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Hepatocyte nuclear factor 4α is a critical factor for the production of complement components in the liver

Carlos Ichiro Kasano-Camones[1] · Satomi Yokota[1] · Maiko Ohashi[1] · Noriaki Sakamoto[1] · Daichi Ito[1] · Yoshifumi Saito[1] · Ryo Uchida[1] · Kazumi Ninomiya[1,2] · Yusuke Inoue[1,2]

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Abstract

The complement system plays an important role in biological defense as an effector to eliminate microorganisms that invade an organism and it is composed of more than 50 proteins, most of which are produced in the liver. Of these proteins, the mRNA expression of *C3* and *Cfb* is known to be positively regulated by the nuclear receptor HNF4α. To investigate whether HNF4α regulates the complement system, we analyzed the hepatic expression of genes involved in the complement activation pathway and membrane attack complex (MAC) formation within the complement system using liver-specific *Hnf4a*-null mice ($Hnf4a^{\Delta Hep}$ mice) and tamoxifen-induced liver-specific *Hnf4a*-null mice ($Hnf4a^{f/f;AlbERT2cre}$ mice). We found that hepatic expression of many complement genes including *C8a*, *C8b*, *C8g*, and *C9* that are involved in formation of the MAC was markedly decreased in $Hnf4a^{\Delta Hep}$ mice and $Hnf4a^{f/f;AlbERT2cre}$ mice. Furthermore, expression of *C8A*, *C8B*, and *C8G* was also decreased in human hepatoma cell lines in which the expression of HNF4α was suppressed, and expression of *C8G* and *C9* was induced in a human immortalized hepatocyte cell line with forced expression of HNF4α. Transactivation of *C8g* and *C9* was dependent on HNF4α expression of HNF4α binding sites, indicating that *C8g* and *C9* are novel target genes of HNF4α. The results suggest that hepatic HNF4α plays an important role in regulation of the complement system, mainly MAC formation.

Keywords HNF4α · Complement components · Membrane attack complex · Liver

Introduction

The major role of the complement system in the innate immune system is removal of microorganisms invading a living body, and the complement system consists of more than 50 serum proteins, cell surface receptors, and regulators (Ricklin *et al.* 2010; Kemper *et al.* 2014). When a pathogen invades a living body, the complement system recognizes the pathogen and a chain reaction of a complement cascade is activated, resulting in eventual destruction of the pathogen. The complement system is activated by three independent pathways; the classical pathway triggered by a pathogen-bound antibody, the lectin pathway triggered by pathogen-bound mannose-binding lectin (MBL), and the alternative pathway triggered by spontaneous formation of C3b. Fragments activated by a proteolysis cascade exhibit various biological activities including opsonization of a pathogen, recruitment of inflammatory immune cells, and pathogen lysis (Gialeli *et al.* 2018; Lopez-Lera *et al.* 2019). Specific complement components work in complement activation: C1q, C1r, C1s, C4, and C2 for the classical pathway; mannose-binding lection-associated serine protease 1 (MASP1), MASP2, MASP3, C4, and C2 for the lectin pathway; and C3 and factors B and D for the alternative pathway. The three pathways commonly result in C3 activation and are followed by formation of the membrane attack complex (MAC) by C5b-C9. The MAC forms a transmembrane pore on the cell surface of pathogens and aged erythrocytes, and water and ions intrude into the cells from the pore, eventually resulting in cell lysis due to dysfunction of the maintenance of osmotic pressure (Morgan *et al.* 2017). Thus, inherited deficiencies of the complement

✉ Yusuke Inoue
yinoue@gunma-u.ac.jp

[1] Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin-Cho, Kiryu, Gunma 376-8515, Japan

[2] Gunma University Center for Food Science and Wellness, Maebashi, Gunma 371-8510, Japan

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proteins are mainly associated with enhanced susceptibility to *Neisseria* infections due to dysfunctional host defense caused by inactivation of the late complement components consisting of C5b-C9 and with autoimmune diseases such as systemic lupus erythematous (SLE) due to deposits of circulating immune complexes caused by inactivation of the early complement components consisting of three independent pathways (Vignesh *et al.* 2017; Schroder-Braunstein and Kirschfink 2019). However, excessive complement activation in response to pathogens causes excessive consumption of complement proteins and persistent inflammation. Thus, several complement inhibitors have been identified. For example, GPI-anchored CD59 in host cells inhibits MAC formation between C5b-8 and C9, resulting in protection of own cells including erythrocytes from invasion by complement activation (Meri *et al.* 1990; Kim and Song 2006). In addition, the complement system has been found to have multiple roles in maintenance of cellular homeostasis including clearance of apoptotic and secondary necrotic cells; in adaptive immunity; and in diseases such as inflammatory diseases, autoimmune disorders, rheumatoid arthritis, asthma, Alzheimer's disease, and cancers (Gullstrand *et al.* 2009; Ricklin *et al.* 2010). In this way, an appropriate balance between complement activators and inhibitors has an important role in body homeostasis to prevent invasion of pathogens and excessive immune reaction.

Many complement proteins circulate in the blood stream and are mainly produced in the liver as well as many serum proteins except immunoglobulin (Vignesh *et al.* 2017). Expression of liver-enriched genes is mainly regulated by liver-enriched transcription factors including HNF1, HNF3 (FOXA), HNF4α, HNF6 (ONECUT), and C/EBP (Schrem *et al.* 2004; Lau *et al.* 2018). Of these, hepatic expression of terminal complement genes including *C5*, *C8a*, *C8b*, *C8g*, and *C9* was shown to be decreased in *Hnf1a*-null mice and an HNF1α binding site was identified in the promoter region of the *C8a* gene (Pontoglio *et al.* 2001). It was also shown that HNF4α positively regulates *C3* and *Cfb* expression (Garnier *et al.* 1996; Shavva *et al.* 2013). In addition, hepatic expression of many coagulation factors including *F5*, *9*, *11*, *12*, and *13b* was shown to be decreased in liver-specific *Hnf4a*-null mice ($Hnf4a^{\Delta Hep}$ mice) and *Hnf4a*-suppressed mouse primary hepatocytes (Inoue *et al.* 2006; Safdar *et al.* 2012). The coagulation system also consists of coagulation factors that are mainly produced in the liver and is an enzymatic cascade as is the complement system and the complement and coagulation systems are intimately connected to each other (Foley 2016), indicating that HNF4α might also be a central regulator in the complement system.

In this study, we investigated whether hepatic HNF4α positively regulates the expression of the complement genes using $Hnf4a^{\Delta Hep}$ mice and tamoxifen-induced liver-specific *Hnf4a*-null mice ($Hnf4a^{f/f;AlbERT2cre}$). We found that hepatic expression of many complement genes was markedly decreased in $Hnf4a^{\Delta Hep}$ mice and that complement *C8g* and *C9* are novel target genes of hepatic HNF4α. These findings reveal that hepatic HNF4α is an important factor in regulation of the complement system including MAC formation.

Materials and methods

Animal Liver-specific *Hnf4a*-null ($Hnf4a^{\Delta Hep}$) mice were kindly provided by Dr. Frank J Gonzalez (Hayhurst *et al.* 2001). To generate tamoxifen-induced liver-specific *Hnf4a*-null ($Hnf4a^{f/f;AlbERT2cre}$) mice (Bonzo *et al.* 2012), $SA^{+/CreER2}$ mice were kindly provided by Dr. Pierre Chambon (Schuler *et al.* 2004). All experiments were performed with 45-d-old male *Hnf4a*-floxed ($Hnf4a^{f/f}$) and $Hnf4a^{\Delta Hep}$ mice, and 13-wk-old tamoxifen-treated $Hnf4a^{f/f}$ and $Hnf4a^{f/f;AlbERT2cre}$ 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.

Cell lines HEK293T (purchased from ATCC in February 2010), HepG2 (purchased from RIKEN Cell Bank in December 2011), and OUMS-29 (Kobayashi *et al.* 2000) (kindly provided from Dr. Nam-Ho Huh, Okayama University) 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/streptomycin (Wako). All cell lines were confirmed to be mycoplasma-free by nuclear staining with Hoechst 33,342 (Thermo Fisher Scientific, Tokyo, Japan). HEK293T and HepG2 were re-authenticated by STR analysis, and OUMS-29 was unique and not cross-contaminated or misidentified because no matched STR profiles of OUMS-29 were found in the Expasy Profile Database (Supplemental Fig. 1).

RNA extraction, reverse-transcription, and quantitative PCR Extracted total RNA from cell lines and livers of $Hnf4a^{\Delta Hep}$ and $Hnf4a^{f/f}$ mice was transcribed using ReverTraAce qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). These cDNA were used for quantitative PCR using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) with the specific primers on a LightCycler 480 system II (Roche, Basel, Switzerland). Levels of mRNA expression were normalized relative to *Gapdh* mRNA as an internal control using ΔΔCt method. Nucleotide sequences of the primers are shown in Supplemental Table 1.

Transfection of dicer-substrate siRNA Dicer-substrate siRNA (DsiRNA; 10 nM) against human *HNF4A* mRNA

and negative control (Integrated DNA Technologies, Tokyo, Japan) were transfected into HepG2 cells with Lipofectamine RNAiMAX (Life Technologies). After 48 h of transfection, total RNA and protein were harvested. Nucleotide sequences for the DsiRNAs duplexes are follows: rArUrGrGrCrCrArArGrArUrUrGrArCrArArCrCrUrGrUrUGC and rGrCrArArCrArGrGrUrUrGrUrCrArArUrCrUrUrGrGrCrCrArUrGrC for HNF4α, and rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArCrGrCrGrUAT and rArUrArCrGrCrGrUrArUrUrArUrArCrGrCrGrArUrUrArArCrGrArC for negative control.

Western blot Whole cell lysates from HepG2 cells treated with siRNA against HNF4α and HNF4α-overexpressed OUMS-29 cells were also prepared as described previously (Matsuo *et al.* 2015). The nuclear extracts, the whole cell lysate, and pull-down samples were diluted with Laemmli sample buffer, incubated at 65 °C for 15 min, fractionated by 10% SDS–polyacrylamide gel electrophoresis. The gels were transferred onto a PVDF membrane (GE Healthcare, Tokyo, Japan). The membrane was incubated for 1 h with PBS containing 0.1% Tween 20 and 5% skim milk, and then incubated for 1 h with anti-HNF4α (Perceus Proteomics, Tokyo, Japan) and anti-γ tubulin antibodies (Sigma). After washing, the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology), and the reaction product was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Construction of luciferase reporter plasmids of the mouse complement promoters The mouse *C8g* (-1895/1) and *C9* (-1992/-1) promoters and the shorter promoters of these genes from the translation start site were amplified from genomic DNA of mouse liver by PCR and cloned into the luciferase reporter vector, pGL4.11 (Promega, Madison, WI). Mutations were introduced into an HNF4α binding site in the *C8g* and *C9* promoters by overlap and inverse PCR-based mutagenesis, respectively. The following primers were used (the mutated HNF4α binding sites were indicated as capital and bold letters): for *C8g* promoter, tggacagtggac**AGA**gacctaggacag and ctgtcctaggtc**TCT**gtccactgtcca; and for *C9* promoter, **AAGGA**acacttagcctaagccaaca and tccaaccaatggagagtcattgtaa. As a positive control, the mouse *Otc* promoter was used (Inoue *et al.* 2002).

Transient transfection and luciferase assays The mouse *Otc*, *C8g*, and *C9* promoters cloned into pGL4.11 with pGL4.74 as an internal control and HNF4α expression plasmid were co-transfected into the cell lines using polyethyleneimine Max (PolyScience, Warrington, PA) as a transfection reagent. After 48 h, the cells were washed with phosphate-buffered saline and promoter activities were measured using Dual-Glo Luciferase Assay System (Promega).

Electrophoretic mobility shift assay (EMSA) EMSA was carried out using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) and nuclear extracts from HepG2 cells. The following double-stranded probes were used (mutations in the HNF4α binding site are indicated as capital and bold letters): the HNF4α binding site at -203/-192 in the mouse ornithine transcarbamylase (*Otc*) promoter (Inoue *et al.* 2002), the HNF4α binding site at -72/-60 in the mouse *C8g* promoter (wild type; tggacagtggactctgacctaggacagtg and cactgtcctaggtcagagtccactgtcca, mutant; tggacagtggac**AGA**gacctaggacagtg and cactgtcctaggtc**TCT**gtccactgtcca), and the HNF4α binding site at -82/-70 in the mouse *C9* promoter (wild type; tccattggttggaccttgacacttagccta and taggctaagtgtcaaggtccaaccaatgga, mutant; tccattggttgga**AAGGA**acacttagccta and taggctaagtgt**TCCTT**tccaaccaatgga). Nuclear extracts (3 μg) and the 5′-biotin labeled probes of the HNF4α binding sites for the *C9* promoter (wild-type) were added and the reaction mixture 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 5′-biotin labeled probe. For supershift analysis, 1 μg each of anti-HNF4α, anti-C/EBPα, and 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 5′-biotin labeled probe. DNA–protein complexes were fractionated by 7% PAGE containing 5% glycerol, and blotted onto a Biodyne B Nylon membrane (Pall, Tokyo, Japan). After washing, DNA–protein complexes were visualized using detection module in the kit on an ImageQuant LAS4000.

Chromatin immunoprecipitation Chromatin immunoprecipitation using liver samples from *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice was performed according to our previous protocol using anti-HNF4α antibody (Perseus Proteomics, Tokyo, Japan) and normal goat IgG (Santa Cruz Biotechnology) (Morimoto *et al.* 2017). Purified DNA was amplified by quantitative PCR using ΔΔCt method. Enrichment of HNF4α binding site was normalized to the input samples compared with normal goat IgG antibody. The following primers were used for real-time PCR: mouse *C8g* promoter containing HNF4α binding site (ctaggatggaccctggcttg and tcacctactgtccgtagcagg), mouse *C9* promoter containing HNF4α binding site (tggtttattgcataatgacact and tttgttcataggtgttggctta), mouse *Otc* promoter containing HNF4α binding site (gaagaggctgggctctgaa and atagagtagggcagggtgcag) as a positive control, and mouse *Hmgcs2* gene without HNF4α binding site (gatccctgggactcacaca and gaatgcacatttatggaggtca) as a negative control.

Statistical analysis All values are expressed as the mean ± standard derivation (S.D.). All data were analyzed by

the Mann–Whitney test for significant differences between the mean values of each group.

Results

Hepatic expression of many complement genes is downregulated in liver-specific Hnf4a-null mice Since HNF4α is a master regulator for maintenance of liver functions and many complement proteins are mainly produced in the liver, the expression profile of hepatic mRNAs encoding complement genes was analyzed in liver-specific *Hnf4a*-null mice (*Hnf4a*$^{\Delta Hep}$ mice) and control *Hnf4a*$^{f/f}$ mice by quantitative RT-PCR (Fig. 1*A*). Hepatic expression of many complement genes including *C1r*, *C1s*, *C2*, *C4*, *C5*, *C6*, *C8a*, *C8b*, *C8g*, and *C9* was downregulated in *Hnf4a*$^{\Delta Hep}$ mice, but no significant difference in expression of *C1qa*, *C1qb*, *C1qc*, and *C7* was observed between *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice. A similar tendency in expression was observed between tamoxifen-induced liver-specific *Hnf4a*-null mice (*Hnf4a*$^{f/f;AlbERT2cre}$ mice) and tamoxifen-treated control mice (*Hnf4a*$^{f/f}$ mice) (Fig. 1*B*). Expression of *C3* was significantly upregulated in *Hnf4a*$^{\Delta Hep}$ mice but was not different in tamoxifen-treated *Hnf4a*$^{f/f}$ and *Hnf4a*$^{f/f;AlbERT2cre}$ mice. Of these downregulated genes, C5b-C9, which are late complement components, form the membrane attack complex (MAC) that leads to cell death by the formation of channels on the plasma membrane. Expression of *C8a*, *C8b*, *C8g*, and *C9* was remarkably decreased in *Hnf4a*$^{\Delta Hep}$ mice and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice. These results indicate that HNF4α is a central regulator of the production of complement components in the liver.

Expression of complement genes by HNF4α in human hepatoma cells and immortal human hepatocytes Since hepatic expression of many complement genes was decreased in *Hnf4a*$^{\Delta Hep}$ mice and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice, expression of *C1-C9* mRNAs was analyzed in human hepatoma-derived HepG2 cells by siRNA knockdown of HNF4α (Fig. 2*A*). Expression of *C2*, *C4*, *C6*, *C8A*, *C8B*, and *C8G* was suppressed by inhibition of the expression of HNF4α, and these results were similar to the results in *Hnf4a*$^{\Delta Hep}$ and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice. Expression of *C1QA*, *C1QB*, *C7*, and *C9* was not detected

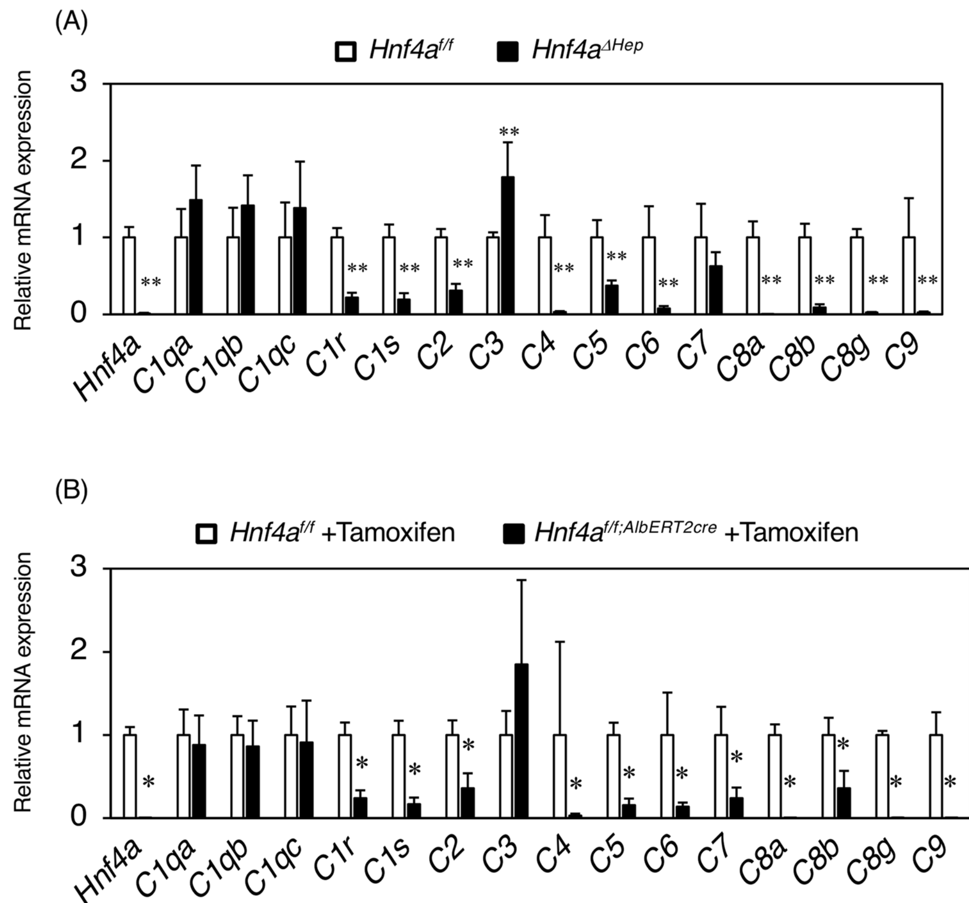

Figure 1. Hepatic expression of complement genes in *Hnf4a*$^{\Delta Hep}$ and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice. Quantitative RT-qPCR for *Hnf4a* and complement mRNAs from total liver RNA of *Hnf4a*$^{f/f}$ *and Hnf4a*$^{\Delta Hep}$ mice (***A***) and tamoxifen-treated *Hnf4a*$^{f/f}$ and *Hnf4a*$^{f/f;AlbERT2cre\ mice}$ (***B***) ($n=5$ for each genotype). The normalized expression in *Hnf4a*$^{\Delta Hep}$ and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice was presented relative to that in *Hnf4a*$^{f/f}$ and tamoxifen-treated *Hnf4a*$^{f/f}$ mice using *Tbp* mRNA as an internal control. Data are mean ± S.D. $*P<0.05$. $**P<0.01$.

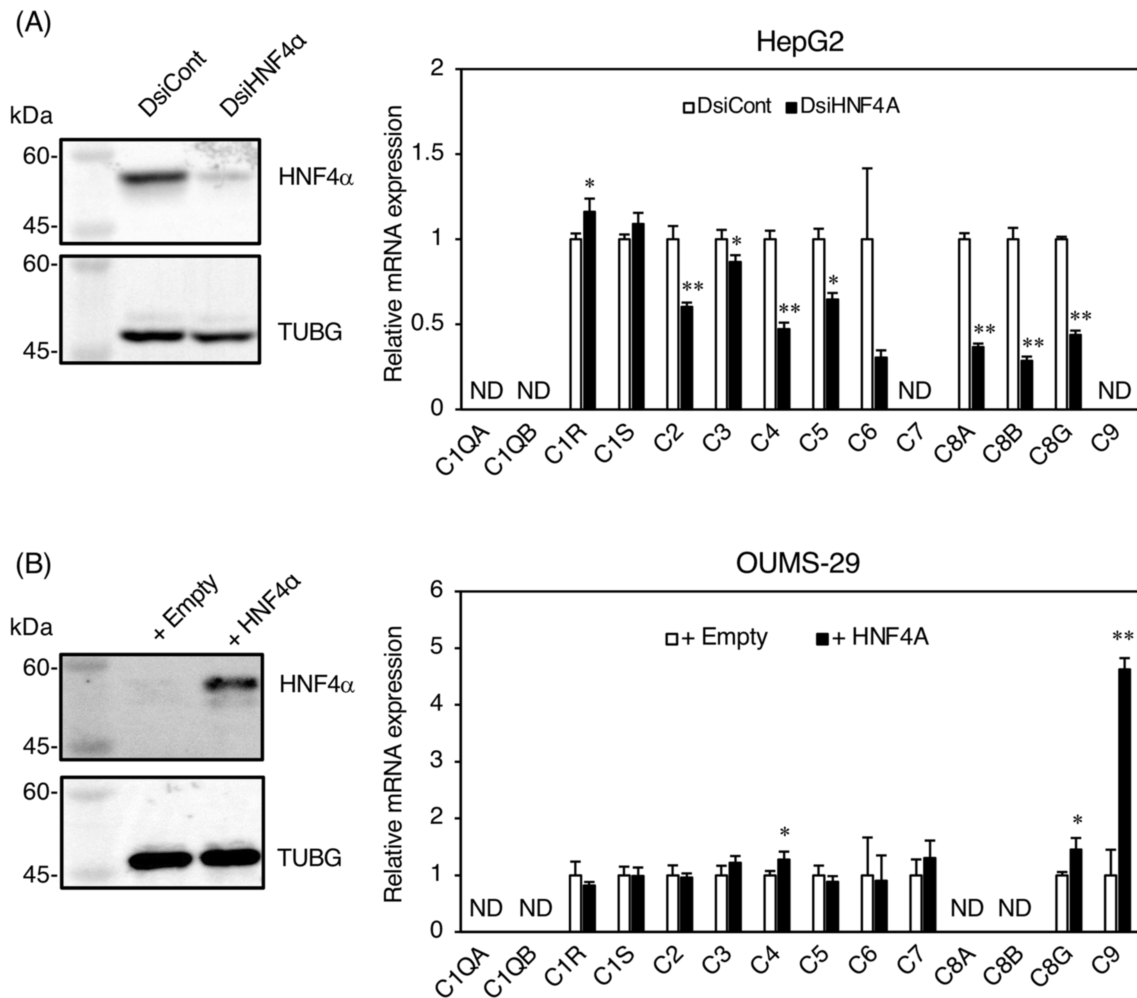

Figure 2. Expression of complement genes by HNF4α in human hepatoma cells and immortalized human hepatocytes. (*A*) Western blot analysis of HNF4α and TUBG protein in human hepatoma cell lines, HepG2 cells treated with negative control of siRNA (siCont) and siRNA for *HNF4A* (siHNF4A) (*left*). Quantitative RT-PCR from total RNA of HepG2 cells treated with siCont and siHNF4A (*right*). The normalized expression in siHNF4A-treated cells is presented relative to that in siCont-treated cells using *GAPDH* mRNA as an internal control. (*B*) Western blot analysis of HNF4α and TUBG protein in human immortalized human hepatocytes, OUMS-29 cells transfected an empty vector (Empty) and HNF4α expression vector (HNF4A) (*left*). Quantitative RT-PCR from total RNA of empty vector and HNF4α expression vector-transfected OUMS-29 cells using *GAPDH* mRNA as an internal control (*right*). Data are mean ± S.D. $*P<0.05$; $**P<0.005$ compared to the cells treated with siCont, or the cells transfected the empty vector. *ND*, not detected.

in HepG2 cells treated with siRNA of control and HNF4α. Since C1Q and C7 are produced in macrophages and dendritic cells and in endothelial cells, respectively (Langeggen *et al.* 2000; Lu and Kishore 2017), C1Q and C7 would be produced in extrahepatic cells such as Kuppfer cells and endothelial cells in the liver and would not be target genes of hepatic HNF4α. However, since C9 is mainly produced in hepatocytes, we investigated whether expression of complement genes is induced by HNF4α in highly differentiated immortalized human hepatocytes, OUMS-29 cells (Kobayashi *et al.* 2000). It was found that the expression of *C8G* and *C9* was significantly induced by HNF4α, but other complement genes were not induced or not detected in HNF4α-overexpressed OUMS-29 cells. Thus, we focused on transcriptional regulation of the *C8g* and *C9* genes by HNF4α.

Direct transactivation of the C8g gene by HNF4α Expression of *C8a*, *C8b*, *C8g*, and *C9* genes, which are components of the MAC, was markedly decreased in *Hnf4a*$^{\Delta Hep}$ mice and human hepatoma cells treated with siRNA for HNF4α. In addition, expression of *C8G* and *C9* genes was increased

by overexpression of HNF4α in immortal human hepatocytes. Thus, promoter analysis was performed to investigate whether HNF4α directly transactivates the *C8g* and *C9* genes. The *C8g* promoter containing the region at -600/-1 from the translation start site was transactivated by HNF4α (Fig. 3*A*). The promoter activity of the *C8g* promoter at -589/-1 was decreased by about half compared to that of the -600/-1 promoter. The promoter activity of the -101/-1 promoter was further decreased and the -44/-1 promoter no longer had promoter activity. Since an HNF4α binding site at -72/-60 and E-box at -595/-590 were predicted in the *C8g* promoter by the JASPAR database, mutations were introduced into these binding sites. The promoter activity of the -600/-1 promoter that contains mutations in the E-box (-600Mut1) was decreased by half compared to that of the wild-type promoter, and the -600/-1 promoter that contains

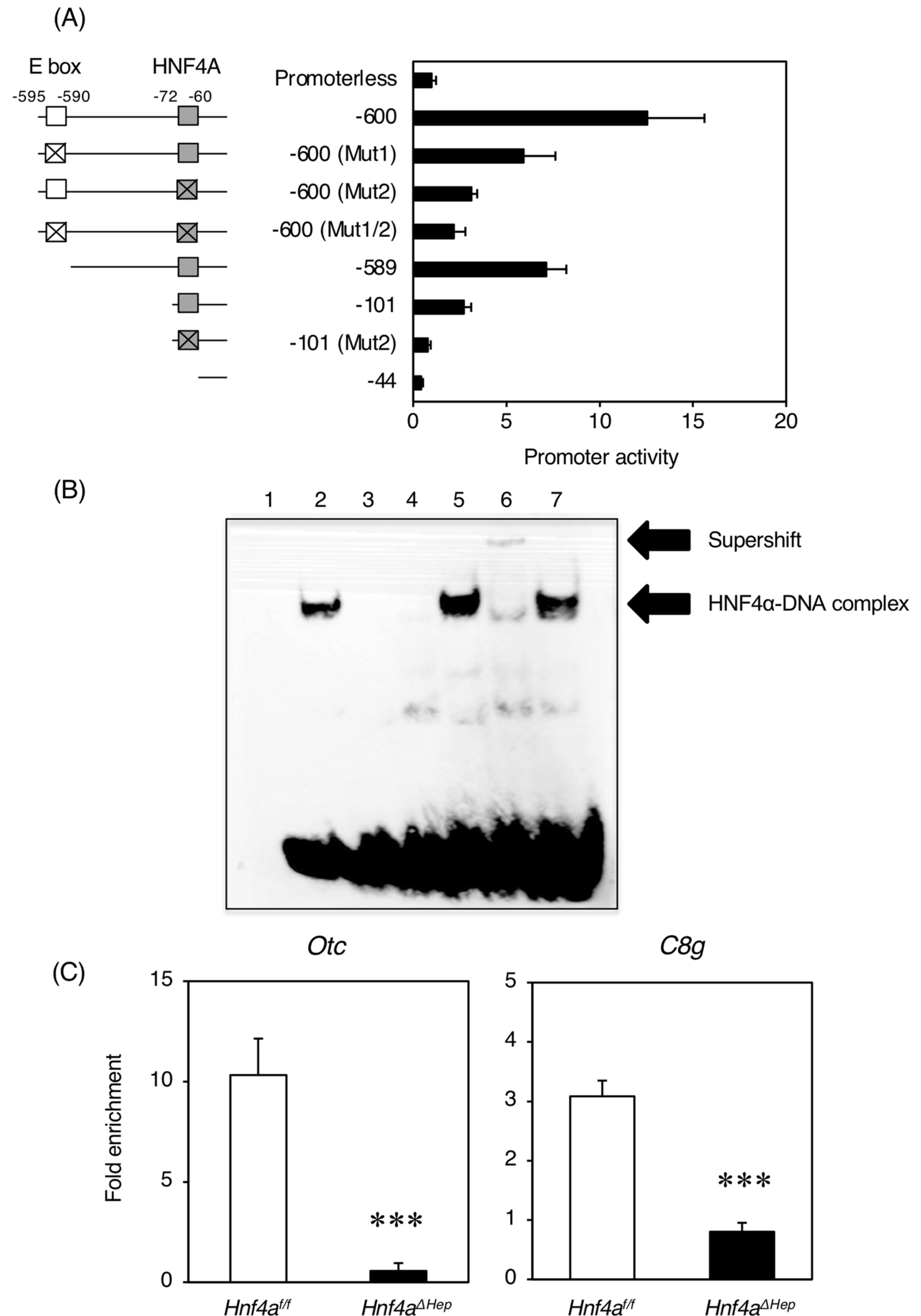

Figure 3. Promoter analysis of the mouse *C8g* gene. (***A***) Promoter activity of the *C8g* gene. The *C8g* promoters from the translation start site were transfected into HepG2 cells. Mutations were introduced into the predicted E box (-595/-590) and HNF4α binding site (-72/-60) in the promoter. (***B***) Electrophoretic mobility shift assay. Biotin-labelled probe carrying the HNF4α binding site in the *C8g* promoter was incubated without nuclear extracts (*lane 1*). Nuclear extracts from HepG2 cells were incubated with biotin-labelled probe carrying the HNF4α binding site in the *C8g* promoter in the absence (*lane 2*) or presence of 50-fold excess amounts of the unlabeled *C8g* and *Otc* probes (*lanes 3 and 4*), and the *C8g* probe that contains mutations in the HNF4α binding site (*lane 5*). For supershift analysis, anti-HNF4α and anti-C/EBPα antibodies were added, respectively (*lanes 6 and 7*). Complex between HNF4α and the probe, and supershifted complex are indicated by the *lower and upper arrows*, respectively. (***C***) Chromatin immunoprecipitation using the livers of *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice with normal goat IgG and anti-HNF4α antibody. The region containing the HNF4α binding site in the *Otc* and *C8g* promoters 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. Data are mean ± S.D. **P* < 0.05 compared to *Hnf4a*$^{f/f}$ mice.

mutations in the HNF4α binding site was further decreased (-600Mut2). When mutations were introduced into both sites (600Mut1/2), the promoter activity was decreased to the same level as that of the -101/-1 promoter. The promoter activity of the -101/-1 promoter that contains mutations in the predicted HNF4α binding site was decreased to the basal level (-101Mut2), indicating that both the E-box and the HNF4α binding site are critical *cis*-elements for transactivation of the *C8g* gene. To determine whether HNF4α can directly bind to the HNF4α binding site in the *C8g* promoter, an electrophoretic mobility shift assay (EMSA) was performed (Fig. 3*B*). Nuclear extracts from HepG2 cells bound to the HNF4α binding site (lane 2, lower arrow). This complex was diminished by the addition of excess amounts of unlabeled *C8g* and *Otc* competitors that contain functional HNF4α binding sites (lanes 3 and 4) but not the *C8g* competitor that contains mutations in the HNF4α binding site (lane 5). Furthermore, the complex was supershifted by an anti-HNF4α antibody (lane 6, the upper arrow) but not by an unrelated anti-C/EBPα antibody (lane 7). Chromatin immunoprecipitation (ChIP) analysis using the livers of *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice indicated that HNF4α in *Hnf4a*$^{f/f}$ mice strongly bound to the *Otc* and *C8g* promoter regions compared to that in *Hnf4a*$^{\Delta Hep}$ mouse livers (Fig. 3*C*), suggesting that HNF4α directly and physiologically binds to the *C8g* promoter in mouse livers.

Direct transactivation of the C9 promoter by HNF4α Promoter analysis of the mouse *C9* gene was also performed in HEK293T cells (Fig. 4*A*). As a positive control, the mouse *Otc* promoter containing two HNF4α binding sites was strongly transactivated by HNF4α (Inoue *et al.* 2002). The *C9* promoter at -1992/-1 and that at -91/-1 from the translation start site were also transactivated by HNF4α. However, the activity of the shorter deletion mutant of the *C9* promoter at -60/-1 was not induced by HNF4α, indicating that a functional HNF4α binding site might exist between -61 and -91 in the *C9* promoter. Since an HNF4α binding site was predicted at -84/-69 in the *C9* promoter by the JASPAR database, mutations were introduced into the predicted HNF4α binding site of the -1992/-1 promoter (-1992/Mut). As expected, the promoter activity of the -1992/Mut promoter was not transactivated by HNF4α (Fig. 4*A*). EMSA showed that nuclear extracts from HepG2 cells bound to the HNF4α binding site (Fig. 4*B*, lane 2, lower arrow). This complex was diminished by the addition of excess amounts of unlabeled *C9* and *Otc* competitors that contain HNF4α binding site (lanes 3 and 4) but not the *C9* competitor that contains mutations in the HNF4α binding site (lane 5). Furthermore, the complex was supershifted by the anti-HNF4α antibody (lane 6, the upper arrow) but not by the anti-PPARβ antibody (lane 7). ChIP using the livers of *Hnf4a*$^{f/f}$ and *Hnf4a*$^{\Delta Hep}$ mice also indicated that HNF4α in *Hnf4a*$^{f/f}$ mice strongly bound to the promoter region compared to that in *Hnf4a*$^{\Delta Hep}$ mouse livers (Fig. 4*C*), suggesting that HNF4α directly and physiologically binds to the *C9* promoters in mouse livers.

Discussion

In this study, we found that hepatic expression of many complement genes is decreased in *Hnf4a*$^{\Delta Hep}$ mice. Promoter activity of the human *C3* gene is dependent on HNF4α expression in HepG2 cells (Shavva *et al.* 2013), but hepatic expression of *C3* mRNA was not decreased in *Hnf4a*$^{\Delta Hep}$ mice and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice and in siRNA against HNF4α-treated HepG2 cells. Thus, the *C3* gene may not be a direct target of HNF4α. On the other hand, decreased expression of *C8a*, *C8b*, and *C8g* was observed in *Hnf4a*$^{\Delta Hep}$ mouse livers and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice and in siRNA against HNF4α-treated HepG2 cells. Hepatic expression of *C9* was significantly decreased in *Hnf4a*$^{\Delta Hep}$ mice and tamoxifen-treated *Hnf4a*$^{f/f;AlbERT2cre}$ mice, but the expression was not detected in siRNA against HNF4α-treated HepG2 cells, indicating that C9 is produced in normal hepatocytes but is not produced in HepG2 cells. Conversely, the expression of *C8A* and *C8B* was not detected, but the expression of *C8G* and *C9* was induced by overexpression of HNF4α in human immortalized hepatocytes.

C8 is composed of three subunits, C8A, C8B, and C8G, and C8A dimerizes with C8G by a disulfide bond (C8A-G), followed by a non-covalent bond with C8B (Steckel *et al.* 1980; Ng *et al.* 1987). Hemolytic activity was shown only by C8A and C8B, and addition of C8G to C8A and C8B significantly elevated the activity, indicating that C8G is not essential for hemolytic activity but enhances the activity (Parker and Sodetz 2002). Of *C8* deficiency in humans, patients with *C8A* and *C8G* deficiencies are abundant in Asians and Africans, but patients with *C8B* deficiency are more common in Caucasians (Sjoholm 2002). C8 proteins, like other complement proteins, are mainly expressed in the liver, but it was reported that the expression of C8G is elevated in the brains of Alzheimer's disease mice and patients (Kim *et al.* 2021). Since treatment with C8G in Alzheimer's disease model mice attenuated neuroinflammation, C8G plays an important role in protection of the brain from inflammation. Furthermore, there was a positive correlation between circulating C8G protein and metabolic dysfunction-associated steatotic liver disease (MASLD) (Shi *et al.* 2024). In this study, we showed that *C8g* is a direct HNF4α target gene, suggesting that the HNF4α-C8G axis might be involved in the pathogenesis of MASLD.

C9 proteins form a transmembrane channel by binding to the C5b-8 complex on the surface of pathogens (Bayly-Jones *et al.* 2017). *C9*-deficient mice had impaired antibody-mediated

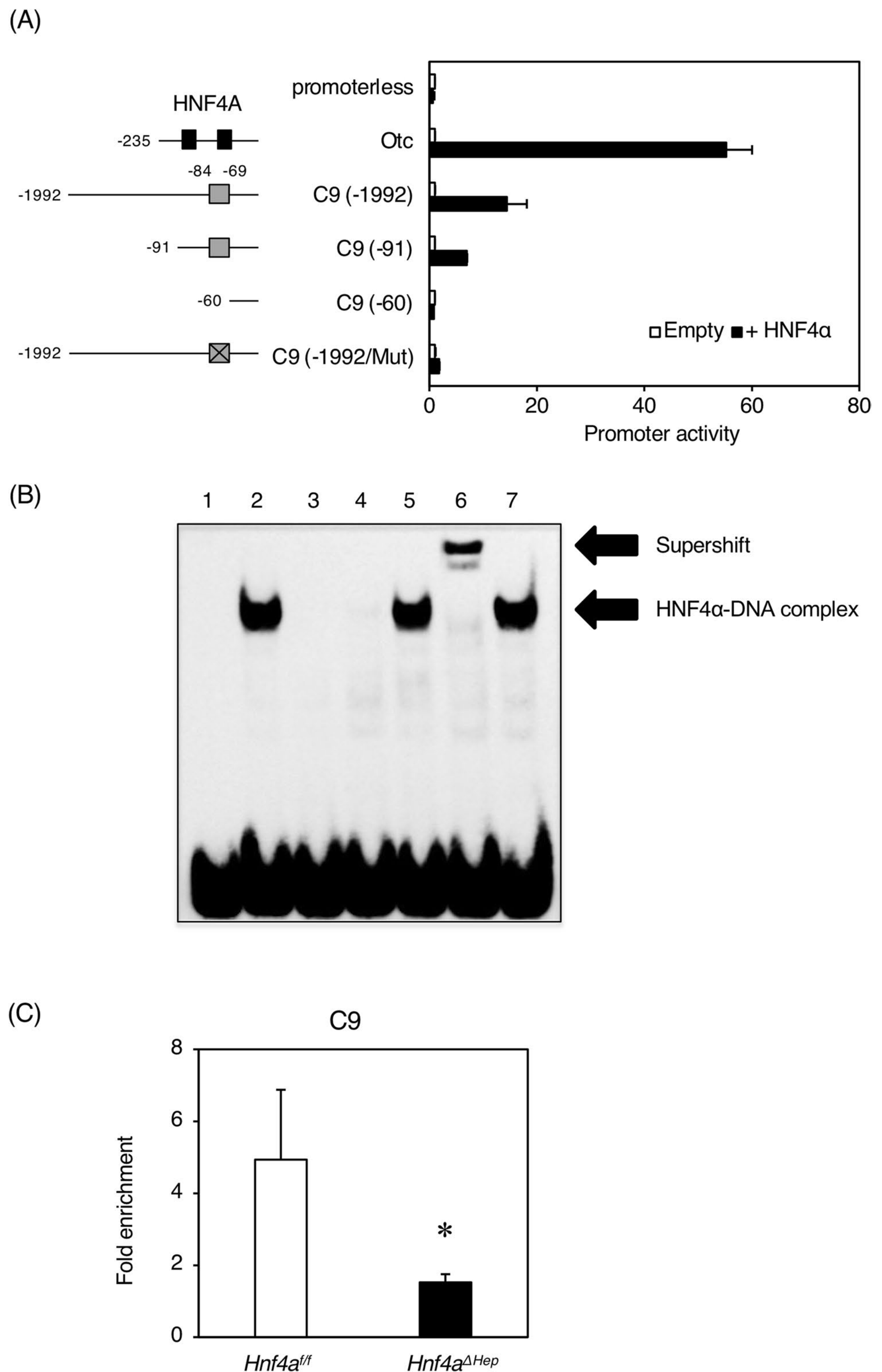

Figure 4. Promoter analysis of the mouse *C9* gene. (***A***) Promoter activity of the *C9* gene. The *C9* promoters from the translation start site were co-transfected with an empty vector (Empty), or HNF4α expression vector (+HNF4α) into HEK293T cells. Mutations were introduced into the predicted HNF4α binding site (-72/-60) in the promoter. (***B***) Electrophoretic mobility shift assay. Biotin-labelled probe carrying the HNF4α binding site in the *C9* promoter was incubated without nuclear extracts (*lane 1*). Nuclear extracts from HepG2 cells were incubated with biotin-labelled probe carrying the HNF4α binding site in the *C9* promoter in the absence (*lane 2*) or presence of a 50-fold excess amounts of the unlabeled *C9* and *Otc* probes (*lanes 3 and 4*), and the *C9* probe that contains mutations in the HNF4α binding site (*lane 5*). For supershift analysis, anti-HNF4α and anti-PPARb antibodies were added, respectively (*lanes 6 and 7*). Complex between HNF4α and the probe and supershifted complex are indicated by the *lower* and *upper arrows*, respectively. (***C***) Chromatin immunoprecipitation using the livers of $Hnf4a^{f/f}$ and $Hnf4a^{\Delta Hep}$ mice with normal goat IgG and anti-HNF4α antibody. The region containing the HNF4α binding site in the *C9* 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. Data are mean ± S.D. *$P < 0.05$ compared to $Hnf4a^{f/f}$ mice.

hemolysis and LPS-induced acute shock, indicating that C9 plays a critical role in complement-mediated hemolysis and inflammation activation (Fu *et al.* 2016). C9 deficiency, which is very rare in Caucasians, is more frequent in Japanese, and some patients exhibit *Neisseria* infections, but most are asymptomatic (Inai *et al.* 1989; Grumach and Kirschfink 2014). Moreover, the serum or plasma level of C9 protein was shown to be increased in various cancers including squamous cell lung cancer, gastric cancer, and colorectal cancer (Chong *et al.* 2010; Murakoshi *et al.* 2011; Narayanasamy *et al.* 2011).

C9 was also suggested to be associated with other diseases. For instance, an increased urinary ratio of C9 to CD59, which inhibits MAC formation, was associated with tubulointerstitial fibrosis in lupus nephritis patients, and C9 protein was elevated in plasma exosomes of Alzheimer's disease patients (Cai *et al.* 2022; Wang *et al.* 2023). Furthermore, hepatic expression of *C9* was decreased in the early stage of NAFLD, with a decreasing trend in the expression with NASH progression (Subudhi *et al.* 2022). Regarding the regulatory mechanism of *C9* expression, expression of HNF4α was inhibited by hepatitis C virus (HCV) infection and the expression of C9 was decreased in plasma of hepatocellular carcinoma (HCC) patients with HCV infection (Aydin *et al.* 2019; Ferrin *et al.* 2014). These findings suggested that *C9* is possibly regulated by HNF4α, but this study proved that *C9* is a novel target gene of HNF4α. In addition, hepatitis B virus-encoded oncogene X protein (HBx) inhibits *C9* transcription via inhibition of transcription factor USF-1 function, resulting in inhibition of MAC formation, and dihydro-stilbene gigantol potently alleviates oxidative stress and inflammation in the liver via decreased expression of *C9* (Xue *et al.* 2020; Baidya *et al.* 2022). Our results provide new insights into the possible involvement of HNF4α in further pathogenesis of various C9-associated diseases.

In summary, our study showed that HNF4α positively regulates the expression of complement genes involved in complement activation and MAC formation and that *C8g* and *C9* are target genes of HNF4α. This study provides insight into the transcriptional mechanism of *C8g* and *C9* by HNF4α, which is expected to lead to a better understanding of the pathogenesis of and therapeutic application for diseases in which these genes have been implicated.

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Data availability All the data presented in the manuscript is provided in the main text and the supplementary file.

Declarations

Conflict of interest The authors declare no competing interests.

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