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Synergistic regulation of hepatic *Fsp27b* expression by HNF4α and CREBH

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Abstract

The CIDE (cell death-inducing DFF45-like effector) family composed of CIDEA, CIDEB, CIDEC/FSP27 (fat-specific protein 27), has a critical role in growth of lipid droplets. Of these, CIDEB and CIDEC2/FSP27B are abundant in the liver, and the steatotic livers, respectively. Hepatocyte nuclear factor 4α (HNF4α) has an important role in lipid homeostasis because liver-specific HNF4α-null mice (*Hnf4a*$^{\Delta Hep}$ mice) exhibit hepatosteatosis. We investigated whether HNF4α directly regulates expression of CIDE family genes. Expression of *Cideb* and *Fsp27b* was largely decreased in *Hnf4a*$^{\Delta Hep}$ mice, while expression of *Cidea* was increased. Similar results were observed only in *CIDEC2*, the human orthologue of the *Fsp27b*, in human hepatoma cell lines in which HNF4α expression was knocked down. Conversely, overexpression of HNF4α strongly induced *CIDEC2* expression in hepatoma cell lines. Furthermore, HNF4α transactivated *Fsp27b* by direct binding to an HNF4α response element in the *Fsp27b* promoter. In addition, *Fsp27b* is known to be transactivated by CREBH that is regulated by HNF4α, and expression of CREBH was induced by HNF4α in human hepatoma cells. Co-transfection of HNF4α and CREBH resulted in synergistic transactivation and induction of *Fsp27b* compared to that of HNF4α or CREBH alone. These results suggest that HNF4α, in conjunction with CREBH, plays an important role in regulation of *Fsp27b* expression.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.05.070.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Keywords

HNF4α; CIDE family; *Fsp27b*; CREBH; Liver

1. Introduction

Hepatocyte nuclear factor 4α (HNF4α) is a member of the nuclear receptor superfamily and is a master regulator of the differentiation status of hepatocytes and binds to about 12% of the genes in human hepatocytes and positively regulates hepatocyte-specific gene expression [1-4]. Liver-specific *Hnf4a*-null mice (*Hnf4a*$^{\Delta Hep}$ mice) were found to exhibit hepatosteatosis, partially due to a defect of VLDL secretion by downregulation of microsomal triglyceride transfer protein (*Mttp*) and many apolipoproteins including *Apob* [5], indicating that HNF4α has an important role in lipid homeostasis in liver.

Among many target genes of HNF4α, the *CIDEB* promoter was transactivated by HNF4α [6,7]. CIDEB is a member of the cell death-inducing DEF45-like effector (CIDE) protein family with CIDEA and CIDEC/FSP27 (fat-specific protein 27) that are involved in growth of lipid droplets. Of these, *Cidea* is induced in livers of HFD-fed and *ob/ob* mice [8,9], and is conversely reduced in mouse livers that are resistant to hepatosteatosis [10]. Thus, CIDEA plays an important role in the development of hepatosteatosis. *Cideb* is abundantly expressed in liver, and *Cideb*-deficient mice are resistant to HFD-induced hepatosteatosis [11]. Furthermore, CIDEB may be a critical factor controlling VLDL secretion by interaction with APOB [12], revealing that CIDEB is essential for the maintenance of hepatic lipid homeostasis. *Fsp27* is induced hepatosteatotic *ob/ob* mouse livers by PPARγ [8]. *Fsp27* was found to exist as two variants, the first identified *Fsp27a*, and a *Fsp27b* with 10 additional amino acids [13]. FSP27B protein is more stable than FSP27A protein, but their biochemical functions are similar. *Fsp27b* is enriched in *ob/ob* mouse livers, and overexpression of *Fsp27b* induced lipid accumulation in mouse liver [13]. Furthermore, cyclin AMP responsive element binding protein H (CREBH) transactivates the *Fsp27b* and induces expression of *Fsp27b* and *CIDEC2*, a human orthologue of *Fsp27b*. Hepatic *Fsp27a/b* was also reported to be transactivated by PPARα, PPARγ, and LXRα through the respective binding sites in the promoter region [8,14-16].

In this study, we investigated involvement of the CIDE family in hepatosteatotic development of *Hnf4a*$^{\Delta Hep}$ mice. Expression of Cidea was increased, while expression of *Cideb* and *Fsp27b* was markedly decreased in *Hnf4a*$^{\Delta Hep}$ mice. Similar results were also obtained in human hepatoma cell lines in which HNF4α was overexpressed and silenced. In addition, HNF4α transactivated *Fsp27b* through a binding site in the promoter region, and transactivation and expression of *Fsp27b/CIDEC2* was strongly induced in the presence of CREBH. These findings reveal that *Fsp27b/CIDEC2* is a novel target gene of HNF4α, and HNF4α and CREBH synergistically regulate *Fsp27b* expression.

2. Materials and methods

2.1. Animal

Liver-specific *Hnf4a*-null (*Hnf4a*$^{\Delta Hep}$) mice were described previously [5]. All experiments were performed with 45-day-old male *Hnf4a*-floxed (*Hnf4a*$^{f/f}$) and *Hnf4a*$^{\Delta Hep}$ mice. Mice were housed in a pathogen-free animal facility under standard 12 h light/12 h dark cycle with ad libitum water and chow. All experiments with mice were carried out under Gunma University Animal Care and Experimentation Committee (Permission number 15–021).

2.2. Cell culture

HEK293T, HepG2, Huh7, and HLE/tet-HNF4A cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, Logan, UT) and 100 units/ml penicillin and 100 μg/mL streptomycin (Wako). Tet-inducible HNF4α expressing HLE cells (HLE/tet-HNF4A) were generated using Tet-On 3G inducible expression system (Takara, Kusatsu, Japan). Expression of HNF4α in HLE/tet-HNF4A cells was induced by the addition of 500 ng/mL of doxycycline (Sigma-Aldrich, Tokyo, Japan) into the medium for 24 h.

2.3. RNA extraction, reverse-transcription, and quantitative PCR

Total RNA extracted from cell lines and mouse livers using Isogen II (Wako) was transcribed to cDNA using ReverTraAce qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). cDNA was used for quantitative PCR using Luna qPCR Master Mix (New England Biolabs, Tokyo, Japan) and PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Tokyo, Japan) with the specific primers on a LightCycler 480 system II (Roche). Levels of mRNA expression were normalized relative to *Gapdh* and *TBP* mRNA as an internal control using ΔΔCt method. Nucleotide sequences of the primers are shown in Supplemental Table 1.

2.4. Transfection of siRNA

Ten nM of siRNA against human *HNF4A* mRNA and negative control (Sigma-Aldrich) were transfected into HepG2 and Huh7 cells with Lipofectamine RNAiMAX (Life Technologies). After 48 h of transfection, total RNA was harvested. Nucleotide sequences for the siRNA duplexes against human *HNF4A* are follows; rGrGrCrArGUrGrCrGUrGrGUrGrGrArCrArAdTdT and UUrGUrCrCrArCrCrArCrGrCrArCUrGrCrCdTdT.

2.5. Transient transfection and luciferase assays

HNF4α and CREBH(N) expression plasmids were transfected in Huh7 cells. At 48 h after transfection, the cells were lysed into Isogen II for RNA extraction. The wild-type *Fsp27a/b* promoters and the *Fsp27b* promoters with the mutated HNF4α and CREBH binding sites were cloned into pGL4.11 (Promega, Tokyo, Japan). These promoters were co-transfected into HEK293T cells with pGL4.74 (Promega) as an internal control and HNF4α, CREBH, PPARα, and RXRα expression plasmids using polyethyleneimine Max (Polyscience, Warrington, PA) as a transfection reagent. After 24 h, 10 μM of Wy, 14,643 (Sigma-Aldrich)

was added to the medium of cells transfected PPARα and RXRα expression plasmids. At 48 h after transfection, promoter activities were measured using Dual-Glo Luciferase Assay System (Promega).

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) and nuclear extracts from HNF4α-overexpressed HEK293T cells. The following double-stranded probes were used; wild-type and the mutated HNF4α binding sites in the ornithine transcarbamylase (*Otc*) [2] and the *Fsp27b* promoters. Nucleotide sequences of the primers are shown in Supplemental Table 2. Nuclear extracts (3 μg) and the 5′-biotin labeled probes of the HNF4α binding sites for the *Fsp27b* promoter (wild-type) were added and the mixture was incubated on ice for 10 min. For competition experiments, a 50-fold excess of unlabeled probe was added to the reaction mixture and the mixture was incubated on ice for 10 min prior to the addition of the labeled probe. For supershift analysis, 1 μg of anti-HNF4α or anti-PPARβ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture, and the mixture was incubated on ice for 10 min after the addition of the labeled probe. DNA-protein complexes were fractionated by 7% PAGE and blotted onto a Biodyne B Nylon membrane (Pall, Tokyo, Japan). DNA-protein complexes were visualized using detection module in the kit on an ImageQuant LAS4000.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP using HepG2 cells and liver samples was performed according to a protocol using anti-HNF4α antibody and normal mouse IgG (Santa Cruz Biotechnology) [3]. Purified DNA was amplified by quantitative PCR using ΔΔCt method. Enrichment of the HNF4α binding site was normalized to the input samples compared with normal IgG antibody. The following primers were used for realtime PCR; *Fsp27b* and *CIDEC2* promoters containing HNF4α binding site, and mouse *Hmgcs2* and human *MIR-194* genes without the HNF4α binding site as negative controls. Nucleotide sequences of the primers are shown in Supplemental Table 3.

2.8. Statistical analysis

All values are expressed as the mean ± standard derivation (S.D.). All data were analyzed by the Mann-Whitney *U* test for significant differences between the mean values of each group.

3. Results

3.1. Hepatic expression of CIDE and perilipin family in Hnf4a$^{\Delta Hep}$ mice

Since *Hnf4a*$^{\Delta Hep}$ mice exhibit fatty livers, hepatic expression of CIDE and perilipin family was investigated in *Hnf4a*$^{\Delta Hep}$ mice. Expression of *Cidea* was increased in *Hnf4a*$^{\Delta Hep}$ mice, while expression of *Cideb* and *Fsp27*, the mouse homologue of human *CIDEC*, was markedly decreased in *Hnf4a*$^{\Delta Hep}$ mice (Fig. 1A). A new variant of *Fsp27*, *Fsp27b* was identified in liver, and *Fsp27b* was induced in hepatosteatotic *ob/ob* mice [13], revealing no significant difference in expression of *Fsp27a*, while *Fsp27b* was significantly reduced in *Hnf4a*$^{\Delta Hep}$ mice. These results indicated that *Fsp27b* may be a novel HNF4α target gene. In addition, expression of *Crebh* encoding a transcription factor that is a positive regulator of

Fsp27b, was decreased by nearly half in *Hnf4a*$^{\Delta Hep}$ mice, in agreement with an earlier study revealing that HNF4α positively regulates *Crebh* expression [17]. Expression of *Plin*2-5 except for *Plin1* was significantly increased in *Hnf4a*$^{\Delta Hep}$ mice (Fig. 1B). Increased *Plin2-5* in *Hnf4a*$^{\Delta Hep}$ mice might be a secondary result of the hepatosteatosis in these mice and independent of direct HNF4α.

3.2. Expression of CIDE family by HNF4α in human hepatoma cells

Expression of CIDE family mRNAs were analyzed by siRNA knockdown of HNF4α in human hepatoma cells (Fig. 2A). Knockdown of HNF4α in HepG2 and Huh7 cells showed that expression of *CIDEA* was not significantly different or not detected, and expression of *CIDEB* was slightly decreased even though the *CIDEB* is transactivated by HNF4α [6,7]. Expression of *CIDEC* that detect both *CICEC1* encoding the human orthologue of the mouse *Fsp27a*, and *CIDEC2* encoding the orthologue of the *Fsp27b*, was significantly decreased by HNF4α knockdown, and expression of *CIDEC1* was significantly decreased by HNF4α knockdown only in HepG2 cells. Furthermore, expression of *CIDEC2* was largely decreased by HNF4α knockdown in both cell lines, indicating that *CIDEC2* may be positively regulated by HNF4α. Overexpression of HNF4α in Huh7 cells induced expression of *CIDEA, CIDEB*, and *CIDEC2* (Fig. 2B). Overexpression of CREBH(N), an active form of CREBH [18], moderately induced expression of *CIDEB*. Expression of *CIDEC1* was strongly induced even though *CIDEC1* expression was not induced by CREBH(N) [13]. Induction of *CIDEC2* by CREBH(N) was much stronger than that of *CIDEC1*. Furthermore, overexpression of both HNF4α and CREBH(N) synergistically induced *CIDEB* and *CIDEC2* compared to HNF4α, or CREBH(N) alone. Thus, expression of *CIDEB* and *CIDEC2* is induced in an HNF4α and CREBH(N)-dependent manner. In addition, a doxycycline-inducible HNF4α expression system was generated using human hepatoma-derived HLE cells (HLE/tet-HNF4A cells). Addition of doxycycline (DOX) did not induce expression of *CIDEA* and *CIDEC1*, but expression of *CIDEB* and *CREBH* was significantly induced by DOX. Notably, expression of *CIDEC2* was largely induced by DOX, indicating that HNF4α is a strong inducer of *CIDEC2*.

3.3. Transactivation of the Fsp27b gene by HNF4α

To investigate whether the *Fsp27b* promoter is transactivated by HNF4α, a JASPAR database search was performed, and a high score HNF4α binding site was found at +922/+934 from the transcription start site of the *Fsp27a* (+1) and the *Fsp27b* (+946) genes with high conservation among the species (Fig. 3A and B). The *Fsp27a* promoter was positively regulated by PPARα through peroxisome proliferator response element (PPRE) at −218/−206 as described previously [15]. As expected, the promoter at −2467/+32 including the PPRE was transactivated by PPARα with RXRα and the ligand for PPARα, Wy-14,643, but this promoter was not transactivated by HNF4α (Fig. 3C). In contrast, the *Fsp27b* promoters at +33/+1026 and + 874/+1026 including the predicted HNF4α binding site were markedly transactivated by HNF4α, but the promoter at +950/+1026 without the HNF4α binding site was not transactivated by HNF4α. The +33/+1026 promoter with the mutated HNF4α binding site resulted in no transactivation by HNF4α, revealing that the *Fsp27b* promoter is transactivated in HNF4α-dependent manner. Furthermore, the +33/+1026 promoter and the promoter with the mutated HNF4α binding site was also transactivated by

PPARα to the same degree, but the +874/+1026 promoter was not transactivated by PPARα, indicating that there may be a PPRE between +1026 and + 873. In addition, there was a binding site for CREBH responsive element (CRE) at +911/+919 just upstream of the HNF4α binding site, and CREBH is known to induce *Fsp27b* expression through this element [13]. When the wild-type *Fsp27b* promoter was used, HNF4α alone and CREBH alone equally transactivated the *Fsp27b* promoter by about 10-fold, and both HNF4α and CREBH strongly transactivated the *Fsp27b* promoter (Fig. 4A). When mutations were introduced into the HNF4α binding site, the promoter activities by HNF4α alone, and both HNF4α and CREBH were largely decreased, while the activity by CREBH alone was also decreased by half, indicating that the HNF4α binding site might be essential for full transactivation by CREBH. When mutations were introduced into the CRE, no difference in the promoter activity by HNF4α was observed compared to the wild-type promoter, but the activity by CREBH was largely decreased. Both HNF4α and CREBH reduced the promoter activity by half. Furthermore, mutations of both the HNF4α binding site and CRE dramatically decreased all promoter activities. These findings reveal that HNF4α and CREBH synergistically transactivate the *Fsp27b* promoter.

3.4. Binding of HNF4α in the Fsp27b promoter

To determine whether HNF4α directly binds to the predicted HNF4α binding site in the *Fsp27b* promoter, electrophoretic mobility shift analysis was performed (Fig. 4B). Nuclear extracts from HNF4α-overexpressed HEK293T cells bound to the identified HNF4α binding sites (lane 2, the lower arrow). This complex was diminished by the addition of unlabeled *Fsp27b* competitor and *Otc* competitor that contains a bonafide HNF4α binding site (lanes 3 and 5), but not the competitor that has mutations in the HNF4α binding site of the *Fsp27b* promoter (lane 4). Moreover, the complex was supershifted by anti-HNF4α antibody (lane 6, the upper arrow), but not the unrelated anti-PPARβ antibody (lane 7). Chromatin immunoprecipitation using the livers of *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice indicated that HNF4α in *Hnf4a*$^{f/f}$ mice bound to the promoter region approximately 6.5-fold strongly compared to *Hnf4a*$^{\Delta Hep}$ mouse livers (Fig. 4C). In addition, HNF4α bound to the predicted HNF4α binding site of the *CIDEC2* promoter in HepG2 cells approximately 6.5-fold strongly compared to IgG control (Fig. 4D), suggesting that HNF4α directly and physiologically binds to the *CIDEC2/Fsp27b* promoters in human and mouse livers.

4. Discussion

We investigated whether CIDE family and perilipin family genes are involved in hepatosteatotic development in *Hnf4a*$^{\Delta Hep}$ mice. *CIDEB* is transactivated by HNF4α [6,7], and expression of *Cideb* was strikingly decreased in *Hnf4a*$^{\Delta Hep}$ mice as expected. However, inhibition of HNF4α slightly suppressed expression of *CIDEB*, and overexpression of HNF4α slightly induced expression of *CIDEB* in human hepatoma cells, indicating that HNF4α may weakly regulate *CIDEDB* expression in human liver. In Huh7 cells, CREBH induced *CIDEB* expression, and both HNF4α and CREBH synergistically induced *CIBEB* expression, indicating that both factors might be essential for *CIDEB* transactivation in human normal hepatocytes. Conversely, expression of *Cidea* was significantly increased in *Hnf4a*$^{\Delta Hep}$ mice. Since *Cidea* expression is induced by PPARα and PPARγ [19], increased

expression of *Cidea* in *Hnf4a*$^{\Delta Hep}$ mice may be due to activation of PPARα and/or PPARγ by endogenous ligands. Curiously, expression of *CIDEA* was induced by HNF4α in Huh7 cells, but no induction was observed in HLE cells, suggesting that *Cidea/CIDEA* would not be a target of HNF4α. Furthermore, expression of *Crebh* was significantly decreased in *Hnf4a*$^{\Delta Hep}$ mice as reported that HNF4α directly regulates *Crebh* [17], and expression of *CREBH* was also regulated by HNF4α in human hepatoma cells. Overexpression of *CREBH* did not induce expression of *CIDEC1* in Huh7 cells [13], but our results showed that *CREBH* strongly induce expression of *CIDEC1*. Since we used probe-based qPCR for detection of *CIDEC1*, our results that *CREBH* upregulates *CIDEC1* should be accurate. Interestingly, it was found that *Fsp27b* is a novel HNF4α target gene, and the HNF4α binding site in the *Fsp27b* promoter is located next to the CREBH binding site, indicating that HNF4α and CREBH might synergistically transactivate the *Fsp27b* through direct protein-protein interaction. Thus, HNF4α would strongly regulate *Fsp27b/CIDEC2* through both direct regulation by HNF4α itself and indirect regulation by a HNF4α-CREBH axis.

Hnf4a$^{\Delta Hep}$ mice exhibit hepatosteatosis, but expression of liver-enriched *Cideb* and steatotic liver-enriched *Fsp27b* was markedly decreased in *Hnf4a*$^{\Delta Hep}$ mice. Conversely, expression of *Cidea* that was predominantly expressed in steatotic livers was increased in *Hnf4a*$^{\Delta Hep}$ mice. Thus, HNF4α would play a central role to promote formation of lipid droplet by upregulation of *Cideb* as a main target and *Fsp27b* as a minor target in normal hepatocytes, that is, hepatic HNF4α is an essential factor required to maintain lipid homeostasis by a dual role to store excessive lipid as lipid droplets through CIDEB and FSP27B proteins, and to produce VLDL by induction of *Apob* and *Mttp* expression from lipid droplets as necessary.

However, it is still questionable whether altered expression of the CIDE family contributes to hepatosteatotic development in *Hnf4a*$^{\Delta Hep}$ mice. In normal liver, expression of *Cideb* is most abundant, but expression of *Cidea* and *Fsp27a/b* is very low [8]. Expression of *CIDEA* is increased by about 5-fold, but expression of *Cideb* and *Fsp27b* is strongly decreased in *Hnf4a*$^{\Delta Hep}$ mice. Thus, these results suggest that the hepatic total CIDE activity in growth of lipid droplets should be decreased in *Hnf4a*$^{\Delta Hep}$ mice, indicating that hepatosteatosis would be improved in *Hnf4a*$^{\Delta Hep}$ mice when only altered expression of CIDE family is considered. Conversely, expression of *Plin2-5* was modestly increased in *Hnf4a*$^{\Delta Hep}$ mice. Perilipins are the most abundant proteins that localize at the surface of lipid droplets, and protect lipid droplets from lipase action [20]. Thus, increased expression of *Plin2-5* might be partly involved in hepatosteatotic development in *Hnf4a*$^{\Delta Hep}$ mice by suppression of lipolysis. However, since many proteins are associated with lipid droplets [21], it is not easy to mechanistically explain hepatosteatotic development in *Hnf4a*$^{\Delta Hep}$ mice by expression change of CIDE and perilipin family. Further analyses are required to elucidate the detailed mechanism of hepatosteatotic development in *Hnf4a*$^{\Delta Hep}$ mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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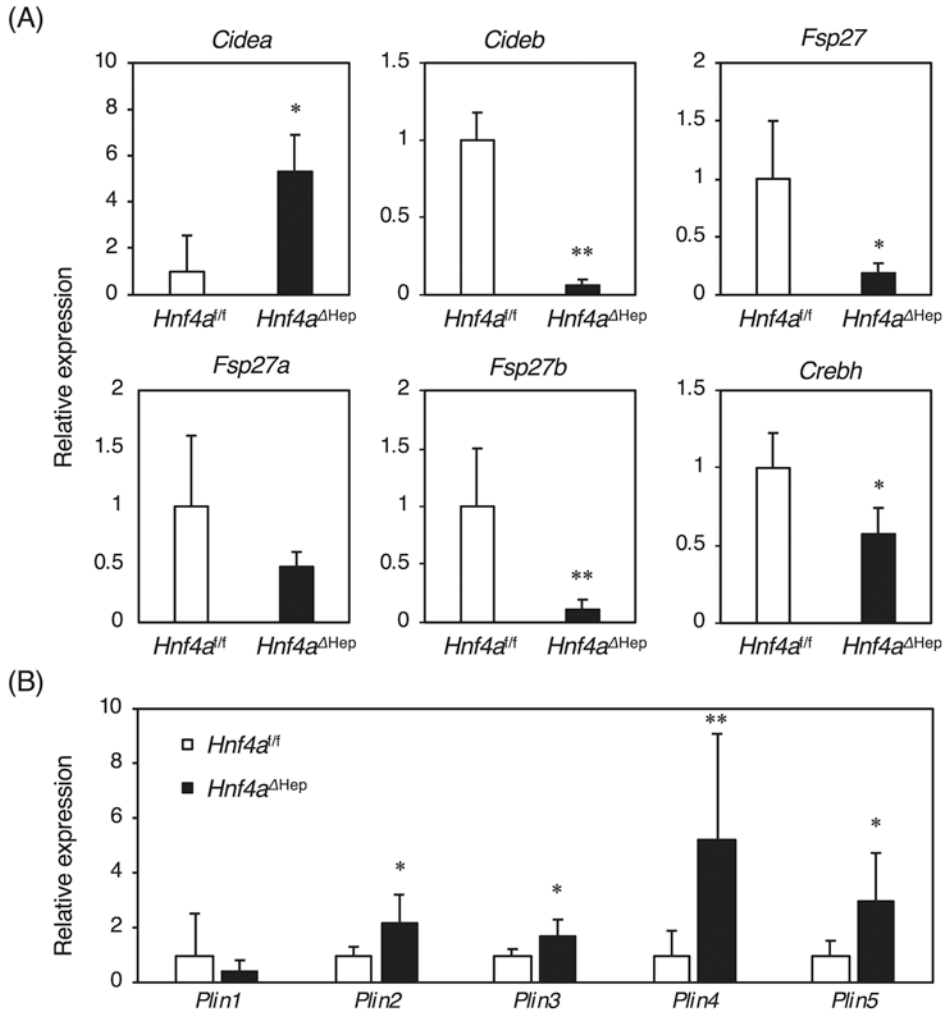

Fig. 1.

Hepatic expression of Cide and Plin family, and *Crebh* in *Hnf4a*$^{\Delta Hep}$ mice. quantitative RT-PCR from total liver RNA of *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice (n = 6 for each genotype). The normalized expression in *Hnf4a*$^{\Delta Hep}$ mice is presented relative to that in *Hnf4a*$^{f/f}$ mice. Data are mean ± S.D. *, $P < 0.05$; **, $P < 0.005$ compared to *Hnf4a*$^{f/f}$ mice.

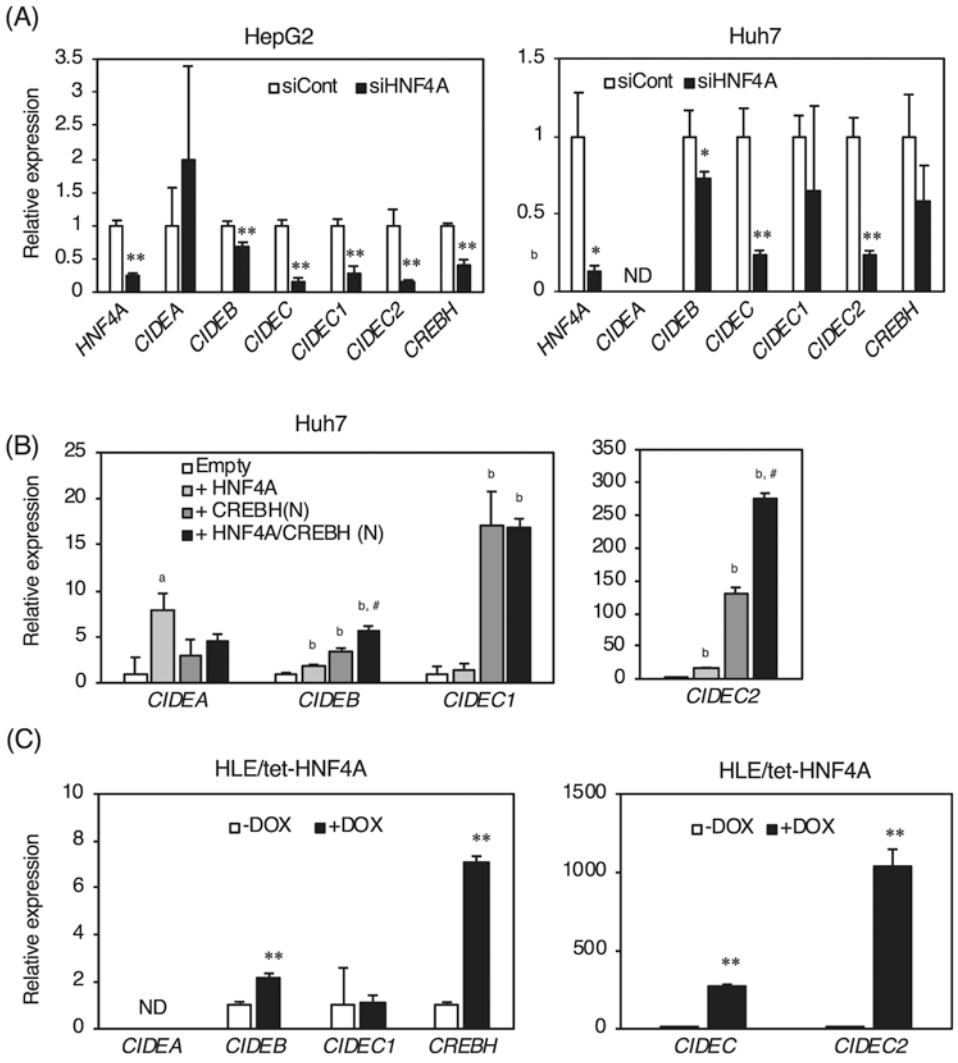

Fig. 2.

Hepatic expression of CIDE family and *CREBH* by HNF4α in human hepatoma cells. (A) quantitative RT-PCR from total RNA of HepG2 and Huh7 cells treated with negative control of siRNA (siCont) and siRNA for *HNF4A* (siHNF4A). (B) quantitative RT-PCR from total RNA of HNF4α-transfected Huh7 cells. (C) quantitative RT-PCR from total RNA of HLE/tet-HNF4A cells treated without or with doxycycline (−DOX, or + DOX) for 24 h. The normalized expression in $Hnf4a^{\Delta Hep}$ mice, HNF4α-transfected cells, and the cells treated with doxycycline is presented relative to that in $Hnf4a^{f/f}$ mice, empty vector-transfected cells, and the cells treated without doxycycline, respectively. Data are mean ± S.D. *, $P < 0.05$; **, $P < 0.005$ compared to the cells treated with negative control of siRNA, the cells transfected an empty, or the cells treated without doxycycline. [a], $P < 0.05$; [b], $P < 0.01$ compared to the cells transfected an empty. *, $P < 0.05$; [#], $P < 0.01$ compared to the cells transfected CREBH(N) vector. ND; not detected.

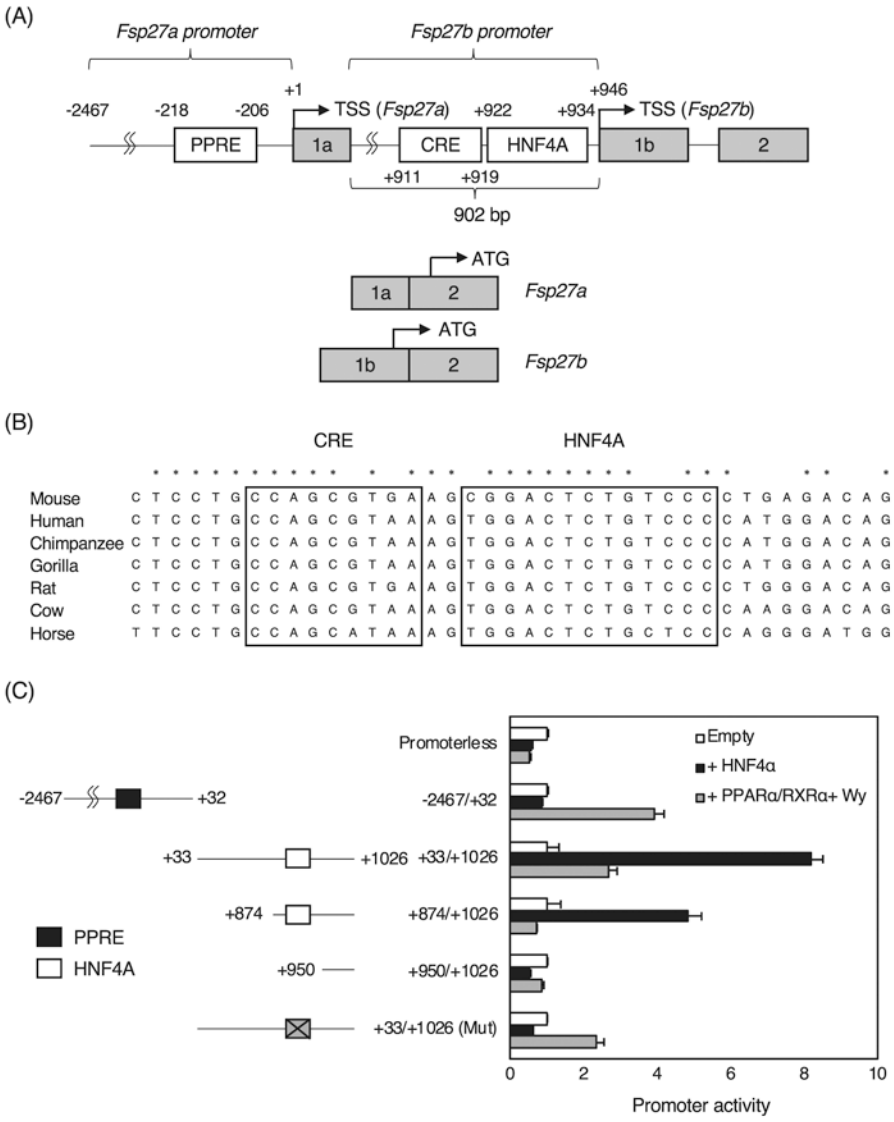

Fig. 3.

Analysis of the *Fsp27a/b* promoters. (A) Schematic structure of the *Fsp27* promoter. Transcription start site of the *Fsp27a* and *Fsp27b* gene is shown as +1 and + 946, respectively. (B) Sequence alignment of the proximal promoter of the *Fsp27b* gene in mouse, human, chimpanzee, gorilla, rat, cow, and horse. CREBH-responsive element (CRE) and predicted HNF4α binding site are encircled as boxes. Completely conserved nucleotides among the species are shown as asterisk. (C) Promoter activity of the *Fsp27* gene in HEK293T cells. The *Fsp27* promoters were co-transfected with empty vector, HNF4α expression vector, and PPARα/RXRα expression vectors treated with 10 μM of Wy-14,643 into HEK293T cells. Mutations were introduced into the HNF4α in the +33/+1,026 promoter (gray square with a cross). HNF4α binding site and PPRE are shown as open and solid squares, respectively. The normalized activity ± S.D. was presented as relative activity based on empty vector-transfected cells. ND; not detected.

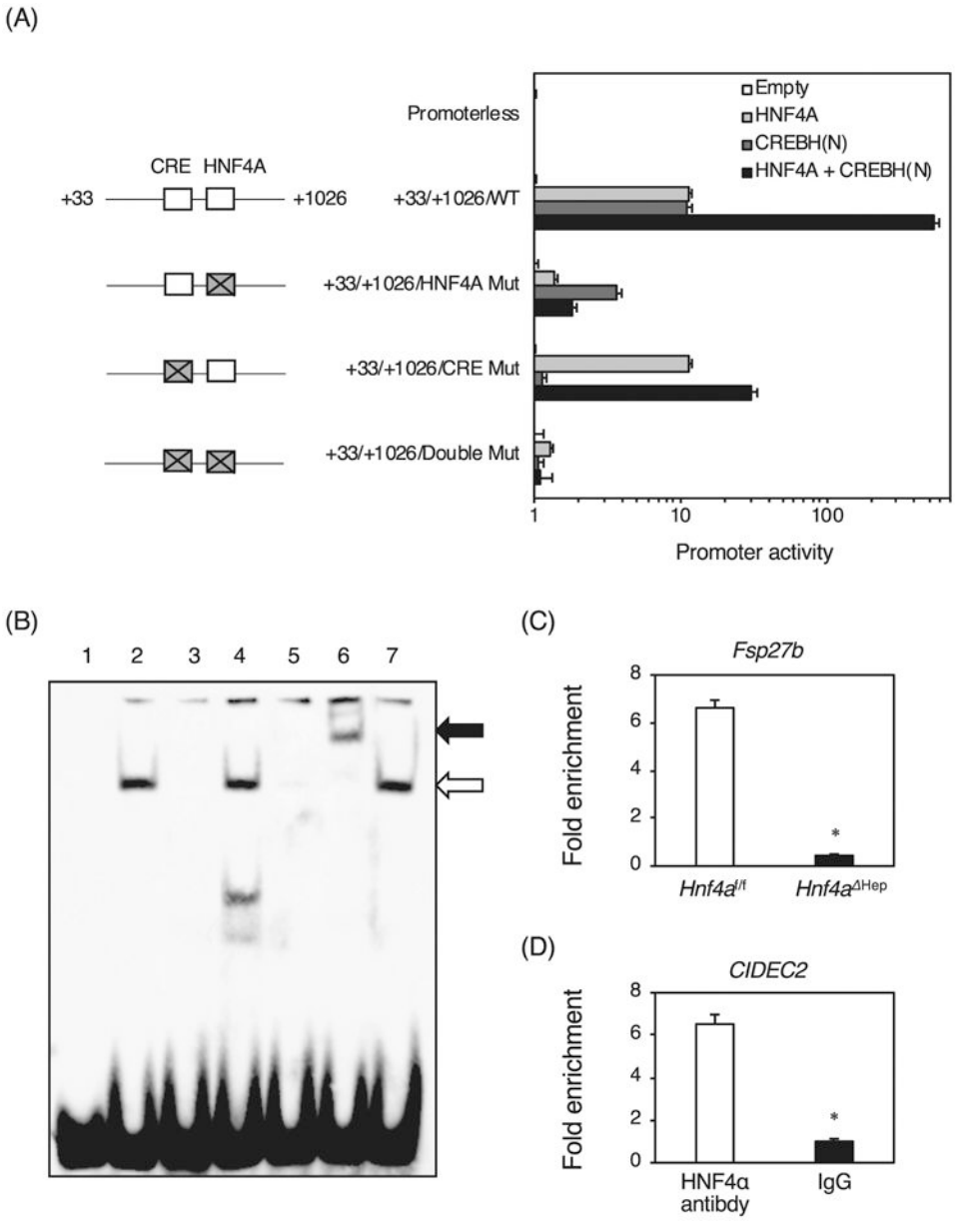

Fig. 4.

Promoter analysis of the *Fsp27b* promoter by HNF4α and CREBH, and identification of an HNF4α binding site in the *Fsp27b* promoter. (A) Promoter activity of the *Fsp27b* gene. The *Fsp27b* promoters were co-transfected with empty vector, HNF4α expression vector, and N-terminus-deleted activated CREBH expression vector, and both HNF4α and CREBH(N) expression vectors into HEK 293T cells. Mutations were introduced into the HNF4α and CREBH biding sites (CRE) (gray square with a cross). (B) EMSA. Nuclear extracts were incubated with biotin-labeled probe carrying the HNF4α binding sites in the *Fsp27b* promoter in the absence (lane 2) or presence of the unlabeled *Fsp27b* probe (lane 3), the mutated *Fsp27b* probe (lane 4), and the *Otc* probe (lane 5). For supershift analysis, anti-HNF4α and anti-PPARβ antibodies were added, respectively (lanes 6 and 7). Complex between HNF4α and the probe and supershifted complex are indicated by the lower and

upper arrow, respectively. (C) Chromatin immunoprecipitation using the livers of *Hnf4a*$^{\Delta Hep}$ and *Hnf4a*$^{f/f}$ mice with anti-HNF4α antibody and normal goat IgG. The region containing the HNF4α binding site in the *Fsp27b* promoter and the region without an HNF4α binding site in the *Hmgcs2* gene were amplified. The data from qPCR was normalized relative to the input and expressed as fold enrichment over data from IgG control. (D) Chromatin immunoprecipitation using HepG2 cells were performed with anti-HNF4α antibody and normal goat IgG. The region containing an HNF4α binding site in the human *CIDEC2* gene and the region without HNF4α binding site in the human *MIR194* gene were amplified. Data are mean ± S.D. *, $P < 0.05$ compared to *Hnf4a*$^{f/f}$ mice and HNF4α antibody.