Hairy and Enhancer of Split 6 Prevents Hepatic Lipid Accumulation Through Inhibition of \textit{Pparg2} Expression

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Peroxisome proliferator-activated receptor gamma (PPAR\textsubscript{\gamma}) is a master regulator for white adipocyte differentiation and lipid storage. The increased level of hepatic PPAR\textsubscript{\gamma}2 isoform reprograms liver for lipid storage and causes abnormal fat accumulation in certain pathophysiologic conditions. The current study aimed to investigate a role of transcriptional repressor hairy and enhancer of split 6 (HES6) in the regulation of \textit{Pparg2} expression and hepatic steatosis induced by diet. Liver-specific overexpression of \textit{Hes6} using adenovirus reduced \textit{Pparg2} messenger RNA levels by 90\% and hepatic triglyceride accumulation by 22\% compared to the levels in mice injected with an adenoviral empty vector with Western diet feeding. In sharp contrast, silencing \textit{Hes6} gene expression using short hairpin RNA increased hepatic lipid accumulation and \textit{Pparg2} messenger RNA levels by 70\% and 4-fold, respectively. To locate hepatocyte nuclear factor 4 alpha (HNF4\textalpha) binding site(s), through which repressional activity of HES6 is mediated, a 2.5-kb \textit{Pparg2} promoter-driven luciferase reporter was constructed for transient transfection assays. Subsequently, chromatin immunoprecipitation and electrophoretic mobility shift assays were performed. An HNF4\textalpha binding consensus sequence was identified at 903 base pairs upstream from the transcription start site of \textit{Pparg2}. Deletion or point mutation of the sequence in a luciferase reporter containing the \textit{Pparg2} promoter abolished HNF4\textalpha-mediated activation in HeLa cells. Chromatin immunoprecipitation and electrophoretic mobility shift assays further confirmed direct recruitment and binding of HNF4\textalpha to the site. Gene expression analysis with liver samples from subjects with nonalcoholic steatohepatitis suggested that the axis of the \textit{Hes6–Hnf4a–Pparg2} transcriptional cascade is also responsible for hepatic fat accumulation in humans. \textbf{Conclusion:} HES6 represses \textit{Pparg2} gene expression, thereby preventing hepatic lipid accumulation induced by chronic Western diet feeding or pathophysiologic conditions. (\textit{Hepatology Communications} 2017;1:1085–1098)

\textbf{Introduction}

The liver is the major organ for synthesizing and delivering triglycerides in the form of very low density lipoprotein (VLDL) to other peripheral tissues, such as muscle and adipocytes, for energy use or storage.\textsuperscript{(1)} Fat accumulation in the liver is considered the initial step before proceeding into nonalcoholic steatohepatitis (NASH). Under certain pathophysiologic conditions, such as insulin resistance or obesity, lipid homeostasis in the liver can be disrupted and hepatic steatosis ensues. A subsequent insult presented by inflammation, oxidative stress, or dietary factors would advance benign steatosis into the next disease stage, which is NASH.\textsuperscript{(2)} Those insults can originate from other peripheral sources, such as macrophages, adipocytes, and intestinal gut bacteria.\textsuperscript{(3,4)} Unlike the underlying mechanisms of how these second hits strike, pathways involved in hepatic lipid accumulation have been relatively well studied and documented.\textsuperscript{(5,6)}

\textbf{Abbreviations:} Ad, adenovirus; Adnull, adenovirus without an insert; cDNA, complementary DNA; ChIP, chromatic immunoprecipitation; CIDEC, cell death inducing DNAse like effector C; CoA, coenzyme A; Creb\textsubscript{H}, cyclic-adenosine monophosphate-response-element binding protein H; EMSA, electrophoretic mobility shift assay; Fsp27, fat specific protein 27; Hes6, hairy and enhancer of split 6; Hnf4\textalpha, hepatocyte nuclear factor 4 alpha; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Ppar, peroxisome proliferator activated receptor; RAR, retinoic acid receptor; sh, short hairpin; Shp, small heterodimer partner; TG, triacylglycerol; VLDL, very low density lipoprotein; WD, Western diet.

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Circulating fatty acids from white adipose tissues mainly contribute to hepatic fat accumulation rather than fatty acids from de novo synthesis.\(^{(2)}\) One of the suggested driving forces for this fat accumulation is induction of the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPAR\(_\gamma\)), especially the PPAR\(_\gamma\)2 (hepatocyte-specific isoform)-associated lipogenic program.\(^{(7,8)}\) In a recent study, Matsusue et al.\(^{(9)}\) reported that PPAR\(_\gamma\) directly activates expression of fat-specific protein 27 (Fsp27/CIDEc) in the liver, thereby promoting hepatic steatosis. As a lipid droplet binding protein, FSP27 has been reported to protect lipid droplets from enzymatic hydrolysis and maintain triacylglycerol (TG) storage in adipose tissues.\(^{(10-12)}\) This functional property of the protein is a major mechanism underlying hepatic steatosis induced by PPAR\(_\gamma\).\(^{(9)}\)

The orphan nuclear hormone receptor small heterodimer partner (Shp, Nr0b2) is involved in bile acid, lipid, and glucose metabolism. Shp\(^{-/-}\) mice displayed a protective phenotype against diet-induced obesity and hepatic steatosis.\(^{(13,14)}\) We have shown that the deletion of Shp directly derepresses transcriptional activity of PPAR\(_\gamma\), thereby inducing the expression of its target genes, especially those involved in fatty acid oxidation, such as carnitine palmitoyltransferase 1 alpha and acyl-coenzyme A (CoA) oxidase 1.\(^{(14)}\) The loss of Shp also resulted in lower expressions of Pparg2 and Fsp27 in the liver, which is mediated through a transcriptional cascade, including a novel repressor hairy and enhancer of split 6 (HES6).\(^{(15)}\)

The mouse Hes6 gene was cloned in an attempt to isolate transcriptional regulators in muscle and neuronal differentiation using degenerate primers targeting basic domain helix-loop-helix transcription factors.\(^{(16)}\) Unlike other basic helix-loop-helix proteins, HES6 promotes neuronal differentiation without DNA binding. This may be achieved by inhibition of HES1-mediated transcription, which negatively regulates muscle and neuronal differentiation, through direct protein–protein interaction.\(^{(17-19)}\) The term Hes originated from its structural homology of the Drosophila hairy and enhancer of split proteins.\(^{(17)}\) Other than the described muscle and neuronal function, HES6 plays roles in hepatic lipid metabolism.\(^{(20)}\) In one of the proposed roles, HES6 interacts with nuclear hormone receptor hepatocyte nuclear factor 4 alpha (HNF4\(_\alpha\)) to repress the expression of Pparg, which encodes a positive regulator in lipogenic and adipogenic programs. Our recent study revealed that nuclear receptor Shp and retinoic acid receptor (RAR) coordinately regulate Hes6 gene expression.\(^{(15)}\) Overexpression or activation of hepatic RAR by adenovirus or all trans retinoic acid treatment increased expression of Hes6 and reduced Pparg2 and Fsp27 gene expression, which inhibits hepatic lipid accumulation.

In the current study, we investigated the effect of HES6 on Pparg2 gene expression and hepatic steatosis in mice through adenovirus-mediated overexpression and silencing and identified a responsive sequence, mediated through HNF4\(_\alpha\), on the Pparg2 promoter region by using transient transfection and gel electrophoretic mobility shift assays.

**Materials and Methods**

**ANIMAL EXPERIMENTS**

Male C57BL/6N mice (Harlan, Indianapolis, IN) were used throughout this experiment. To overexpress...
Hes6 in mouse liver, adenovirus containing Hes6 (AdHes6) complementary DNA (cDNA) was injected into 4-month-old mice fed a Western diet (WD) (TD.88137; Envigo, Madison, WI) for 2 months in advance through the tail vein in a dose of $1 \times 10^9$ plaque-forming units per mouse. To silence Hes6 expression in mouse liver, adenovirus expressing short hairpin RNA (shRNA) targeting the Hes6 gene was delivered into 3-month-old mice fed chow with the same dose. The mice were maintained on a WD for an additional 2 weeks, and their blood and tissues were collected after overnight fasting. All mice were housed in the accredited pathogen-free facility at Northeast Ohio Medical University on a 12-hour light–dark cycle and with free access to diets and water. All animal experiments were based on the protocols reviewed and approved by the Institutional Animal Care and Use Committee of Northeast Ohio Medical University.

CONSTRUCTION OF ADENOVIRUSES

The mouse Hes6 cDNA clone was purchased from Open Biosystems (Dharmacon, Lafayett, CO) and subcloned into a pacAd5 cytomegalovirus shuttle vector to generate AdHes6 based on the protocols provided by the manufacturer (Cell Biolabs Inc., San Diego, CA). In order to design shRNA for mouse Hes6, small interfering RNA Wizard and BLOCK-iT RNAi Designer (InvivoGen) were used. The designed oligonucleotides were synthesized, and annealed double-stranded DNAs were subcloned into a pacAd5 cytomegalovirus shuttle vector to generate four AdshHes6 constructs. After testing the efficacy of silencing in Hepa1-6 cell lines, a construct containing the targeting sequence 5'-GATCCGCACGGATCAACGAGAGTCTTTCAAGAGAAGACTCTCGTTGATCCGTGCTTTTTTG-3' was chosen for further animal experiments. Adenoviruses were propagated in 293T cell lines and purified using CsCl gradient centrifugation. Viral titration was performed using the Adeno-X Rapid Titer Kit (Clontech Laboratories, Inc., Mountain View, CA).

HEPATIC LIPID EXTRACTION AND LIVER STAINING

Lipid was extracted from liver using chloroform, methanol, and a phosphate-buffered saline mixture (2:1:0.75 ratio). Extracted lipid in the chloroform layer was dried overnight and dissolved in a solvent consisting of 95% isopropanol and 5% Triton X-100. TG concentration was determined by a commercial kit (Thermo Fisher Scientific, Rockford, IL) and normalized to wet liver weight. Hematoxylin and eosin and Oil Red O staining have been described.(15)

WESTERN BLOT ANALYSIS

Western blotting was performed with protein extracts from liver as described.(21) Anti-HES6 antibody (Thermo Fisher Scientific) was used in a 1:500 dilution. After visualizing HES6 proteins, the membrane was stripped for β-actin antibody incubation (1:1,000 dilution; Novus Biologicals, Littleton, CO).

MOUSE HEPATOMA CELLS TRANSDUCED WITH ADENOVIRAL CONSTRUCTS

The Hepa1-6 mouse hepatoma cell line was transduced with AdHes6 or AdshHes6 to test the effect of HES6 on lipid accumulation in vitro. Cells were split into six-well plates and maintained in Dulbecco’s modified Eagle’s medium plus 10% bovine growth serum (GE Life Sciences, Logan, UT) until 90% confluency. Cells at 90% confluency were treated with adenoviral constructs (adenovirus without an insert [Adnull], AdHes6, or AdshHes6) at $1 \times 10^{10}$ plaque-forming units/mL in triplicates. Cells were further maintained for 3 days and replenished with fresh media containing 1 mM free fatty acids (palmitic acid and oleic acid, 1:2) plus 1% albumin. Cells were harvested 24 hours later for Hes6 expression or TG analysis. Intracellular TG was extracted using ethanol saponification.(22) Accumulated TG was quantified by comparing with values obtained from cells in media without free fatty acids and normalized to protein content.

RNA ISOLATION AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from livers and white adipose tissues of experimental mice or cultured Hepa 1-6 cells using Trizol reagents (ThermoFisher Scientific). RNA isolated from human liver tissues of normal patients and patients with NASH have been described.(23) cDNA was synthesized from the isolated RNA using PrimeScript RT Master Mix (Clontech). Quantitative PCR (qPCR) analysis was performed as described.(21) Gene-specific primer sequences were
obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank) and reported publications (Supporting Table S2).

TRANSIENT TRANSFECTION AND CHROMATIN IMMUNOPRECIPITATION

Luciferase reporter genes driven by mouse Pparg2 promoters were constructed by insertion of PCR-amplified promoters into pGL3 reporter plasmid (Promega, Madison, WI). All plasmids and transfection assays used in this study have been described.\(^{(15)}\) HeLa and Hepa1-6 were maintained in Dulbecco’s modified Eagle’s medium plus 10% bovine growth serum. A chromatin immunoprecipitation (ChIP) assay was also performed to identify recruitment of HNF4\(_a\) and HES6 to the Pparg2 promoter region using mouse liver as described.\(^{(15)}\)

ELECTROPHORETIC MOBILITY SHIFT ASSAY

To test direct binding of HNF4\(_a\) to a specific site on the Pparg2 promoter, an electrophoretic mobility shift assay (EMSA) was performed using a radiolabeled DNA probe as described.\(^{(24)}\) HNF4\(_a\) protein was expressed using the TNT coupled reticulocyte lysate system (Promega, Madison, WI). Anti-HNF4\(_a\) antibody (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Full sequences of the DNA probes used in this study are described in Supporting Table S3.

STATISTICS

Values are presented as means ± SEM. The Student \(t\) test was used to compare two different groups. \(P\) values less than 0.05 were considered significantly different between groups.

Results

OVEREXPRESSION OF Hes6 AMELIORATES HEPATIC LIPID ACCUMULATION INDUCED BY A WESTERN DIET

Our previous study suggested that a novel transcriptional cascade was responsible for hepatic lipid metabolism.\(^{(15)}\) We showed that all-retinoic acid treatment or overexpression of a hepatic RAR induced Hes6 gene expression in mouse liver, this inhibits lipogenic programs and facilitates faster lipid mobilization, thereby protecting the liver from lipid accumulation induced by a WD. In the current study, we explored a direct role of HES6 in the transcriptional cascade and hepatic lipid metabolism. To test the effect of overexpression of Hes6 in mouse liver, C57BL/6 mice fed the WD for 2 months were injected with AdHes6 or Adnull through the tail vein and maintained for an additional 2 weeks under the WD regimen. The AdHes6-injected mice displayed less pale liver than the Adnull-injected control mice (Fig. 1A, left panel). Liver histology indicated that Hes6 overexpression markedly attenuated hepatic steatosis developed by WD feeding (Fig. 1A, center panel). Their triglyceride level was significantly less than that of the control mice (Fig. 1A, right panel). A similar pattern was observed with hepatic cholesterol level, although statistical significance was not manifested (Supporting Fig. S1A). However, levels of serum TG and cholesterol were not affected by Hes6 overexpression. AdHes6 injection clearly induced Hes6 messenger RNA (mRNA) and protein expressions by around 2-fold in the liver but minimally in white adipose tissue, demonstrating its liver-specific expression (Fig. 1B; Supporting Fig. S2A). Hes6 overexpression significantly inhibited the expression of its downstream target genes Pparg2 and Fsp27.\(^{(15)}\) However, the mRNA levels of its upstream transcription factors Shp and Rar\(_a\) were not affected. We observed a comparable amount of food intake and body weight change between the two groups after virus injection (Supporting Fig. S3A).

To determine whether the reduction of hepatic lipid accumulation observed in an animal model is a direct consequence of Hes6 overexpression, we overexpressed the Hes6 gene in Hepa1-6 cells, a mouse hepatoma cell line, using AdHes6 and measured intracellular TG concentration after free fatty acid challenge. AdHes6 transduction efficiently increased Hes6 expression and decreased intracellular TG levels, which is in accord with the results from the animal study (Fig. 1C).

SILENCING Hes6 EXACERBATES FAT ACCUMULATION IN THE LIVER

To explore the effect of Hes6 silencing on hepatic fat accumulation and expression of genes in the transcriptional cascade, AdshHes6 was constructed and delivered into 3-month-old wild-type C57BL/6 mice.
Immediately after adenoviral injection, the mice were subjected to WD feeding for 2 weeks. The adenovirus injection effectively reduced the Hes6 mRNA and protein expressions by 65% and 53%, respectively, in the mouse liver, which resulted in increased mRNA expressions of Pparg2 and Fsp27 but not Pparg1, Shp, and Hnf4a (Fig. 2B; Supporting Fig. S2B). In contrast to the overexpression, silencing resulted in significant hepatic fat accumulation after the 14-day WD feeding regimen, as demonstrated by gross morphology and Oil Red O staining (Fig. 2A, left panel). Both triglyceride and cholesterol levels were significantly increased in the liver of AdshHes6-injected mice compared to those of their Adnull-injected counterparts (Fig. 2A; Supporting Fig. S1B). However, serum TG and cholesterol levels, food intake, and body weight changes were comparable between the two groups as observed in AdHes6 injection (Supporting Figs. S1B, S2B).
We also tested Hes6 silencing in Hepa1-6 cells using AdshHes6. The infection with AdshHes6 strongly blunted Hes6 mRNA expression and caused significantly higher TG accumulation in the cells when compared to Adnull infection (Fig. 2C). Along with the result from Hes6 overexpression, this in vitro observation suggests that HES6 directly regulates TG accumulation in hepatocytes.

**HNF4α AND HES6 DIRECTLY REGULATE EXPRESSION OF THE Pparg2 GENE**

Martinez-Jimenez et al.\(^{(20)}\) reported that HNF4α directly up-regulates the expression of Pparg and that Hes6 inhibits the expression through an active and direct repression on HNF4α transcriptional activity.
However, they presented mRNA levels of *Pparg1* while performing ChIP and transient transfection assays were performed with *Pparg2* promoter, generating ambiguities in the regulation of *Pparg* gene transcription by HES6 and HNF4α. As the two *Pparg* genes are regulated by two separate promoters, we performed a transient transfection assay using mouse 2.5-kb *Pparg2* promoter-driven luciferase reporter to test direct activation of *Pparg2* by HNF4α. Cotransfection of *Hnf4a* expression plasmid significantly activated the reporter gene, and the addition of *Hes6* inhibited HNF4α-mediated transactivation (Fig. 3A). In contrast to our earlier study, SHP failed to inhibit HNF4α transcriptional activity, suggesting a strong promoter specificity in the formation of the SHP/HNF4α or HES6/HNF4α complex. To confirm the direct effect of HNF4α on the 2.5-kb promoter activity, we constructed a *Pparg2* promoter lacking the potential binding site (Fig. 3B, also refer to Fig. 4). ChIP analysis on the *Pparg2* promoter region from mouse livers using indicated antibodies. Left, –0.25 kb region for the SHP/HNF4α binding sites. Right, –3.0 kb region for the negative control. All the values are presented with SEM. Abbreviations: CDM8, backbone expression plasmid vector; IgG, immunoglobulin G; RLU, relative luciferase unit.

### IDENTIFICATION OF THE HNF4α BINDING SITE RESPONSIBLE FOR *Pparg2* GENE EXPRESSION

In order to locate the HNF4α binding site, additional deletion reporter constructs were generated (Fig. 4, section D2-D4) and used in a transient transfection assay. The D3 deletion mutant failed to respond to *Hnf4a* cotransfection, indicating that a potential HNF4α binding site is in the D3 region (Fig. 5, left panel). Based on the consensus HNF4α sequences identified by protein binding microarrays (Fig. 5B, Consensus), we located the potential HNF4α binding sequence in the D3 region (bold and underlined letters in Figs. 4 and 5B, line S3). Interestingly, the previously reported potential binding sequence (Fig. 5B, line S1) is different from the consensus. To confirm HNF4α specificity for the identified sequence, base mutations were introduced into the sequence as...
depicted in Fig. 5B (S3mut) on the 2.5-kb \textit{Pparg2} reporter (D3mut) and transfection assays were performed in HeLa cells. The introduction of mutations completely abolished the induction of reporter gene activity with \textit{Hnf4a} cotransfection (Fig. 5A, right panel). EMSA was performed to further examine direct binding of HNF4\textsubscript{a} using 30-mer oligonucleotides containing the sequence as a probe (Fig. 5C). HNF4\textsubscript{a} expressed in reticulocytes clearly formed a shifted radiolabeled band (lane 2), which was further shifted by anti-HNF4\textsubscript{a} antibody (lane 5). The band was significantly diminished by competition with a 10-fold excess of cold self-probe (lane 3) but not with cold probe with the mutations (lane 4). A probe containing an earlier reported sequence failed to compete the HNF4\textsubscript{a} binding activity away (lane 6). All these results clearly indicate that the newly identified sequence is truly a key element regulating \textit{Pparg2} gene expression by HNF4\textsubscript{a} and HES6, thereby controlling hepatic lipid accumulation. As the HNF4\textsubscript{a} response sequence is conserved between human PPARG2 and mouse \textit{Pparg2} promoter,\textsuperscript{(29)} we quantified mRNA levels of genes in the proposed transcriptional cascade in the previously studied livers of patients with NASH who had significantly higher hepatic TGs than normal subjects.\textsuperscript{(23)} Along with the reported higher TG values, these liver samples exhibited higher inflammatory and fibrotic gene expression, typical characteristics of
NASH, than normal samples (Fig. 6A). Although statistical significance was lacking due to a small sample size, the patients with NASH displayed a similar pattern of gene expression observed from mouse studies: lower HES6 expression and higher PPARG2 and CIDEC gene expression than those from normal subjects, as shown in Fig. 6B, suggesting that the regulatory role of HES6 and PPARG2 in the development of nonalcoholic fatty liver disease (NAFLD) is conserved, at least in human and mouse.

PPARγ2 ACTIVATES BOTH Fsp27a and Fsp27b TRANSCRIPTION IN DIET-INDUCED HEPATIC STEATOSIS

A recent study reported that the liver predominantly produces FSP27β, which contains 10 more amino acids at the N-terminal of the originally reported FSP27 (FSP27α). According to the study, the transcription of Fsp27b starts at 950 base pairs downstream...
of the original Fsp27a and is directly regulated by cyclic-
adenosine monophosphate–response-element
binding protein H (CREBH), whereas Fsp27a is
regulated by PPARγ. The suggested role of CREBH
and FSP27β in hepatic fat accumulation by fasting has also
been observed in ethanol-induced hepatic steatosis.(31)
Thus, we tested the involvement of
Creb3l3 and Fsp27b in diet-induced hepatic steatosis using
quantitative PCR analysis. We found a 6-month WD regi-
men strongly up-regulated hepatic Pparg2, Fsp27a,
and Fsp27b expressions and significantly reduced Hes6
mRNA level (Fig. 7A). As noted in the report, Fsp27b
expression was several hundred-fold higher than the
Fsp27a expression, confirming Fsp27b as the major
hepatic isoform (data not shown). However, the WD
failed to induce Cleb3l3 (a gene encoding CREBH)
expression in the liver. These results strongly suggest
that PPARγ2, not CREBH, is the major activator for
both Fsp27a and Fsp27b expressions in the
development of diet-induced hepatic steatosis. In addi-
tion, Hes6 overexpression in the liver repressed the
mRNA levels of Fsp27a and Fsp27b, while knockdown
by shHes6 markedly induced these levels (Fig. 7B). In
contrast, the expression of Creb3l3 was affected margin-
ally by these modifications of Hes6 expression. Moreover,
the level of CREB3L3 mRNA was reduced in the livers of
patients with NASH, arguing against its potential role
in development of NAFLD (Fig. 7C). In our model sys-
tem where hepatic steatosis was manipulated by WD or
Hes6 expression, PPARγ2 appears to be the main activa-
tor for Fsp27b expression and hepatic fat accumulation,
whereas CREBH affects the pathway minimally, if any,
in response to those challenges. CREBH may be an
important regulator for the hepatocyte-specific expres-
sion of Fsp27b and TG mobilization during a fed–fast
cycle. Induction of PPARγ2 may synergistically increase
Fsp27b promoter activity in conjunction with CREBH
DNA binding during WD feeding.

FIG. 6. Conserved regulation of PPAR2 gene expression by
HES6 in patients with NASH. Total RNAs were extracted from
liver biopsy of normal subjects and subjects with NASH (n = 8).
(A) Subjected to real-time qPCR to assess mRNA expression of
inflammation-related genes. (B) Expression of genes in the pro-
posed cascade including HES6 and PPAR2. Expression levels
in patients with NASH were compared to those in normal sub-
jects and plotted with SEM. *P < 0.05; **P < 0.01. Abbrevia-
tions: ACTA2, smooth muscle actin alpha 2; COL1A2, collagen
type I alpha 2 chain; IL, interleukin; TNF, tumor necrosis factor.

Discussion
The liver functions as a major organ to uptake fatty
acids from circulation and deliver them along with
newly synthesized ones to peripheral tissues. In certain
pathophysiologic conditions, the liver fails to maintain
homeostasis, which results in lipid accumulation and
leads to development of hepatic steatosis. The progres-
sion of NAFLD was initially proposed as a two-hit
theory in which hepatic steatosis is a prerequisite con-
dition for development of more serious NAFLD, such as
NASH, fibrosis, and cirrhosis.(32) A simple basis for
the development of steatosis is an imbalance between the
output and input of hepatic lipids. The output is
represented by fatty acid oxidation and VLDL secre-
tion and the input by de novo lipogenesis and uptake of
fatty acids from circulation. Our previous studies sug-
gested that orphan nuclear receptor Shp knockout mice
are protected from diet-induced hepatic steatosis due
to increased fatty acid oxidation by relieving its repres-
sion on PPARα target genes. In a subsequent study,
we additionally suggested that Shp is involved in regu-
lating a novel transcriptional cascade, which deter-
mines the fate of hepatic lipid through controlling
lipogenesis and adipogenesis. PPARγ2, a master regu-
lator for adipogenesis and lipogenesis in adipocytes,
was suggested to be a major downstream transcription
factor in the proposed cascade. The expression of the
Pparg2 gene in the liver is repressed in normal condi-
tions, possibly to accommodate quick fat mobilization.
In agreement with this, hepatic expression of \textit{Pparg2} not \textit{Pparg1} is increased in obesity induced by diet or genetic manipulations, and its increase is believed to be an initial step in the development of hepatic steatosis.\cite{8,33,34} Martinez-Jimenez et al.\cite{20} proposed that HES6 and HNF4α directly suppress \textit{Pparg} transcription, and our previous study demonstrated that expression of \textit{Hes6} and \textit{Pparg2} are reciprocally related to each other in mouse models of diet-induced obesity.\cite{15}

Although a regulatory function of HES6 in hepatic \textit{Pparg} transcription has been suggested, its direct involvement in development of steatosis was challenged by the transgenic mouse study in which hepatic overexpression of \textit{Hes6} did not result in any significant changes in lipid accumulation on either the fed or fasted conditions of chow-fed mice.\cite{20} However, a recent report studying animals with liver-specific knockout of Fos-related antigen 1 (\textit{Fra1}), a

![FIG. 7. Expression of hepatic \textit{Fsp27b} and its upstream regulators \textit{Pparg2} and \textit{Creb3l3} in mice and in patients with NASH. Indicated hepatic mRNA levels from (A) 6-month WD-fed WT mice or (B) Hes6-overexpressed (AdHes6) or Hes6-silenced (AdshHes6) mice were quantified and compared to levels from corresponding controls using real-time qPCR. (C) Indicated hepatic gene expressions were also evaluated in normal individuals and patients with NASH. The relative gene expression was plotted with SEM. *\textit{P} < 0.05; **\textit{P} < 0.01; ***\textit{P} < 0.005 compared to control or normal samples.](image)
component of activator protein 1 dimers, emphasized the importance of the transcriptional cascade PPARγ2 and downstream targets in the development of NAFLD by cellular stress. The current study was aimed to understand the regulation of Pparg2 expression by HES6 and HNF4α and critically test the function of HES6 on hepatic lipid accumulation on a WD challenge. Overexpression of Hes6 attenuated hepatic fat accumulation and Pparg2 gene transcription in WD-fed C57BL/6 mice. In contrast, silencing of Hes6 with shRNA exacerbated hepatic fat accumulation and further increased Pparg2 transcription. Activation of Pparg2 promoter by HNF4α and inhibition of the activity by addition of HES6 in transient transfection assays clearly indicated that the two transcription factors directly regulate Pparg2 expression. ChIP and EMSA identified an HNF4α binding site, which is almost identical to the consensus sequence reported by Bolotin et al. and is also conserved in human PPARG2 promoter. Although a strong interaction between HNF4α and HES6 had been manifested and a molecular mechanism of HES6-mediated transcriptional repression has been proposed in myogenesis, how Pparg2 gene is repressed by these two transcription factors remains elusive. In an EMSA assay, HNF4α binding was diminished with HES6 addition, which argues against the proposed repression mechanism and the recruitment of Hes6 on the Pparg2 promoter (data not shown; Fig. 3C). Nonetheless, the data from human subjects with NASH suggest that the proposed transcriptional cascade is also similarly important in hepatic lipid homeostasis in human. Although the liver biopsies from subjects with NASH displayed a significant increase in the expression of inflammatory genes, a direct link between Hes6 expression and NASH development requires further investigation. Unlike Shp−/− mice, the expression of many of the major genes involved in hepatic fatty acid oxidation and synthesis were not affected by overexpression or silencing of Hes6 despite changes in fat accumulation with WD feeding (data not shown). Among the tested genes, stearoyl CoA desaturase 1 was significantly up- or down-regulated by overexpression or silencing of Hes6, respectively (Supporting Fig. S2). As desaturation of fatty acids was demonstrated as a critical step in the hepatic fat accumulation with high-carbohydrate diet feeding, we concluded that stearoyl CoA desaturase 1 also plays a role in the hepatic lipid metabolism affected by the Hes6-Pparg2 cascade along with FSP27β. Even though other important fatty acid synthesis or oxidation genes were not affected by the cascade, clear changes in hepatic fat accumulation in mouse models of HES6 highlight the importance of the role of FSP27 in lipid droplet protection and the development of hepatic steatosis. As evidenced by a recent observation, the rates of fatty acid esterification into hepatic TGs are dependent on the levels of plasma fatty acids. Our study emphasizes that hepatic fatty acid uptake and subsequent TG use represented by TG hydrolysis or VLDL production are crucial factors determining the development of hepatic steatosis. Therefore, induction of Fsp27 and stearoyl CoA desaturase 1 by the Hes6–Hnf4a–Pparg2 axis must be sufficient to result in significant fat accumulation in the liver without an increase in de novo fatty acid synthesis in our animal models.

A recent study identified the liver-specific Fsp27 transcript (Fsp27b), a longer version of original Fsp27 (Fsp27a), which encodes an additional 10 amino acids at its N-terminal end under the control of a basic leucine zipper transcription factor, CREBH. The study argued strongly against an earlier report that has shown regulation of hepatic Fsp27 expression by PPARγ. However, an expression profile of mRNAs in the regulatory axis revealed that hepatic CREBH was not induced in the settings of WD feeding, Hes6 gene silencing, and NASH, where fat accumulation and induction of Pparg2 and Fsp27 expressions were evident. Thus, PPARγ2 not CREBH plays a more critical role in Fsp27 expression and subsequent fat accumulation in these contexts. Although further study is required, CREBH appears to be an important cofactor for maintaining expression of Fsp27b in a liver-specific manner, and induction of Pparg2 activates transcription of both isoforms of Fsp27 effectively as shown in our results. Therefore, HES6, a novel transcriptional repressor of Pparg2 expression, should be considered an important regulator for hepatic fat metabolism in response to a variety of physical and environmental conditions.

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