N=3-4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (B) *SREBP1c*^{-/-} and *SREBP1c*^{+/+} mice were fasted for 24 h and then refed for 12 h. Both fasted and refed mice were sacrificed at ZT3. The levels of SREBP1c and CRY1 mRNAs were determined by qRT-PCR and normalized to TBP mRNA levels. Data are represented as mean \pm SD, $N=3-4$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).

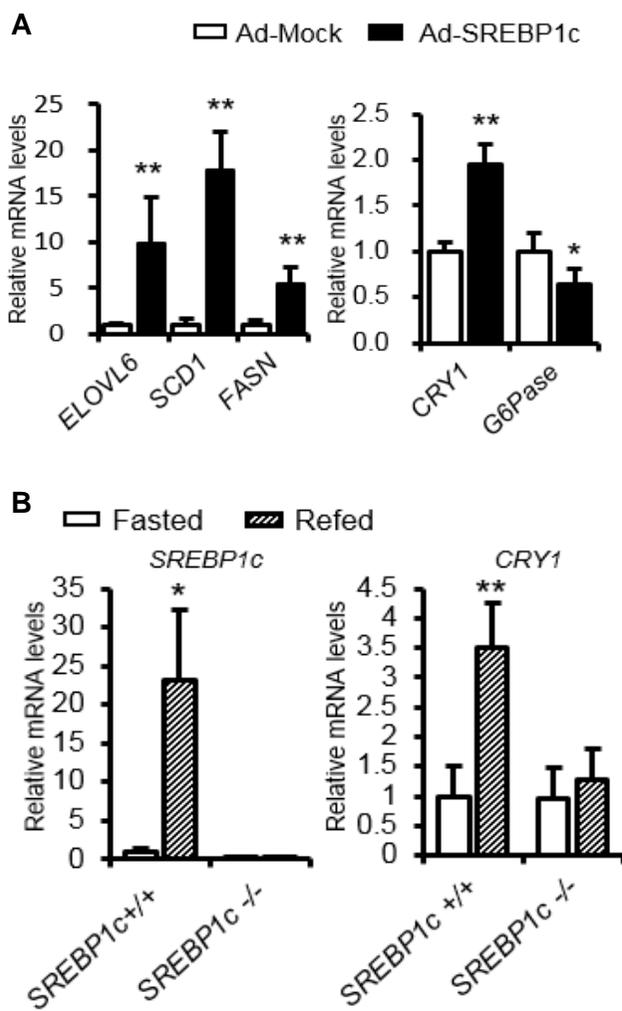
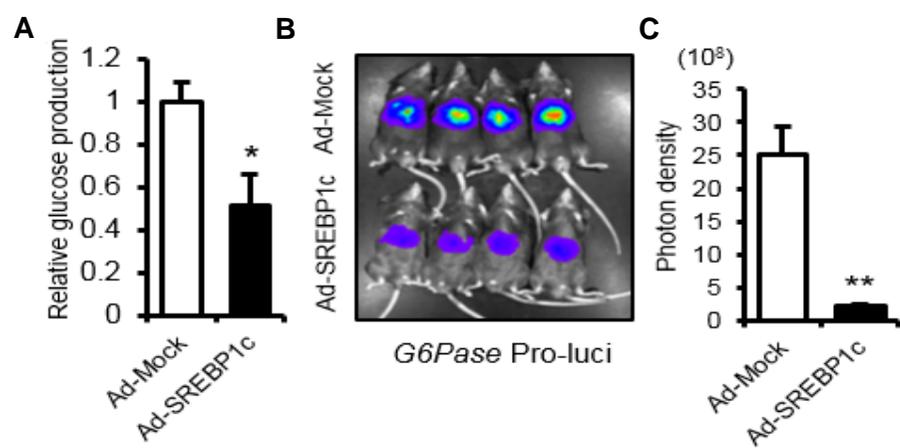


Figure 19. SREBP1c suppresses hepatic gluconeogenesis

(A) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-SREBP1c. Relative glucose secretion was measured using a glucose oxidase (GO) kit as described in Methods. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$ (Student's *t*-test). (B) *C57BL/6* mice were infected with Ad-G6Pase-luc and either Ad-Mock or Ad-SREBP1c. (C) The effect of hepatic SREBP1c overexpression on G6Pase promoter activity was measured by optical *in vivo* imaging analysis and converted to photon density.



activity of the *G6Pase* gene *in vivo* (Figure 19B, 19C). These findings led us to investigate the effect of SREBP1c on blood glucose level *in vivo*. Pyruvate tolerance test demonstrated that the adenoviral overexpression of SREBP1c significantly decreased blood glucose level following pyruvate injection (Figure 20A, 20B). Accordingly, *SREBP1c*^{-/-} mice showed higher blood glucose than *SREBP1c*^{+/+} mice (Figure 20C, 20D). Thus, these results suggest that hepatic SREBP1c would suppress gluconeogenesis, potentially by modulating gluconeogenic gene expression.

To examine whether CRY1, a novel target gene of SREBP1c, might modulate hepatic gluconeogenic gene expression, I suppressed CRY1 expression via siRNA in rat hepatoma H4IIE cells. Downregulation of CRY1 increased the expression of G6Pase and PEPCK genes (Figure 21A), which are crucial for hepatic gluconeogenesis. On the contrary, hepatic CRY1 overexpression decreased the expression of G6Pase and PEPCK genes in mouse primary hepatocytes (Figure 21B). To confirm these observations, I measured pyruvate-induced blood glucose level from *CRY1*^{+/+} and *CRY1*^{-/-} mice. As shown in Figure 22A and 22B, *CRY1*^{-/-} mice shows higher blood glucose level than *CRY1*^{+/+} mice. To test whether CRY1 could be a key mediator of the inhibition of hepatic gluconeogenesis by SREBP1c, I performed glucose production assays in primary hepatocytes. As indicated in Figure 23, SREBP1c overexpression significantly suppressed glucose production, while suppression of CRY1 with siRNA in SREBP1c-overexpressing hepatocytes rescued the ability to produce glucose. To establish whether the SREBP1c-CRY1 signaling

Figure 20. SREBP1c regulates pyruvate induced blood glucose level.

(A and B) *C57BL/6* mice were infected with Ad-Mock or Ad-SREBP1c and subjected to the PTT (A), as described in Methods. All mice were fasted at ZT10 and performed PTT at ZT3. The result was converted by the area-under-the curve (AUC) analysis (B). Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). (C and D) Pyruvate tolerance test (C) was performed in *SREBP1c*^{-/-} and *SREBP1c*^{+/+} mice. All mice were fasted at ZT10 and performed PTT at ZT3. Results were converted to AUC values (D). Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).

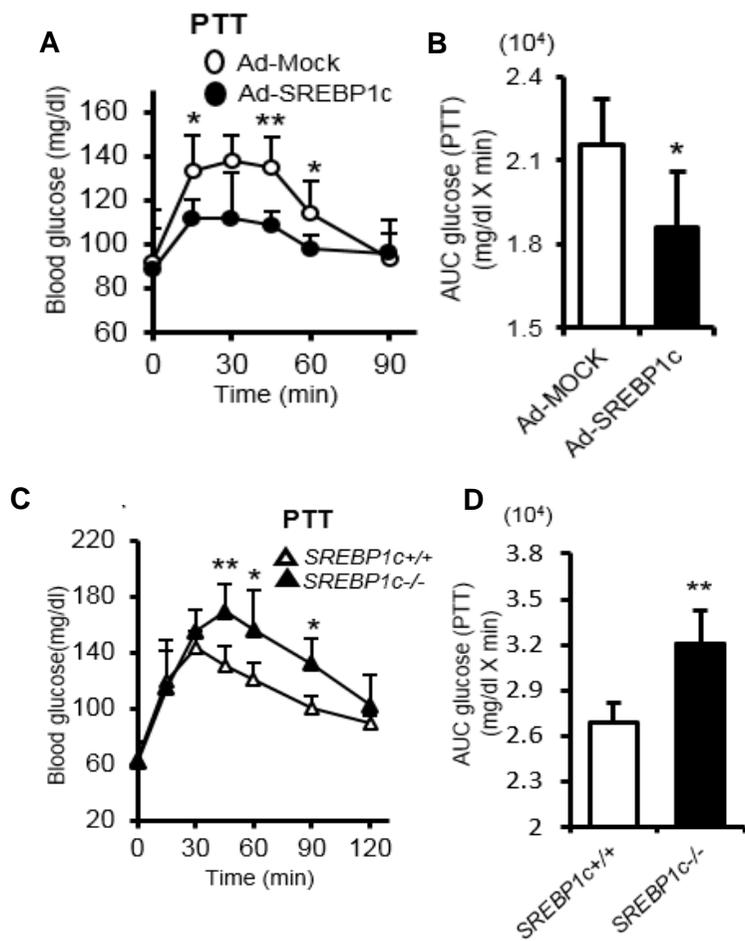


Figure 21. CRY1 regulates gluconeogenic gene expression.

(A) H4IIE cells were transfected with siCON or siCRY1. Relative PEPCK and G6Pase mRNA levels were determined by qRT-PCR and normalized to cyclophilin mRNA levels. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test). (B) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1. Relative PEPCK and G6Pase mRNA levels were determined by qRT-PCR and normalized to TBP mRNA levels. Data are represented as mean \pm SD, $N=3$ for each group. $*P < 0.05$ (Student's *t*-test).

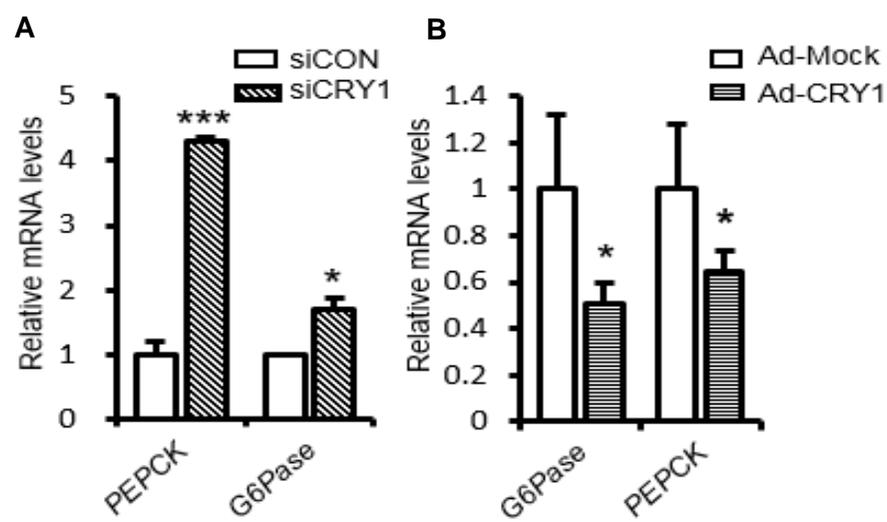


Figure 22. *CRY1*^{-/-} mice showed low level of glucose upon pyruvate challenge.

(A and B) Pyruvate tolerance test (A) was performed in *CRY1*^{-/-} and *CRY1*^{+/+} mice.

All mice were fasted at ZT10 and performed PTT at ZT3. Results were converted to

AUC values (B). Data are represented as mean \pm SD, $N=7$ for each group. * $P < 0.05$,

** $P < 0.01$ (Student's *t*-test).

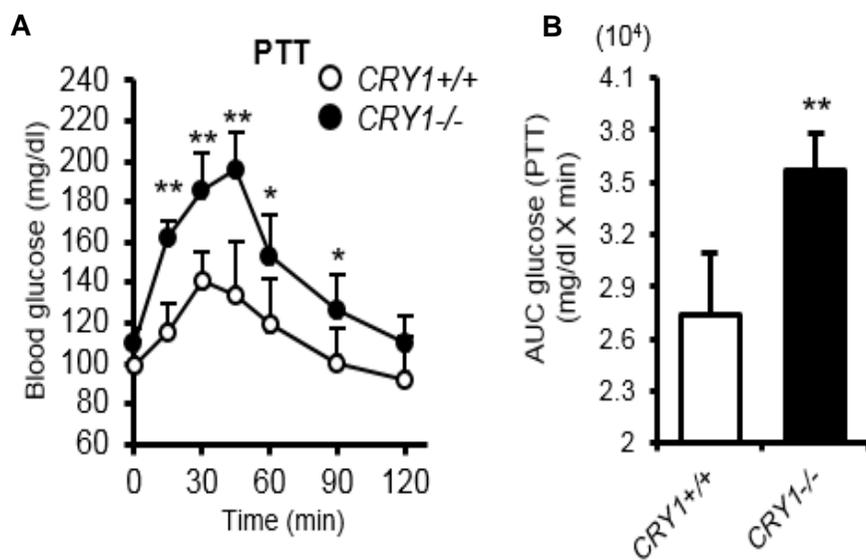
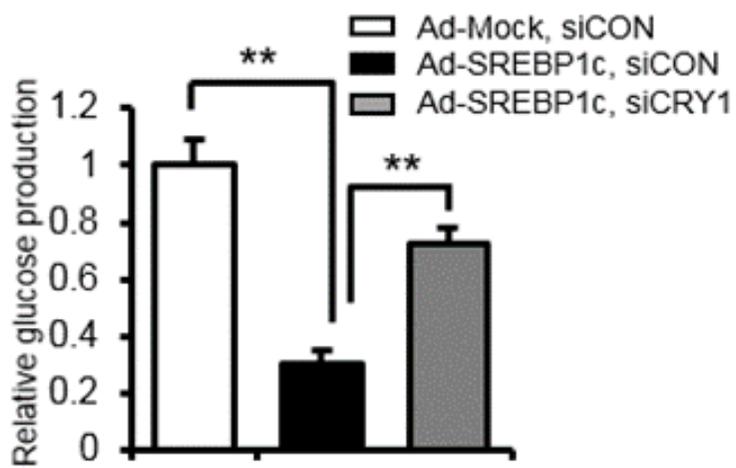


Figure 23. The SREBP1c-CRY1 signaling pathway regulates gluconeogenesis *in vitro*.

Mouse primary hepatocytes were infected with Ad-Mock or Ad-SREBP1c and transfected with either siCON or siCRY1. Relative glucose production was measured using a glucose oxidase (GO) kit. Data are represented as mean \pm SD, $N=4$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).



pathway could indeed repress hepatic glucose production *in vivo*, CRY1 was adenovirally overexpressed in the liver of *SREBP1c^{-/-}* mice. While *SREBP1c^{-/-}* mice showed higher blood glucose level than *SREBP1c^{+/+}* mice during the pyruvate tolerance test, *SREBP1c^{-/-}* mice with CRY1 overexpression exhibited an attenuated level of blood glucose, comparable to that of WT mice (Figure 24A, 24B). These data strongly indicate that the SREBP1c-CRY1 signaling pathway could inhibit hepatic gluconeogenesis *in vivo*.

CRY1 regulates FOXO1 protein

To decipher the underlying mechanism(s) by which insulin-induced CRY1 could repress hepatic gluconeogenesis, I focused on FOXO1, as its regulatory effects on insulin signaling and gluconeogenesis are well established. In mouse primary hepatocytes, the level of the FOXO1 protein was decreased by CRY1 overexpression (Figure 25A) while FOXO1 mRNA levels were not altered (Figure 25B). These data indicated that CRY1 might modulate the level of the FOXO1 protein, probably, independent of the FOXO1 mRNA. Furthermore, the level of the FOXO1 protein was enhanced in CRY1-suppressed hepatocytes (Figure 25C, 25D). In accordance with these *in vitro* data, the FOXO1 protein level was higher in the liver of *CRY1^{-/-}* mice than in the liver of the *CRY1^{+/+}* mice, whereas levels of the FOXO1 mRNA were not different between *CRY1^{+/+}* and *CRY1^{-/-}* mice (Figure 26A, 26B).

Figure 24. The SREBP1c-CRY1 signaling pathway regulates gluconeogenesis *in vivo*.

(A and B) Pyruvate tolerance test (A) in *SREBP1c^{+/+}* mice injected with Ad-Mock and in *SREBP1c^{-/-}* mice injected with either Ad-Mock or Ad-CRY1. Results were converted to AUC values (B) to assess the effect of *CRY1* overexpression in *SREBP1c^{-/-}* mice. All mice were fasted at ZT 10 and performed PTT at ZT 3. Data are represented as mean \pm SD, $N=4-5$ for each group. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

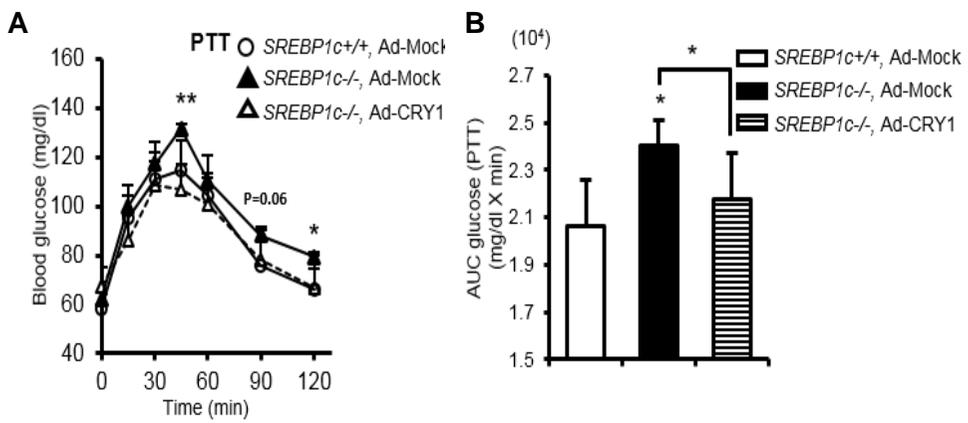


Figure 25. CRY1 regulates FOXO1 protein level *in vitro*.

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The expression profiles of FOXO1 were analyzed at the protein level (A) using western blotting and at the mRNA level (B) using qRT-PCR. Data are represented as mean \pm SD, $N=4$ for each group. $*P < 0.05$ (Student's t -test). (C and D) H4IIE cells were transfected with siCON or siCRY1. Immunocytochemical analysis (C) of endogenous FOXO1. DAPI, 4', 6-diamidino-2-phenylindole. Endogenous FOXO1 and CRY1 protein levels were analyzed using western blotting (D).

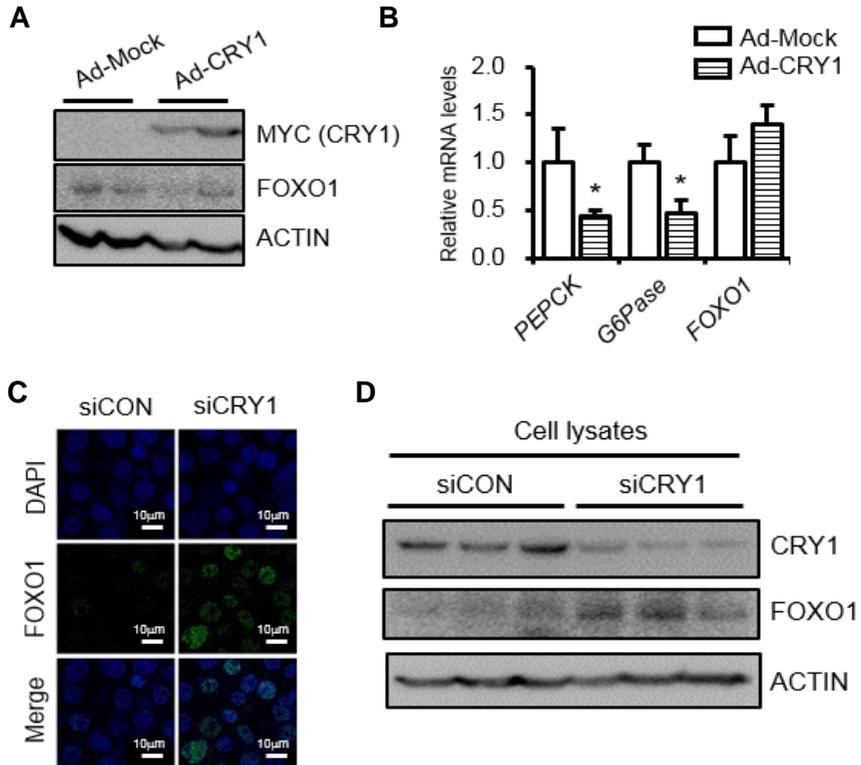
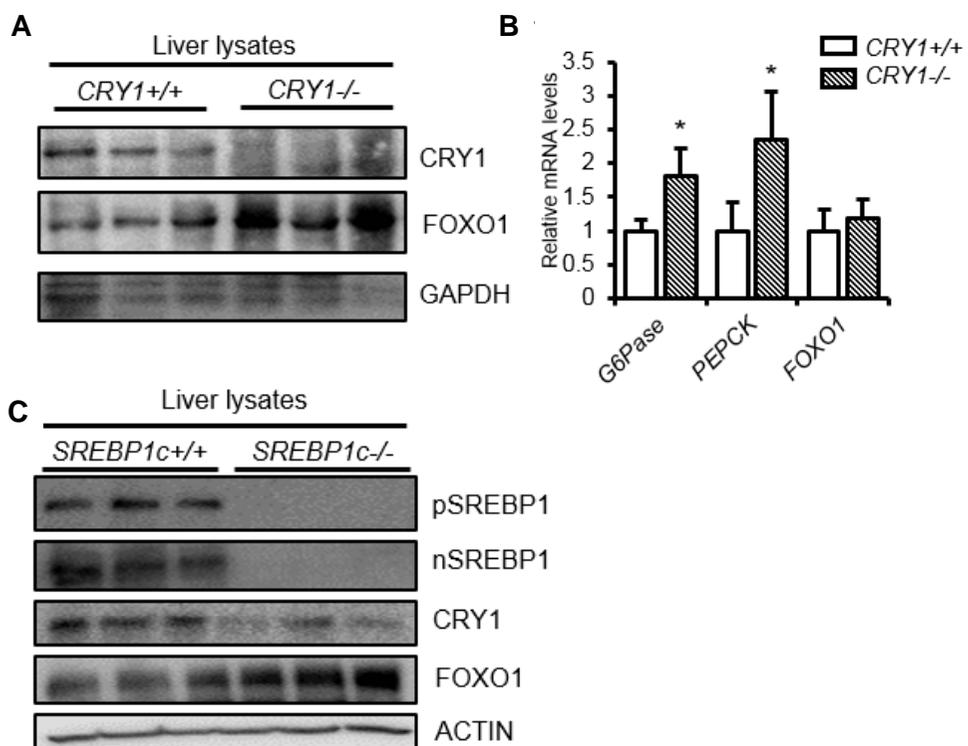


Figure 26. FOXO1 protein level is elevated in SREBP1^{-/-} and CRY1^{-/-}.

(A and B) The expression patterns of FOXO1 protein in the liver of *CRY1*^{+/+} and *CRY1*^{-/-} mice were analyzed by western blotting (A) and qRT-PCR (B). Relative mRNA levels were determined using qRT-PCR and normalized to the levels of the TBP mRNA. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test). (C) Expression of CRY1 and FOXO1 proteins in the liver of *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice was analyzed by western blotting.



Moreover, compared to the levels of proteins observed in the livers of the *SREBP1c*^{+/+} mice, the amount of the FOXO1 protein was higher and the level of the CRY1 protein was lower in the liver of *SREBP1c*^{-/-} mice (Figure 26C). To verify that CRY1 could inhibit hepatic gluconeogenesis via FOXO1 modulation, the effects of CRY1 and/or FOXO1 suppression on gluconeogenic gene expression were examined. Increased expression of G6Pase and PEPCK genes by CRY1 suppression was abolished when the FOXO1 gene was downregulated by siRNA (Figure 27), indicating that CRY1 could alleviate hepatic gluconeogenesis via suppression of the FOXO1 protein. Therefore, these *in vivo* and *in vitro* data suggest that hepatic FOXO1 protein could be regulated by CRY1.

FOXO1 protein is decreased by insulin-activated CRY1

FOXO1 translocation from the nucleus to the cytoplasm by AKT is a well-known mechanism by which insulin acutely inhibits hepatic glucose production (Rena et al., 1999). As insulin upregulates CRY1 that, in turn, downregulates the FOXO1 protein (Figure 14C and 25A), I investigated the time course of these events by examining the expression profiles of the FOXO1 and CRY1 proteins in insulin-treated primary hepatocytes. As shown in Figure 28A and 28B, AKT phosphorylation was clearly induced in cells treated with insulin for a relatively short period (0.5~4 h). Concomitantly, FOXO1 phosphorylation was also increased by

Figure 27. CRY1 inhibits gluconeogenesis via FOXO1 signaling pathway.

H4IIE cells were co-transfected with siCRY1 and/or siFOXO1. Relative mRNA levels were normalized to the cyclophilin mRNA level. Data are represented as mean \pm SD, $N=3$ for each group. # $P < 0.05$, ## $P < 0.01$ versus siCRY1, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus siCON (Student's t -test).

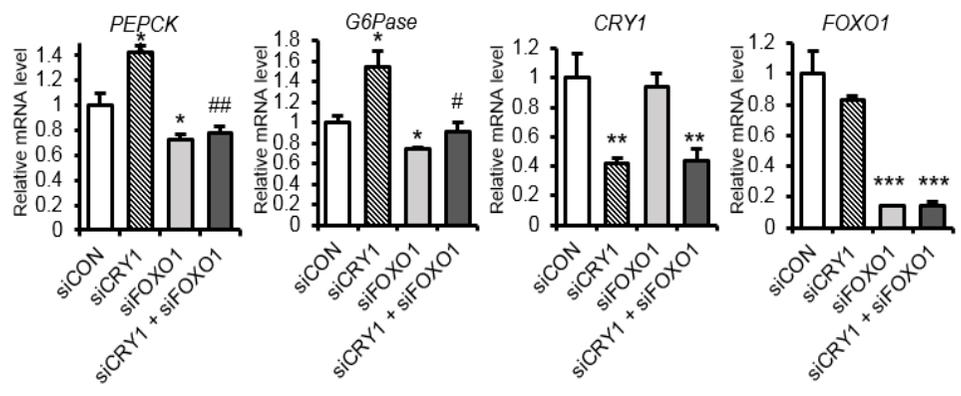
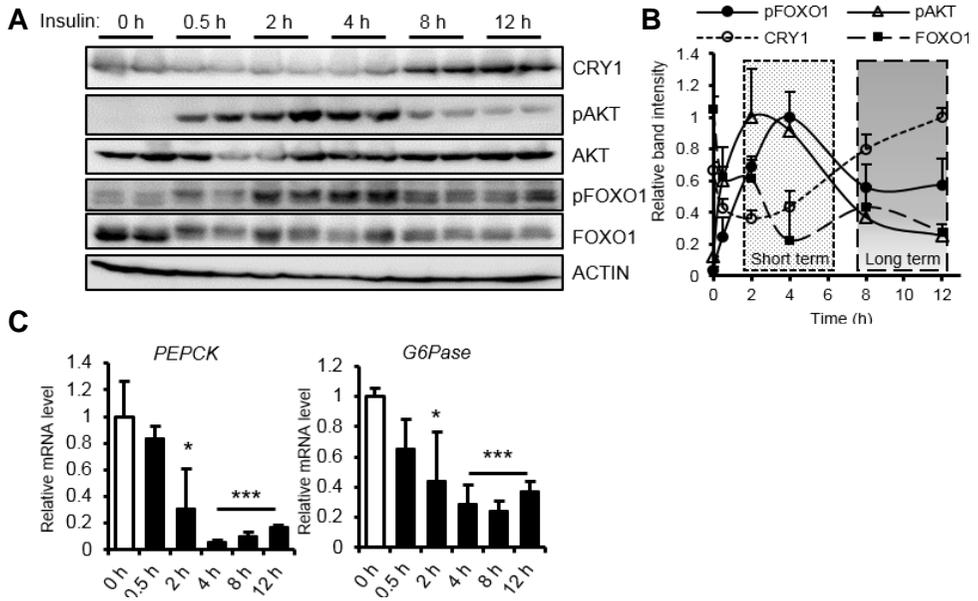


Figure 28. CRY1 is induced in long term insulin action with inhibition of gluconeogenesis.

(A, B, and C) Mouse primary hepatocytes were treated with insulin for different periods. Protein levels (A) were determined with western blotting and the results were converted to the band intensity graph (B). mRNA levels (C) were analyzed by qRT-PCR. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t -test).



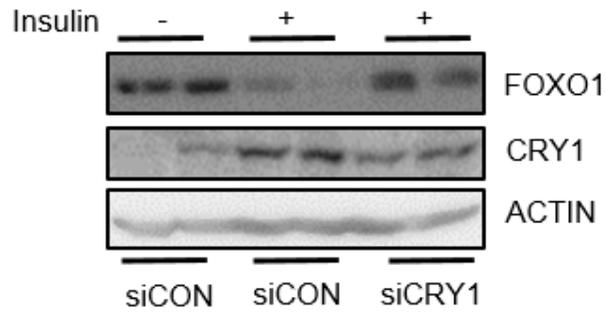
insulin. However, phosphorylation levels of AKT and FOXO1 were gradually and substantially decreased by a long-term (8~12 h) incubation with insulin, implying that FOXO1 translocation from the nucleus to the cytoplasm by AKT might be more pronounced after a short exposure to insulin rather than following a long-term insulin treatment. Intriguingly, in hepatocytes treated with insulin for long periods, the level of the CRY1 protein was markedly increased, while that of the total FOXO1 protein was decreased, indicating that the amount of the CRY1 protein appears to be inversely related to the total quantity of the FOXO1 protein. Moreover, the expression of PEPCK and G6Pase genes was repressed after either a long-term or a short-term insulin treatment (Figure 28C). These data suggest that reduction in the FOXO1 protein level might be involved in the suppression of hepatic gluconeogenesis as a result of a delayed effect of insulin treatment.

Next, I explored whether CRY1 could modulate the decrease in FOXO1 protein in insulin-treated hepatocytes. To address this, the expression of CRY1 was suppressed by siRNA with or without an insulin treatment. As shown in Figure 29A, long-term insulin challenge led to a decrease in FOXO1 protein, while suppression of CRY1 protein restored the level of the FOXO1 protein. In the presence of insulin, decreased levels of PEPCK and G6Pase mRNA were also substantially restored by CRY1 knockdown in hepatocytes (Figure 29B). Together, these data indicate that CRY1 could repress the expression of hepatic gluconeogenic genes via reduction of the FOXO1 protein level during the long-term insulin action.

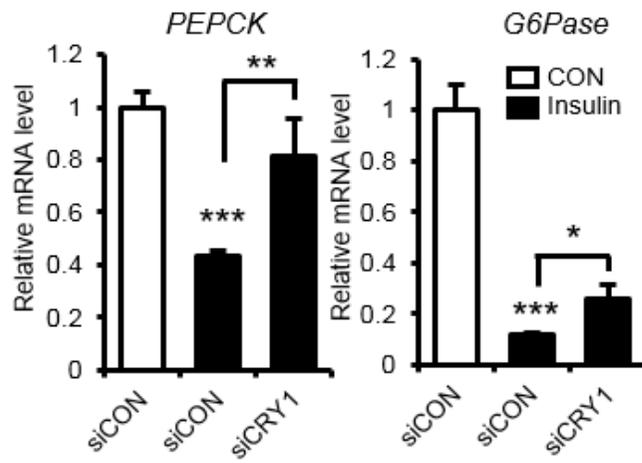
Figure 29. Insulin sustainably suppresses gluconeogenesis via CRY1.

(A and B) H4IIE cells were transfected with siCON or siCRY1 and treated with 10 nM insulin for 12 h. Protein levels (A) were analyzed by western blotting and relative mRNA levels (B) were determined by qRT-PCR and normalized to the cyclophilin mRNA level. Data are represented as mean \pm SD, $N=3$ for each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t -test).

A



B



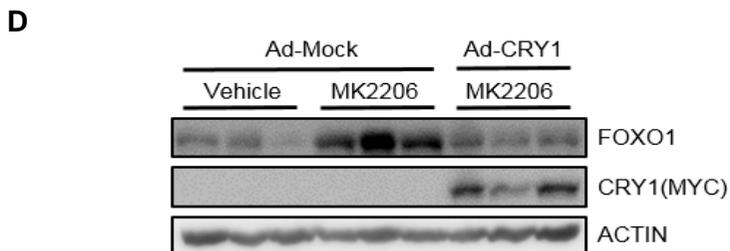
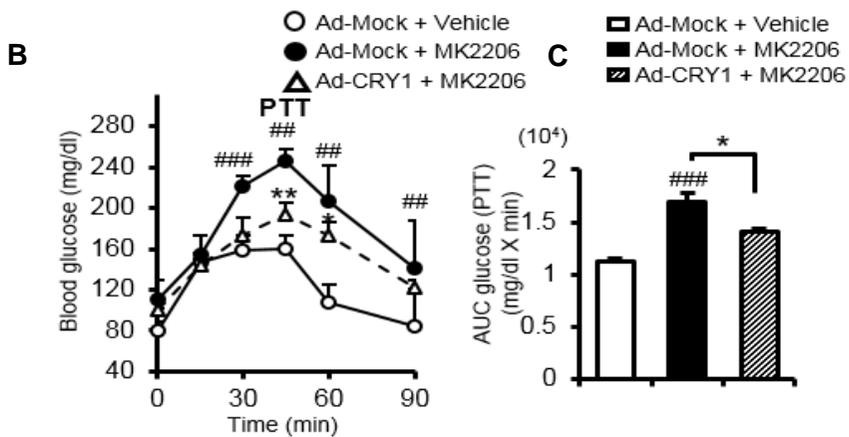
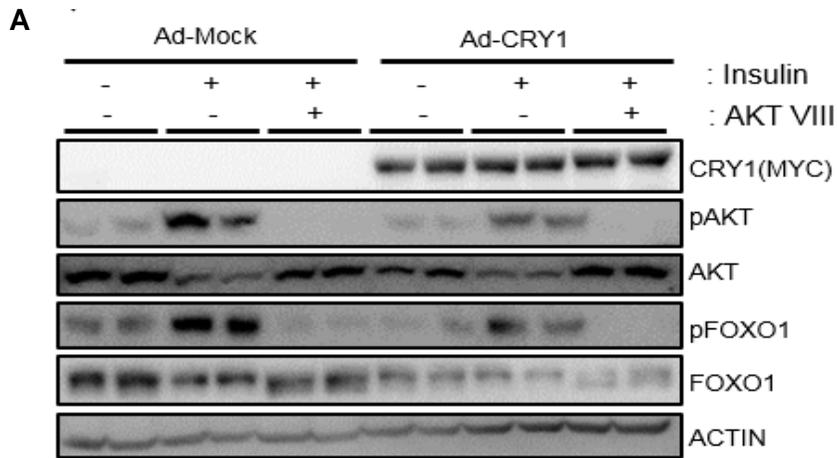
To test whether enhanced CRY1 could suppress hepatic gluconeogenesis even in the absence of a short-term insulin action, I employed AKT inhibitors. In primary hepatocytes, insulin increased phosphorylation levels of both AKT and FOXO1, while a co-treatment with the AKT inhibitor AKTVIII blocked phosphorylation of both proteins, as expected (Figure 30A). However, in Ad-CRY1 overexpressing hepatocytes, the total FOXO1 level was decreased in insulin and/or the AKT inhibitor treated cells, implying that CRY1 could downregulate FOXO1 protein independent of FOXO1 phosphorylation (Figure 30A). In order to confirm this observation *in vivo*, I tested another AKT inhibitor, MK2206, in mice. As expected, administration of MK2206 significantly increased blood glucose level upon pyruvate challenge; however, adenoviral CRY1 overexpression in mice significantly attenuated blood glucose level even in the presence of MK2206 (Figure 30B, 30C). It is noteworthy that the level of the FOXO1 protein was greatly augmented by MK2206, whereas CRY1 elevation suppressed FOXO1 protein expression *in vivo* (Figure 30D). Taken together, these data clearly indicate that CRY1-dependent FOXO1 reduction may contribute to the suppression of hepatic gluconeogenesis independent of AKT activation.

CRY1 stimulates proteasomal degradation of FOXO1

Since CRY1 overexpression appeared to decrease the level of the FOXO1

Figure 30. CRY1 suppresses FOXO1 protein level independent of AKT activity.

(A) Mouse primary hepatocytes were infected with Ad-Mock and Ad-CRY1, and then treated with insulin (10 nM) or insulin (10 nM) and AKTVIII (5 μ M) for 12 h. Protein levels were determined with western blotting. (B, C and D) *C57BL/6* mice were infected with Ad-Mock or Ad-CRY1 and subjected to the pyruvate tolerance test (B) with or without the AKT inhibitor MK2206. MK2206 (30 mg/kg) was given by oral gavage 10 min before the pyruvate tolerance test. All mice were fasted at ZT 10 and performed PTT at ZT 3. Results were converted to AUC values (C). After the pyruvate tolerance test, hepatic protein levels (D) were analyzed by western blotting. Data are represented as mean \pm SD, $N=5-7$ for each group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus Ad-Mock + vehicle control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Ad-Mock + MK2206 control (Student's t -test).

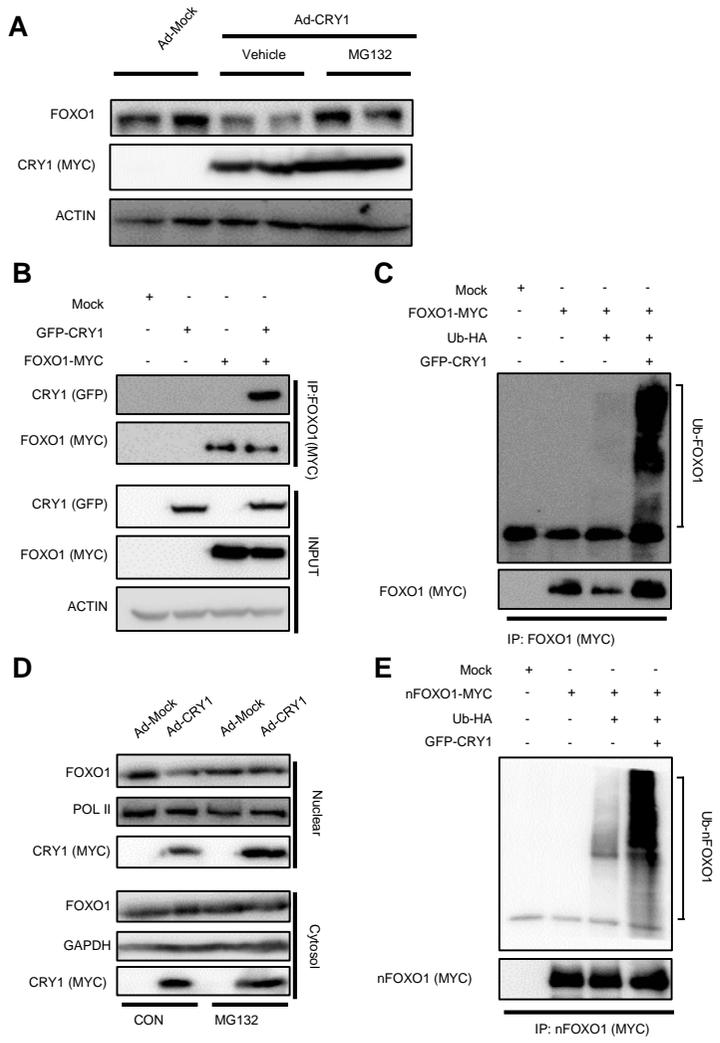


protein, but not the FOXO1 mRNA, I investigated whether the downregulation of the FOXO1 protein proceeds via proteasomal degradation. As shown in Figure 31A, the reduction in the FOXO1 protein by CRY1 overexpression was alleviated by MG132 treatment, indicating that the regulation of FOXO1 protein by CRY1 may be, at least in part, dependent on proteasomal degradation. When I tested physical interaction between FOXO1 and CRY1 proteins, co-immunoprecipitation assays revealed that CRY1 could associate with FOXO1 protein (Figure 31B). Then, I examined whether CRY1 might induce FOXO1 degradation via the ubiquitination-proteasome pathway. As shown in Figure 31C, CRY1 overexpression dramatically promoted FOXO1 poly-ubiquitination, implying that CRY1 could potentiate FOXO1 degradation, probably, through protein-protein interactions.

To explore the subcellular location of FOXO1 degradation induced by CRY1, levels of the nuclear and cytosolic FOXO1 protein were investigated. As shown in Figure 31D, the nuclear fraction of the FOXO1 protein was decreased by CRY1 overexpression, whereas incubation with MG132 blocked this decrease. At the same time, levels of cytosolic FOXO1 were not altered upon CRY1 overexpression irrespective of the presence of MG132. Consistent with these results, poly-ubiquitination of the nuclear form of the FOXO1 mutant protein (nFOXO1-MYC) was greatly augmented by CRY1 (Figure 31E). Therefore, it is plausible that the degradation of the FOXO1 protein via poly-ubiquitination is stimulated by CRY1 in the nucleus

Figure 31. CRY1 accelerates ubiquitin-mediated FOXO1 degradation

(A) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The cells were treated with 20 μ M MG132 or vehicle for 4 h. Total cell lysates were analyzed by western blotting with indicated antibodies. (B) HEK293T cells were transfected with GFP-CRY1 and/or FOXO1-MYC expression vectors. Co-immunoprecipitation with an anti-MYC antibody and western blotting were performed with the indicated antibodies. IP, immunoprecipitation. (C) COS-1 cells were co-transfected with plasmids encoding FOXO1-MYC, GFP-CRY1, and Ubiquitin-HA. After transfection, the cells were treated with MG132 (20 μ M) for 6 h and then the cell lysates were subjected to immunoprecipitation with an anti-MYC antibody followed by western blotting with indicated antibodies. IP, immunoprecipitation. (D) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1. After infection, the cells were treated with MG132 (20 μ M) for 4 h. Nuclear and cytosolic fractions were isolated and analyzed by western blotting with indicated antibodies. (E) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, GFP-CRY1, and Ubiquitin-HA. After transfection, the cells were challenged with MG132 (20 μ M) for 6 h. The cell lysates were subjected to immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation.



CRY1 is involved in MDM2-mediated FOXO1 degradation

Among several ubiquitin E3 ligases of the FOXO1 protein (Fu et al., 2009; Kato et al., 2008), I found that the MDM2 ubiquitin E3 ligase was involved in the CRY1-mediated FOXO1 degradation. As shown in Figure 32A, MDM2 suppression markedly rescued the level of the FOXO1 protein in CRY1-overexpressing cells, implying that MDM2 may participate in the CRY1-dependent FOXO1 reduction. To study the role of CRY1 in MDM2-mediated FOXO1 degradation, I tested whether CRY1 might regulate the subcellular localization of MDM2. Wild type CRY1 and cytosolic CRY1 (Δ NLS-CRY1) did not change the subcellular location of the nuclear MDM2 (Figure 32B). However, I revealed that CRY1 potentiates the association between FOXO1 and MDM2 (Figure 32C).

In another experiment, I explored if CRY1 could modulate MDM2-mediated FOXO1 degradation. As shown in Figure 33A and 33B, CRY1 overexpression promoted MDM2-mediated poly-ubiquitination of the nuclear form of FOXO1 protein (Figure 33A), whereas CRY1 suppression attenuated FOXO1 poly-ubiquitination by MDM2 (Figure 33B). These data indicate that CRY1 would participate in MDM2-induced FOXO1 degradation and repress FOXO1-mediated hepatic glucose production.

Figure 32. CRY1 represses FOXO1 protein through intensifying MDM2 and FOXO1 binding.

(A) Mouse primary hepatocytes were infected with Ad-Mock or Ad-CRY1 and/or siCON or siMDM2. Total cell lysates were analyzed by western blotting with indicated antibodies. (B) HEK293T cells were transfected with FLAG-MDM2 with GFP-WT CRY1 or GFP-cytosolic CRY1 (Δ NLS CRY1) and immunocytochemical analysis of FLAG-MDM2 and GFP was carried out. DAPI, 4',6-diamidino-2-phenylindole. (C) HEK293T cells were transfected with FLAG-MDM2, nFOXO1-MYC, and GFP-CRY1 expression vectors. Total cell lysates were subjected to co-immunoprecipitation with an anti-MYC antibody followed by western blotting with indicated antibodies. IP, immunoprecipitation.

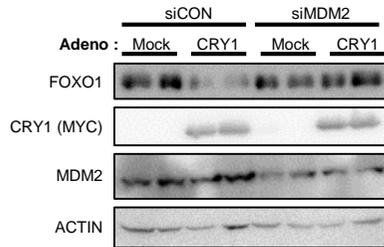
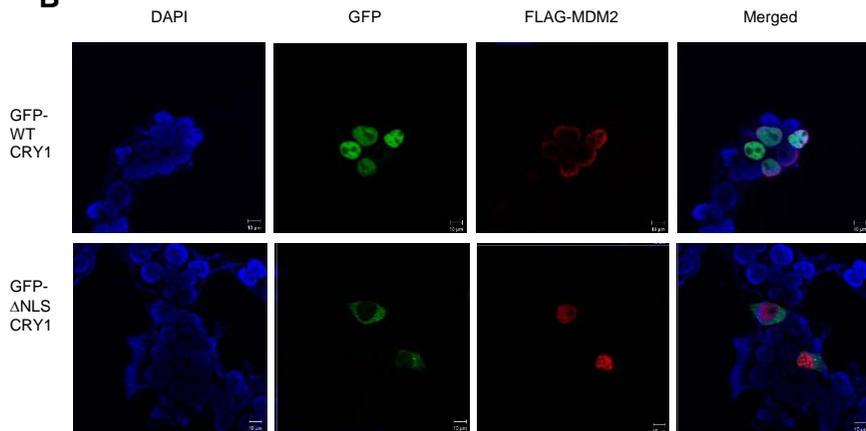
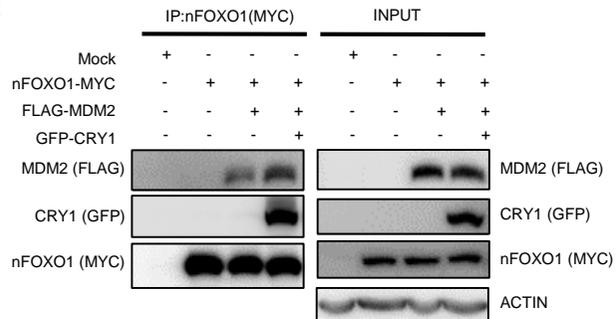
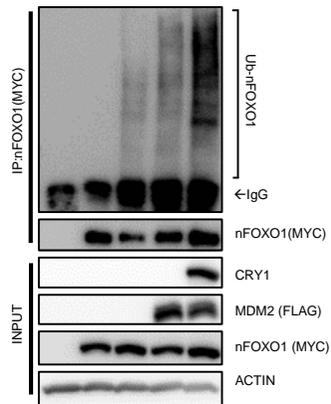
A**B****C**

Figure 33. CRY1 is involved in MDM2-mediated FOXO1 ubiquitination

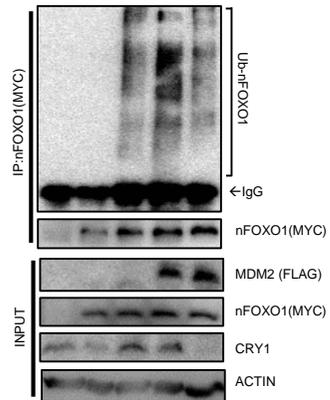
(A) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, FLAG-MDM2, FLAG-CRY1, and Ubiquitin-HA. After transfection, the cells were challenged with MG132 (20 μ M) for 6 h. Cell lysates underwent immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation (B) COS-1 cells were co-transfected with plasmids encoding nFOXO1-MYC, FLAG-MDM2, Ubiquitin-HA, and siCRY1. Cells were treated with MG132 (20 μ M) for 6 h. Cell lysates were subjected to immunoprecipitation with an anti-MYC antibody. IP, immunoprecipitation.

A

Mock	+	-	-	-	-
nFOXO1-MYC	-	+	+	+	+
Ub-HA	-	-	+	+	+
FLAG-MDM2	-	-	-	+	+
FLAG-CRY1	-	-	-	-	+

**B**

Mock	+	-	-	-	-
nFOXO1-MYC	-	+	+	+	+
Ub-HA	-	-	+	+	+
FLAG-MDM2	-	-	-	+	+
siCRY1	-	-	-	-	+



CRY1 mitigates hyperglycemia in *db/db* mice

In the liver of obese animals such as *db/db* and DIO (diet-induced-obesity) mice, SREBP1c level is elevated while hepatic gluconeogenesis is not repressed (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000; Yoon et al., 2010). To explore which process(es) might be dysregulated in the regulation of hepatic gluconeogenesis, I have examined mRNA and protein levels for SREBP1c-CRY1 axis and gluconeogenic genes in diabetic animals. Similar to previous reports (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000; Yoon et al., 2010), the mRNA levels of SREBP1c and gluconeogenic genes were elevated in *db/db* mice (Figure 34A, 34B). However, hepatic CRY1 protein was greatly decreased in *db/db* mice (Figure 34B). Similarly, DIO mice exhibited elevated SREBP1c and gluconeogenic genes whereas CRY1 was not activated (Figure 34C, 34D). To test the idea that dysregulated CRY1 protein might mediate hyperglycemia with enhanced FOXO1 protein in diabetic animals, CRY1 was adenovirally overexpressed in the liver of *db/db* mice. As shown in Figure 35A, the level of blood glucose was decreased by CRY1 overexpression. Moreover, ectopic CRY1 expression reduced the levels of FOXO1 protein as well as gluconeogenic gene expression in *db/db* mice (Figure 35B, 35C). These results propose that CRY1 could ameliorate hyperglycemia by repressing the level of FOXO1 protein in *db/db* mice.

Figure 34. SREBP1c is activated while CRY1 is not elevated in *db/db* and DIO mice.

(A, and B) Ten-week-old male *db/+* and *db/db* mice were sacrificed in fed states at ZT3. The relative mRNA levels of various hepatic genes (A) were determined by qRT-PCR analyses and normalized to the TBP mRNA level. Data are represented as mean \pm SD, $N=4$ for each group. * $P < 0.05$ versus *db/+* group. (Student's *t*-test). Protein levels (B) were determined with western blotting. (C and D) Eight-week-old *C57BL/6* mice were fed a NCD or HFD for 8 weeks. Hepatic gene expression levels (D) were determined by qRT-PCR and normalized by the level of the TBP mRNA. $N=5$ in each group. Hepatic protein levels (E) were analyzed by western blotting

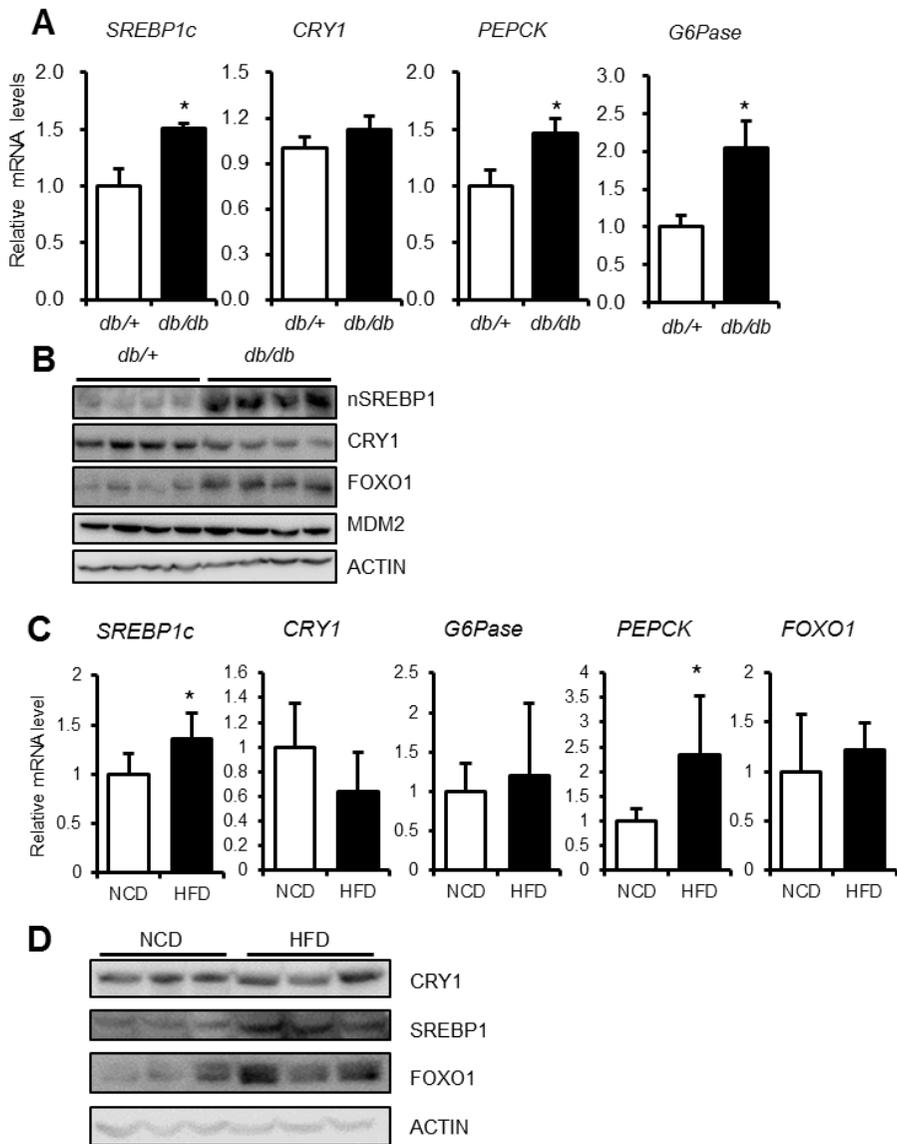
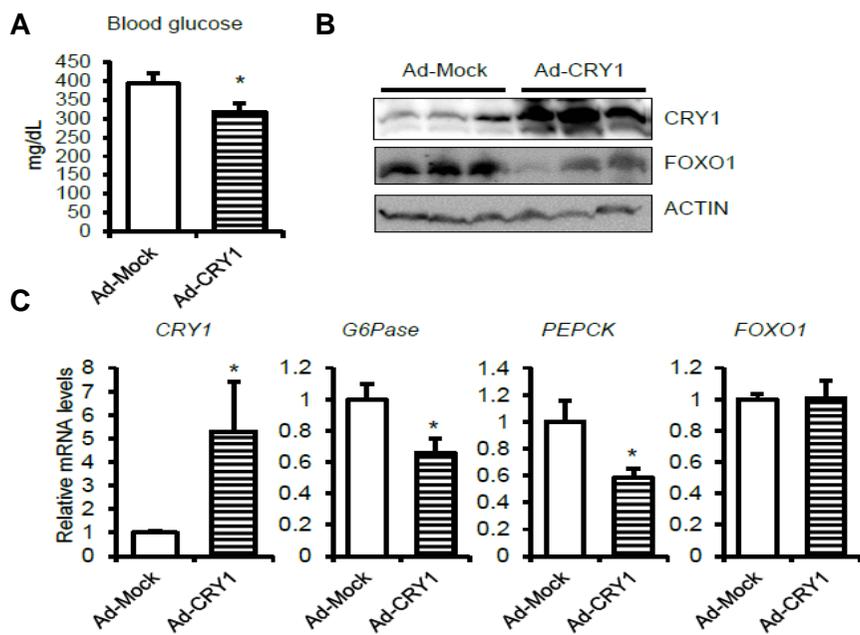


Figure 35. CRY1 alleviates gluconeogenesis in *db/db* mice

(A, B, and C) Ten-week-old male *db/db* mice were infected through the tail vein with adenovirus encoding GFP or CRY1 (adenoviral dose of 2×10^{10} viral particles per mouse). The blood glucose levels (A) were measured in ad libitum at ZT3. After all of the mice were sacrificed at ZT3, hepatic protein levels (B) were analyzed by western blotting, and the relative mRNA levels (C) were determined by qRT-PCR analyses and normalized to the TBP mRNA level. Data are represented as mean \pm SD, $N=5$ for each group. $*P < 0.05$ versus Ad-Mock group. (Student's *t*-test).



Discussion

As a major anabolic hormone, insulin stimulates lipogenesis and represses gluconeogenesis in the liver. Following insulin exposure, lipogenesis is upregulated by SREBP1c, and the expression of SREBP1c target genes such as FASN, SCD and ELOVL6 is thus induced (Chu et al., 2013; Kim et al., 1998a; Ponugoti et al., 2010). In contrast, insulin blocks hepatic gluconeogenesis through AKT-mediated phosphorylation of FOXO1 and PGC1 α (Gross et al., 2008; Li et al., 2007), both are major regulators of gluconeogenic genes including PEPCK and G6Pase. Here, I propose that the SREBP1c-CRY1 signaling pathway plays an important role to inhibit hepatic gluconeogenesis under anabolic state. Accumulating evidences from hepatic gluconeogenic gene expression, *in vitro* glucose output assays, time kinetics of insulin signaling cascades, and pyruvate tolerance test, which reflects both hepatic glucose output and peripheral glucose disposal, have consistently suggested the idea that maintenance of SREBP1c-induced CRY1 is crucial to prevent unnecessary hepatic gluconeogenesis during insulin action. In this regard, it has been reported that single nucleotide polymorphisms (SNPs) of SREBP1c and CRY1 genes are associated with hyperglycemia in human (Harding et al., 2006; Kelly et al., 2012).

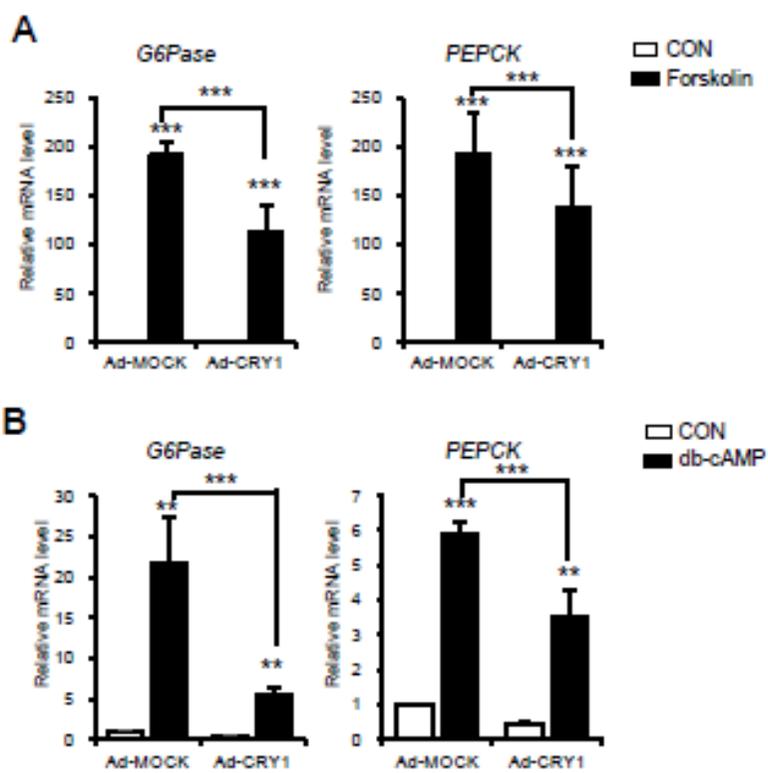
Similar to previous reports (Beaven et al., 2013; Han et al., 2015; Kakuma et al., 2000), the expression of SREBP1c and gluconeogenic genes was increased in the liver of diabetic *db/db* mice (Figure 34A, 34B, 34C). Unexpectedly, the level of hepatic CRY1 protein was reduced in *db/db* mice. In this work, I have demonstrated

that ectopic overexpression of CRY1 in *db/db* mice alleviated hepatic gluconeogenesis by reducing FOXO1 protein (Figure 35A, 35B, 35C). Moreover, I have shown that hepatic CRY1 could attenuate blood glucose level by decreasing FOXO1 protein, independent of AKT activity (Figure 30A, 30B, 30C, 30D). Although it remains to be elucidated how elevated SREBP1c fails to increase CRY1 in the liver of *db/db* mice, it is very likely that increased FOXO1 protein might be resulted from reduced hepatic CRY1 protein in *db/db* mice.

It has been reported that CRY1 seems to suppress hepatic glucose production through interfering glucagon signaling (Hirota et al., 2012; Zhang et al., 2010). CRY1 interacts with GR (Lamia et al., 2011) and the α subunit of the glucagon receptor (Zhang et al., 2010), which are involved in the regulation of gluconeogenesis. Nonetheless, specific roles of CRY1 during the nutrient-rich state after exposure to insulin have not been clearly elucidated. To explore whether CRY1 might repress gluconeogenesis by inhibiting glucagon signaling, CRY1-overexpressing primary hepatocytes were treated with forskolin or db-cAMP to mimic the stimulation of glucagon signaling pathways. In primary hepatocytes, CRY1 partially repressed gluconeogenic gene expression in the presence of forskolin or db-cAMP (Figure 36A, 36B), implying that in addition to the glucagon signaling pathway there may be another signaling cascade, regulated by CRY1, which suppresses hepatic glucose production. Thus, CRY1 is appears to be involved in multiple regulatory pathways that control hepatic gluconeogenesis in response to insulin and glucagon.

Figure 36. Gluconeogenic gene expression is partially attenuated by CRY1 overexpression upon incubation with forskolin and db-cAMP

(A and B) Mouse primary hepatocytes were adenovirally infected with Ad-Mock or Ad-CRY1. The cells were treated with 10 μ M forskolin (A), 5 μ M db-cAMP (B), or vehicle (CON) for 4 h. PEPCK and G6Pase mRNA levels were determined using qRT-PCR and normalized to the level of the TBP mRNA. The values represent the mean \pm SD (N=3 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 (Student's t-test).



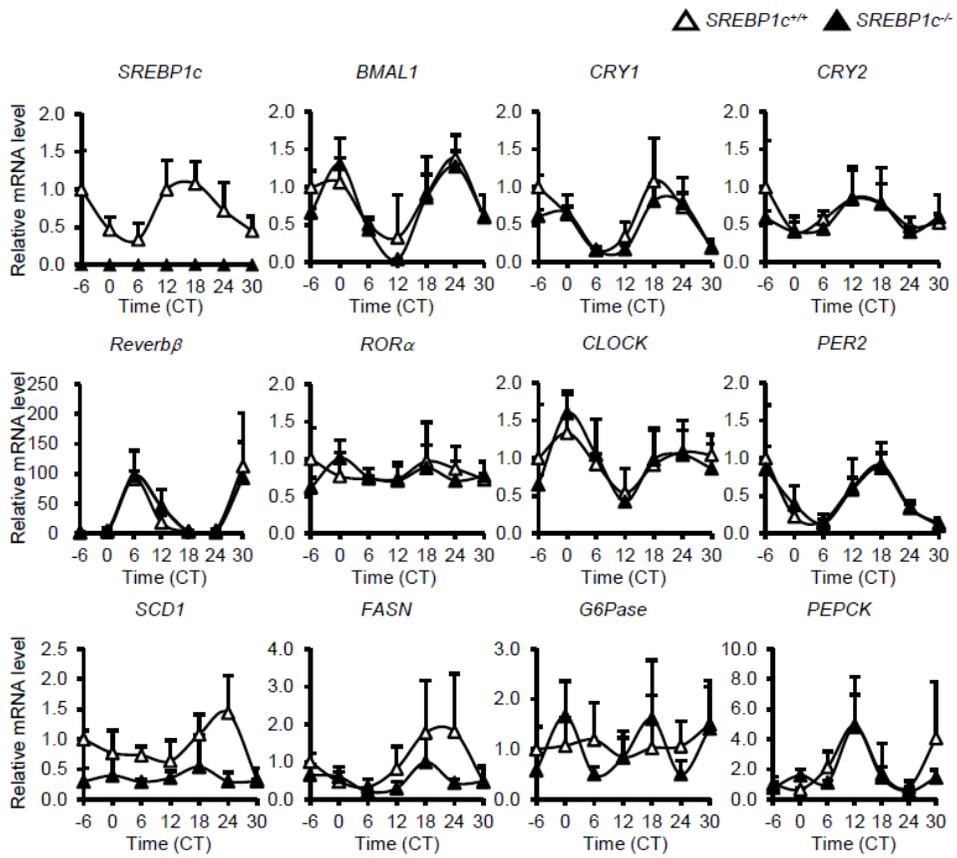
Activation of FOXO1-mediated gluconeogenesis is inhibited by insulin. AKT, a key downstream molecule of the insulin-activated signaling, phosphorylates FOXO1, which then is translocated from the nucleus to the cytoplasm through its association with the 14-3-3 protein (Zhao et al., 2004). In primary hepatocytes, FOXO1 phosphorylation was rapidly increased by insulin. However, hepatic gluconeogenic programming is persistently and efficiently suppressed regardless of the decreased FOXO1 phosphorylation at the late stage of insulin action. Intriguingly, hepatic CRY1 expression was enhanced at relatively late periods of insulin action (Figure 28A, 28B). Furthermore, in primary hepatocytes, a long-term insulin treatment downregulated FOXO1 expression, while suppression of CRY1 rescued FOXO1 protein levels as well as gluconeogenic gene expression (Figure 29A, 29B). It is noteworthy that CRY1 overexpressing mice showed a decrease of blood glucose level as well as of FOXO1 protein when AKT activity was pharmacologically repressed with AKT inhibitor MK2206 (Figure 30A, 30B, 30C, 30D). Collectively, our *in vitro* and *in vivo* data suggest that the CRY1-dependent FOXO1 degradation would be one of crucial mechanisms to attenuate hepatic gluconeogenesis for the long-term insulin action. Therefore, these observations prompted us to propose that the AKT-mediated FOXO1 phosphorylation provides an acute response during early insulin response, whereas SREBP1c-mediated CRY1 regulation would be a more durable process leading to the repression of futile hepatic gluconeogenesis throughout the anabolic state.

CRY1 is one of the key proteins in the circadian negative feedback loop. I observed that CRY1 levels were regulated by insulin and SREBP1c *in vivo* and *in vitro*. Given that hepatic circadian clock gene expression is altered in the STZ-injected insulin-deficient rats (Yamajuku et al., 2012), I investigated circadian clock gene oscillations in the liver of *SREBP1c^{+/+}* and *SREBP1c^{-/-}*. As shown in Figure 37, I did not observe any significant differences in hepatic circadian clock gene oscillations between *SREBP1c^{+/+}* and *SREBP1c^{-/-}* mice, implying that insulin-activated SREBP1c could stimulate *CRY1* gene expression in liver, probably, independent of circadian clock gene oscillations. Also, I cannot exclude the possibility that SREBP1c-induced CRY1 might contribute to minor roles for hepatic circadian oscillation in *SREBP1c^{-/-}* mice because it has been reported that *CRY1^{-/-}* mice exhibit fewer changes in circadian oscillations compared to *CRY1^{-/-}CRY2^{-/-}* double mutant mice (van der Horst et al., 1999). Furthermore, it is possible that remaining SREBP1a and/or SREBP2 activity in *SREBP1c^{-/-}* mice might maintain intact circadian clock gene oscillations and this homeostatic regulation needs to be addressed in future studies (Im et al., 2009; Liang et al., 2002). Nonetheless, hepatic CRY1 gene expression is clearly upregulated by SREBP1c in the postprandial condition.

As SREBP1c could simultaneously regulate both gluconeogenesis and lipogenesis, it is plausible to suggest that hepatic SREBP1c would effectively coordinate the anabolic pathways by upregulating fatty acid metabolism and

Figure 37. Expression profiles of various genes in the liver of *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice

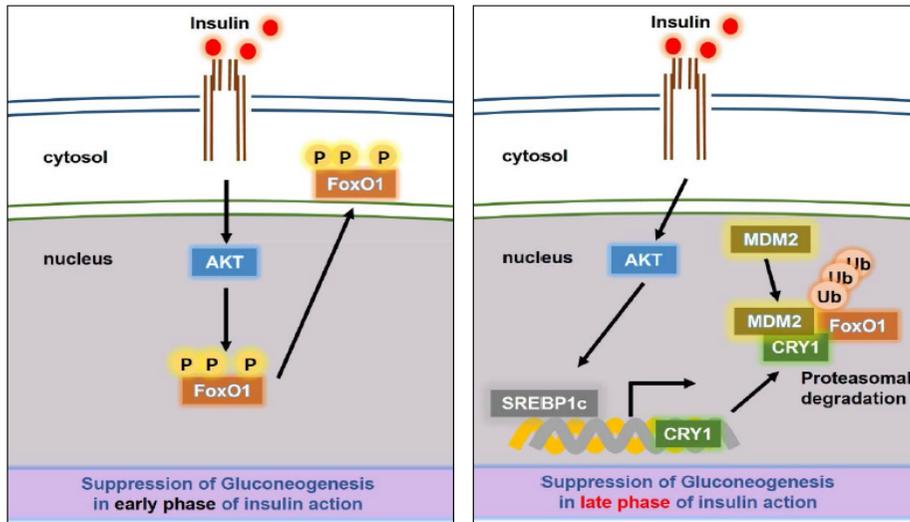
Livers were isolated every 6 h from *SREBP1c*^{+/+} and *SREBP1c*^{-/-} mice fed with normal chow diet and kept under 12 h: dark, 12 h: dark cycle for 1 week. Relative mRNA levels were determined by qRT-PCR and normalized by the level of the TBP mRNA. N=3-4 at each time point.



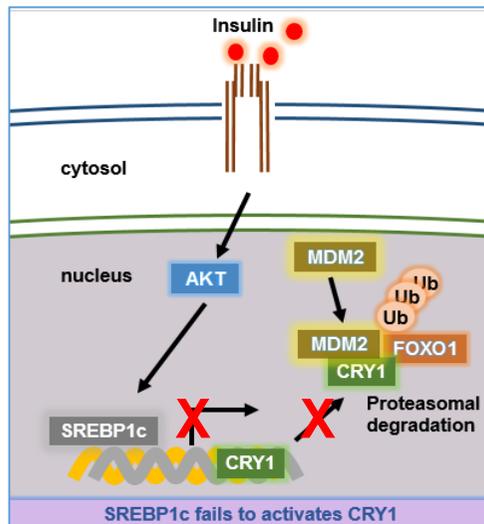
downregulating glucose metabolism upon insulin signaling with different target genes. Our study is the first report to reveal the role of SREBP1c in *CRY1* activation, which appears to be crucial in the regulation of hepatic glucose metabolism in the anabolic state. Based on the circadian oscillatory gene expression profile in *SREBP1c*^{-/-} mice, the SREBP1c protein may not actively govern hepatic circadian clock. However, increased expression of hepatic *CRY1* during the postprandial state is primarily regulated by the insulin-activated SREBP1c, which eventually leads to the suppression of glucose production via FOXO1 degradation (Figure 38). Although the roles of hepatic *CRY1* in energy metabolism need to be investigated further, our data provide an important clue to understand the molecular mechanisms that link hepatic SREBP1c and glucose homeostasis in physiological and pathological conditions.

Figure 38. Schematic diagram of the proposed model of chapter 2.

Normal Condition



Diabetic Condition



Conclusion and perspectives

1. Altering the hepatic circadian clock gene by feeding period restriction is associated with hepatic lipid and glucose metabolism, but not with body weight change

The harmony of the peripheral circadian clock and the central circadian clock is involved in diverse metabolic homeostasis. Therefore, it is likely that dysregulated circadian clocks are closely linked with metabolic disorders such as diabetes, obesity and cardiovascular disease. The central circadian clock is regulated by light signal whereas the peripheral circadian clock is controlled by food intake and diverse hormones. Interestingly, it has been reported that shift workers are prone to have high body mass index and cardiovascular events, indicating that unsynchronized circadian clock might participate in metabolic dysregulation (Aronoff et al., 2001; Colles et al., 2007; Tholin et al., 2009). In this aspects, it has been proposed that circadian clock regulation might be a potential therapeutic approach to cure for obesity or metabolic disorders.

As the prevalence of obesity continues to rise, its contribution to mortality increases. In particular, obesity-induced insulin resistance is one of the key factors for the development of metabolic diseases such as hypertension, atherosclerosis, and type 2 diabetes. The primary causes of obesity is simply expressed by higher energy intake compared to energy expenditure. To solve the relationship with

unsynchronized circadian clock and obesity, I analyzed two experimental groups that have equal amounts of energy intake with different circadian clock regulation by feeding period restriction.

In chapter 1, I have demonstrated that an unsynchronized circadian clock with the same amounts of energy intake had no effects on body weight change. Because rodents, including experimental mice, are nocturnal animals who consume more food in night time than day time, I have provided the night time feeding group with the equal amounts of food as the day time feeding group. Compared to the ad libitum group, the day time feeding and night time feeding group are offered restricted calorie intake with both NCD and HFD. Intriguingly, obtained data reveal that the same amounts of food intake with different feeding periods do not affect the body weight change regardless of NCD or HFD.

I also found out that lipid and glucose metabolisms were altered in liver upon feeding period restriction. The expression patterns of certain genes belonging to hepatic lipogenesis, gluconeogenesis, and lipid oxidation were influenced by feeding period alteration. Moreover, the levels of serum triacylglyceride, cholesterol, and glucose were changed, indicating that alteration of peripheral circadian clock genes is important for metabolic regulation. Although the mechanisms of how peripheral circadian clocks could regulate metabolic change are still to be elucidated, the results from chapter 1 is important for understanding the relationship between circadian clock and metabolic regulation.

2. Hepatic SREBP1c is a mediator of feeding dependent CRY1 gene alteration

Accumulating evidences have suggested that key metabolic regulators are involved in the modulation of circadian clock gene expression. For example, PPAR γ and its coactivator PGC1 α activate BMAL1 transcription and regulate circadian oscillation (Lamia et al., 2009; Schmutz et al., 2010; Wang et al., 2008a). Moreover, ROR α and Rev-erb α directly and inversely regulates BMAL1 gene expression to maintain circadian oscillation (Raspe et al., 2002; Tini et al., 1995). However, it has not been fully understood whether and how feeding or nutrient rich states might control circadian clock core genes in peripheral tissues.

In chapter two, I have shown that SREBP1c activates CRY1 gene expression. SREBP1c is expressed in a precursor form, which is bound to the ER membrane. Under insulin signaling, SREBP1c precursor is processed by site 1 protease and site 2 protease, and the truncated SREBP1c is located to the nucleus to activate its target gene such as FASN, SCD1 and itself. Further, SREBP1c is post-translationally activated with feeding signals accompanied with hormonal changes. CRY1 promoter contains both SRE motif and E-BOX motif which are binding motifs of SREBP1c. Also, E-box motif is a target element of BMAL1 and CLOCK heterodimer, which are the core regulators of circadian clock oscillation (Ramsey et al., 2007). I have demonstrated that SREBP1c stimulates CRY1 gene expression through SRE

sequence(s), not E-BOX motif. Luciferase assays performed with WT, 3XSRE mutants, and E-BOX have suggested that SRE sequences in the CRY1 promoter are target sites of SREBP1c. Because SREBP1c may not compete with BMAL1 and CLOCK at the E-box motif of CRY1 promoter, it appears that SREBP1c might have a little effects on circadian clock gene oscillation (Figure 37).

3. SREBP1c-CRY1 axis participates in inhibition of gluconeogenesis in postprandial state

SREBP1c is a well-known transcription activator that regulates lipid biosynthetic pathway in postprandial states. Recent data have also suggested that SREBP1c might participate in the suppression of hepatic gluconeogenesis (Lee et al., 2007; Yamamoto et al., 2004). As a transcriptional activator, SREBP1c directly regulates lipogenesis by binding to target gene promoters. However, the molecular mechanism by which transcriptional activator SREBP1c may suppress gluconeogenic genes are not fully elucidated.

In chapter two, I have revealed a novel signaling cascades of SREBP1c to mediate inhibition of hepatic gluconeogenesis. In liver, feeding or insulin mediated CRY1 activation by SREBP1c leads to degradation of FOXO1 protein, which is a crucial activator of hepatic gluconeogenesis. In primary hepatocytes, overexpression of SREBP1c downregulated glucose output and lowers PEPCK and G6Pase gene

expression. Overexpression of CRY1, as a novel target gene of SREBP1c, also repressed gluconeogenesis, whereas knock down of CRY1 led to increase in gluconeogenic gene expression. Among various signaling cascades that regulate gluconeogenesis, FOXO1 was regulated by CRY1. I have shown that CRY1 would act as a scaffold protein by binding with FOXO1 and MDM2 Ubiquitin E3 ligase MDM2 mediated FOXO1 ubiquitination, accompanied with FOXO1 protein degradation in liver. In addition, SREBP1c KO mice showed higher levels of blood glucose, and FOXO1 protein, compared to those of WT mice. Furthermore, CRY1 overexpression in SREBP1c KO mice alleviated pyruvate induced blood glucose level. CRY1 KO mice also exhibited increased FOXO1 protein and expression of gluconeogenic genes, concomitant with higher blood glucose level.

FOXO1 is a well-known transcription factor regulated by posttranslational modification. Under insulin signaling, insulin activated AKT phosphorylates FOXO1 protein, which accelerates the translocation of FOXO1 into cytoplasm (Nielsen et al., 2008; Tzivion et al., 2011; Wang et al., 2006). The key regulatory mechanism of insulin mediated suppression of gluconeogenesis has been reported that AKT mediated FOXO1 translocation. In this study, I have revealed a novel pathway that insulin sustainably suppresses hepatic gluconeogenesis by SREBP1c-CRY1. Insulin mediated FOXO1 phosphorylation occurs at an early time point whereas CRY1 is induced at the late stage of insulin action. I have discovered that CRY1 plays a key role to decrease FOXO1 protein upon insulin. To exclude the possibility that the effect

of AKT on FOXO1 phosphorylation, I have used AKT inhibitors. In both *in vitro* and *in vivo* experiments, AKT inhibitors intensified the stability of FOXO1 protein. In contrast, CRY1 overexpression dramatically lowers FOXO1 protein level with or without AKT inhibitor, implying that CRY1 would act as an inhibitor of FOXO1, independent of AKT activity.

Although AKT mediated FOXO1 phosphorylation is one of the major inhibitory mechanisms of hepatic gluconeogenesis, here, I proposed that SREBP1c mediated CRY1 induction would be another crucial signaling pathways that participates in the suppression of gluconeogenesis in the postprandial state.

4. Hyperglycemia is exacerbated by dysregulation of hepatic SREBP1c-CRY1 signaling pathway

Hepatic insulin signaling activates *de novo* lipogenesis for storage excess energy, and inhibits hepatic gluconeogenesis for maintaining blood glucose level. SREBP1c, one of key molecule in insulin signaling pathway, participates in both activation of *de novo* lipogenesis and inhibition of gluconeogenesis. Although hepatic SREBP1c activity is increased in obese animals, the inhibitory effect of SREBP1c on gluconeogenesis was not observed.

Here, I found out that SREBP1c was increased in HFD fed mice and *db/db* mice, while CRY1 was not elevated in both obese mice models. To address the

question which limited CRY1 might be a cause of hyperglycemia in obesity, I have investigated the effects of CRY1 overexpression on glucose metabolism. In *db/db* mice CRY1 overexpression lowered blood glucose level as well as hepatic gluconeogenic gene expression, implying that insufficient CRY1 may not be enough to block FOXO1 activity for normalizing blood glucose level in obese animals. Of course, it is necessary to further investigate why activated SREBP1c fails to increase CRY1 gene expression in obese animals. Moreover, it is likely that various approaches with CRY1 modulation, including stability control, gene expression regulation, chromatin structure modulation, and epigenetic management, will be possible as a therapeutic target.

Hyperglycemia is one of the many characters of obesity and diabetes. Lowering the blood glucose level is an important therapeutic approach for diabetes. Here, I have suggested that the peripheral circadian clock is important for metabolic regulation but not for body weight gain. Although obesity is associated with various metabolic complications, it appears that the primary cause of obesity is correlated with the amounts of food intake, but not circadian clock mediated metabolic changes. I have also elucidated a novel pathway that circadian clock is involved in the regulation of hepatic glucose metabolism. Under normal physiological condition, SREBP1c-CRY1 mediated FOXO1 degradation contributes to suppress gluconeogenesis. During this regulation, SREBP1c stimulated CRY1 expression and CRY1 acts as a scaffold protein by binding with MDM2 and FOXO1, leading to

FOXO1 degradation. Under pathophysiological conditions such as obesity, SREBP1c fails to activate CRY1 gene expression in liver, and as a consequence, gluconeogenesis is still highly activated. It is noteworthy that various approaches to modulate SREBP1c-CRY1 signaling pathway will give the opportunity for overcoming high blood glucose levels. Taken together, the characterization of the interaction between peripheral circadian clock and hepatic metabolism would be crucial for maintaining homeostasis of energy balance.

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국문 초록

최근 연구결과에 따르면 밤과 낮의 변화에 따라 발생하는 일주기성은 개체의 행동 뿐 아니라 분자신호전달에도 중추적인 역할을 담당한다. 또한, 정교하게 조절되는 일주기성은 몸의 에너지 항상성 유지에도 깊이 연관되어 있음이 보고되었다. 이러한 측면에서 일주기성이 유지되지 못하는 경우 포유동물은 비만 및 다양한 대사질환을 유발할 수 있다. 대표적인 에너지 대사 조직인 지방조직, 근육조직, 간조직의 일주기성은 지방생합성, 포도당생합성 및 지방 산화 작용 등을 조절한다. 또한, 중추조직과 말초조직의 일주기성이 일치하지 않는 야간 근로자의 경우 대사질환이 자주 발생하는 경향이 보고되고 있다. 그러나 일주기성의 생리적 및 병리적 역할과 체내 에너지 대사조절과의 상호 관계에 대해서는 분자수준의 연구가 부족한 상황이다.

본 연구를 통해 중추조직과 말초조직의 일주기성이 일치하지 않을 경우 간조직의 포도당 및 지방대사는 변화하는 반면 체중에는 영향을 미치지 않음을 발견하였다. 같은 양의 음식물을 다른 시간대에 섭취하는 조건에서 말초조직의 일주기성은 섭취 시간에 따라 변화하는 것을 관찰한 반면, 중추조직의 일주기성은 변하지 않는다는 것을 관찰할 수 있었다. 간조직에서 대표적인 일주기성 유전자인 BMAL1, CLOCK, PER2는

음식 섭취시간대에 따라 그 발현이 변함을 관찰하였다. 간조직의 지방대사, 포도당대사, 지방 산화에 연관된 유전자들 또한 음식섭취 시간대의 변화에 따라 발현양상이 다르게 조절되었다. 이와 같은 현상은 저칼로리 음식물이나 고칼로리 음식물을 섭취한 야생형 생쥐 모두에서 관찰되었다. 본 연구를 통하여 체중증가에 있어 음식물 섭취 시간대의 변화는 보다는 음식물의 섭취량이 핵심적임을 확인할 수 있었다.

본 학위 논문연구 동안 본인은 음식섭취와 인슐린에 의해 증가되는 CRY1 유전자가 포도당 생합성을 억제함을 발견하였다. 전사인자인 SREBP1c는 음식섭취에 의해 활성화되어 간조직에서 지방생합성을 관장하는데, 간세포의 SREBP1c가 음식섭취에 의해 CRY1의 발현을 증가 시킴을 발견하였다. 간세포주에 CRY1을 과발현 시켰을 경우 포도당생합성 유전자인 PEPCK, G6Pase의 발현이 억제되었고, FOXO1의 단백질량이 감소하였다. 간세포주에서 CRY1은 인슐린을 8시간 이상 처리했을 경우 증가되었고 이상의 연구를 통하여 CRY1은 인슐린이 지속적으로 포도당 생합성을 억제하는 과정 중 중요한 매개자의 역할을 수행하는 것으로 추정된다. 흥미롭게도, SREBP1c에 의해 증가된 CRY1은 FOXO1의 단백질 분해 기전을 촉진시켰으며, FOXO1의 E3 ubiquitin ligase인 MDM2와의 결합을 강화시키는 Scaffold protein의 역할을 담당할 수 있음을 발견하였다. 이와 관련하여, 간에서 SREBP1c가 증가되어 있음이 보고된

db/db 생쥐와 고지방성식이 유도 비만 생쥐 모델에서 CRY1의 발현이 동반하여 증가하지 않았으며, 그 결과 포도당생합성이 증가될 수 있음을 발견하였다. 그러나 *db/db* 생쥐에 CRY1을 과발현 시킨 경우 혈당을 낮추는 동시에 간에서 포도당생합성 관련 유전자들의 발현이 억제됨을 관찰하였다.

본 연구의 결과들을 종합하여 살펴볼 때, 일주기성 유전자는 간조직의 에너지대사를 능동적으로 조절하는 것으로 생각된다. 다양한 일주기성 유전자 가운데 SREBP1c에 의해 증가하는 CRY1은 FOXO1의 단백질 분해 과정 유도를 통해 간세포 내 포도당생합성 유전자의 발현을 억제할 수 있다. 또한, 체중 증가는 말초기관의 일주기성 유전자의 발현 변화에 의한 것이 아니라 섭취한 음식물의 에너지 양에 의해 결정되는 것으로 추정된다. 그러므로 적절한 SREBP1c-CRY1 신호전달 경로의 조절은 체내 에너지대사 항상성 유지에 핵심적인 신호전달 경로 중 하나임을 제안한다.

주요어: 비만, 당뇨, 일주기성, SREBP1c, CRY1, FOXO1, MDM2, PEPCK, G6Pase, 포도당생합성, 인슐린 신호전달경로

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