学 位 論 文

IGF1 suppresses cholesterol accumulation in the liver of

growth hormone deficient mice via the activation of ABCA1

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RESEARCH ARTICLE | *Role of Gut Microbiota, Gut-Brain and Gut Liver Axes in Physiological Regulation of Inflammation, Energy Balance, and Metabolism*

IGF1 suppresses cholesterol accumulation in the liver of growth hormone-deficient mice via the activation of ABCA1

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Fukunaga K, Imachi H, Lyu J, Dong T, Sato S, Ibata T, Kobayashi T, Yoshimoto T, Yonezaki K, Matsunaga T, Murao K. IGF1 suppresses cholesterol accumulation in the liver of growth hormone-deficient mice via the activation of ABCA1. *Am J Physiol Endocrinol Metab* 315: E1232–E1241, 2018. First published August 21, 2018; doi:10.1152/ajpendo.00134.2018.—Recently, several clinical studies have suggested that adult growth hormone (GH) deficiency that also has low concentration of IGF1 is associated with an increased prevalence of fatty liver (FL). ATP-binding cassette transporter A1 (ABCA1) is a pivotal regulator of lipid efflux from cells to apolipoproteins and plays an important role on formation of FL. In this study, we determined the effects of IGF1 on ABCA1 expression in GH-deficient mice to clarify its effects on FL. Western blotting, real-time PCR, and a luciferase assay were employed to examine the effect of IGF1. The binding of FoxO1 to the *ABCA1* promoter was assessed by chromatin immunoprecipitation (ChIP) assay. Cholesterol accumulation was analyzed by Oil Red O stain and cholesterol content measurement. We confirmed that IGF1 upregulated the ABCA1 expression. The activity of a reporter construct containing the *ABCA1* promoter was induced by IGF1, and this effect was blocked by LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K). Constitutively active Akt stimulated the *ABCA1* promoter activity, and a dominant-negative mutant of Akt or mutagenesis of the FoxO1 response element abolished the effect of IGF1. A ChIP assay indicated that FoxO1 mediated IGF1 transcriptional activity by directly binding to the *ABCA1* promoter region. For in vivo experiments, we used an inhibitor for the GH receptor (Pegvisomant) to reduce the IGF1 level. A high-fat diet induced FL in mice (C57BL/6J) given Pegvisomant treatment. IGF1 treatment stimulated ABCA1 expression to improve cholesterol accumulation in these mice. These results show that the PI3K/Akt/FoxO1 pathway contributes to the regulation of ABCA1 expression in response to IGF1 stimulation that suppressed FL in GH-deficient mice.

ABCA1; adult growth hormone deficiency; fatty liver; IGF1

INTRODUCTION

GH (growth hormone) is secreted throughout life and is important to maintain various metabolic, physical, and mental functions in adulthood (18, 24). Adult growth hormone deficiency (AGHD) is characterized by an increase in visceral adipocytes, a dyslipidal profile, early atherosclerosis, and an increase in mortality (18, 24). Several clinical studies have suggested that AGHD increases the prevalence of fatty liver, nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steatohepatitis (NASH) (23). In addition, insulin-like growth factor-1 (IGF1) as well as GH have been shown to play an essential role in preventing the formation of fatty liver. One report indicated that GH replacement therapy greatly improved NASH in a patient with AGHD (41). There have been a few reports on the effect of IGF1 administration on liver diseases, and one report indicated that GH-deficient rats with NASH show an improved liver following the administration of IGF1 (similar to what is observed with the administration of GH) (29). However, the mechanism for how this occurs is not yet known.

ATP-binding cassette transporter A1 (ABCA1), a 254-kDa cytoplasmic membrane protein, is a pivotal regulator of lipid efflux from cells to apolipoproteins. In reverse cholesterol transport, ABCA1 plays an important role (15). ABCA1 was identified as a mutated protein in Tangier disease, and the absence of ABCA1 induces high-density lipoprotein (HDL) deficiency and the deposition of sterols in tissues. In addition, patients with Tangier disease have elevated plasma triglyceride (TG) levels and usually develop fatty liver disease. Indeed, there is an inverse correlation between plasma TG concentrations and dysfunctional ABCA1 alleles (11). Liu et al. (21) reported that TG secretion from hepatocytes increases when ABCA1 function is absent or diminished, suggesting that ABCA1 and fatty liver disease are also closely related. A fatty liver is associated with decreased expression of ABCA1 protein and increased lipid accumulation in the liver, whereas decreased hepatic lipid contents and increased cholesterol efflux were observed in ABCA1 overexpression (22, 48).

Although fatty liver/NAFLD/NASH are important comorbidities in AGHD, the exact roles of IGF1 in hepatic cholesterol accumulation have not been clarified yet. In this study, we determined the effects of IGF1 on ABCA1 expression in GH-deficient mice to clarify its effects on lipid metabolism.

MATERIALS AND METHODS

Cell culture. The HepG2 cells were obtained from American Type Culture Collection company. The present experiments were performed using cell passages 9~35, the cells being trypsinized every 7

days. These cells were cultured in DMEM (Life Technologies, Tokyo, Japan) containing 5.6 mmol/l glucose and supplemented with 10% heat-inactivated fetal bovine serum (Dainippon Pharmaceutical Co., Ltd. Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were incubated in humidified 5% CO_2 at 37°C. When 80% confluent, the cells were washed twice and incubated with 0.5% fetal bovine serum DMEM medium for 6 h. Then the cells were treated with varying doses of IGF1 (R&D Systems) for either 24 h or for different time points (0, 5, 15, 30, and 60 min) before harvesting as described previously (7). The protocol used in this experiment was reviewed and approved by the Kagawa University Institutional Animal Care and Use Committee.

Western blot analysis. The 15 μg of proteins were separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane for immunoblotting. After blocking with skim milk, the membrane was incubated with either anti-ABCA1 antibody (1:200; Santa Cruz), anti-GAPDH antibody (1:5,000; Biomol Research), anti-IGF1R antibody (1:50; Santa Cruz), phosphor-specific FoxO1 polyclonal antibody (1:1,000; Cell Signaling Technology), or FoxO1 antibody (1:1,000; Cell Signaling Technology) as described previously (27). The antigen-antibody complexes were visualized by ECL (GE Healthcare).

Real-time PCR. PCR was performed with a volume of 20 μl in LightCycler (Bio-Rad). The sequences of the forward and reverse primers used for amplifying the *ABCA1* gene were 5'-TTGAACT-TCTGGGCAAATG-3' and 5'-TGGGGATGCCTTCAAACAC-3', respectively. Each set of PCR reactions included water as a negative control and 5 dilutions of the standard. Known amounts of DNA were then diluted to make the standards, and a regression curve of crossing points versus concentration was generated with LightCycler as described previously (27). GAPDH was used as the housekeeping standard.

Transfection of HepG2 cells and luciferase reporter gene assay. We used a construct (*pABCA1*-LUC) containing the *ABCA1* promoter obtained using PCR and its mutant (*pABCA1*-mut-LUC) in which FoxO response sequence (FRS) (GGAAAACAAA) was mutated as previously described (49). Purified ABCA1 promoter plasmid (0.5 μg/well) was transfected into HepG2 cells using Lipofectamine (Life Technologies). Transfected cells were maintained in medium containing 0.5 μg/ml IGF1 for 24 h with or without pretreatment with LY294002 (LY; 10 μmol/l), SB203580 (SB; 1 μmol/l), H-89 (1 μmol/l), or bisindolylmaleimide I (1 μg/ml) to inhibit separately the phosphatidylinositol 3 kinase (PI3K), p38 mitogen-activated protein kinases (p38-MAPK), protein kinase A (PKA), or protein kinase C (PKC) signaling pathway for 30 min. To check the role of the PI3K/Akt pathway, HepG2 cells were treated with cotransfection of ,ABCA1 promoter plasmid (0.5 μg/well) plus a vector (0.5 μg/well) expressing the constitutively active form of Akt (myristylated Akt lacking the pleckstrin homology domain; Akt-CA), p110 catalytic subunit of PI3K (P110), or an expression plasmid encoding a dominant-negative mutant of Akt (Akt with a K197M mutation; Akt-DN) as previously described (49). To check the role of the FoxO1 in ABCA1 transcription, ABCA1 promoter plasmid (0.25 μg/well) was cotransfected with the vector expressing FoxO1 (0.1 μg/well) into HepG2 cells. All assays were corrected for β-galactosidase activity, and the total amount of protein in each reaction was identical. Aliquots (40 μl) were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-ITTM kit (Active Motif) according to the manufacturer's instructions. Chromatin was immunoprecipitated with 2 μg of either rabbit FoxO1 antibody (Cell Signaling Technology) or negative control IgG. DNA was analyzed by PCR to amplify regions containing the putative FRS on human *ABCA1* promoter by using the following primers: forward 5'-

AATCTCCAAGGCAGTAGGTCG-3' and reverse 5'-GAATCTC-CCTCAGGACGCCAA-3', as described previously (27).

Transfection of small interfering RNA. The small interfering RNAs (siRNAs) were designed to target the following sequences: IGF1R-scrambled siRNA, 5'-CCAAUGUUAAUACAUCCAACU-3' and IGF1R siRNA, 5'-AAACUCUUCUACAAUUACGCA-3', as described previously (49).

Cholesterol content assay. For cholesterol content measurement, we employed a method developed by Shahnaz et al. (37), which is a completely automated fluorimetric method for the determination of cellular cholesterol, consisting of enzymatic hydrolysis of cholesteryl ester to free cholesterol and enzymatic oxidation of free cholesterol in the presence of an indicator substrate to produce a fluorescent product.

In vivo experiment. Eight-week-old male mice (C57BL/6J) were purchased from Clea Japan, Inc. (Osaka, Japan), and mice were maintained in controlled light conditions (14 h light, 10 h dark). The protocol used in this experiment was reviewed and approved by the Kagawa University Institutional Animal Care and Use Committee (Kagawa, Japan). Hormonal stimulation was given as intraperitoneal injection. Eight-week-old mice were divided into three groups ($n = 5$ each): *1*) control [high-fat diet (HFD), 45 kcal % fat, 20 kcal % protein, and 35 kcal % carbohydrate], *2*) HFD + 10 mg·kg^{-1} every other day Pegvisomant [(PEG) growth hormone receptor antagonist obtained from Pfizer], and *3*) HFD + PEG + 2.4 mg·kg^{-1} every day IGF1. After the continuous 4-wk administration of PEG and/or IGF1, we employed biochemical analysis, histological analysis of the liver, and the expression of ABCA1 in the liver of those mice. Whole liver was removed and snap-frozen in liquid nitrogen. Furthermore, to confirm the expression of IGF1R in mouse liver, a mouse fed with normal diet (MF: 25 kcal % fat) or HFD given for 4 wk were used. As for the immunostaining, we used the polymer chain two-step indirect method (Simple Stain Mouse MAX PO kit; Nichirei, Tokyo, Japan) as described previously (32). We used anti-IGF1R antibody (1:50; Santa Cruz) as primary antibody.

Statistical analysis. Data was expressed as mean ± SE. Results were analyzed by one-way ANOVA and Student's *t*-test. A value of $P < 0.05$ was considered as statistically being significant. All experiments were performed at least three times.

RESULTS

IGF1 increases the expression of ABCA1 in HepG2 cells. To analyze the effects of IGF1 on ABCA1 expression, Western blot was used to measure the levels of endogenous ABCA1 in HepG2, the human hepatoma cell line. Exposure of these cells to IGF1 for 24 h increased the abundance of endogenous ABCA1 protein in a dose-dependent manner as compared with that of control cells (Fig. 1*A*).

Real-time PCR results also showed that the IGF1 enhances the expression of *ABCA1* mRNA (Fig. 1*B*). These results clearly show that IGF1 increased the expression of *ABCA1* in HepG2 cells. In addition to this, we inhibited expression of IGF1R using siRNA and found that the absence of IGF1R canceled the stimulatory effect of IGF-1 on ABCA1 protein expression by Western blot analysis (Fig. 1*C*). These findings indicate that induction of ABCA1 by IGF1 in HepG2 cells requires IGF1R.

IGF1 increases ABCA1 promoter activity in HepG2 cells. We measured transcriptional activity of the *ABCA1* gene in HepG2 cells. HepG2 cells transfected with reporter genes were exposed to various concentrations of IGF1. Consistent with the changes observed in the levels of ABCA1 protein and mRNA, IGF1 increased *ABCA1* promoter activity in a dose-dependent manner (Fig. 2*A*). To clarify the signal transduction mechanism involved in the stimulatory effects of IGF1 on *ABCA1* pro-
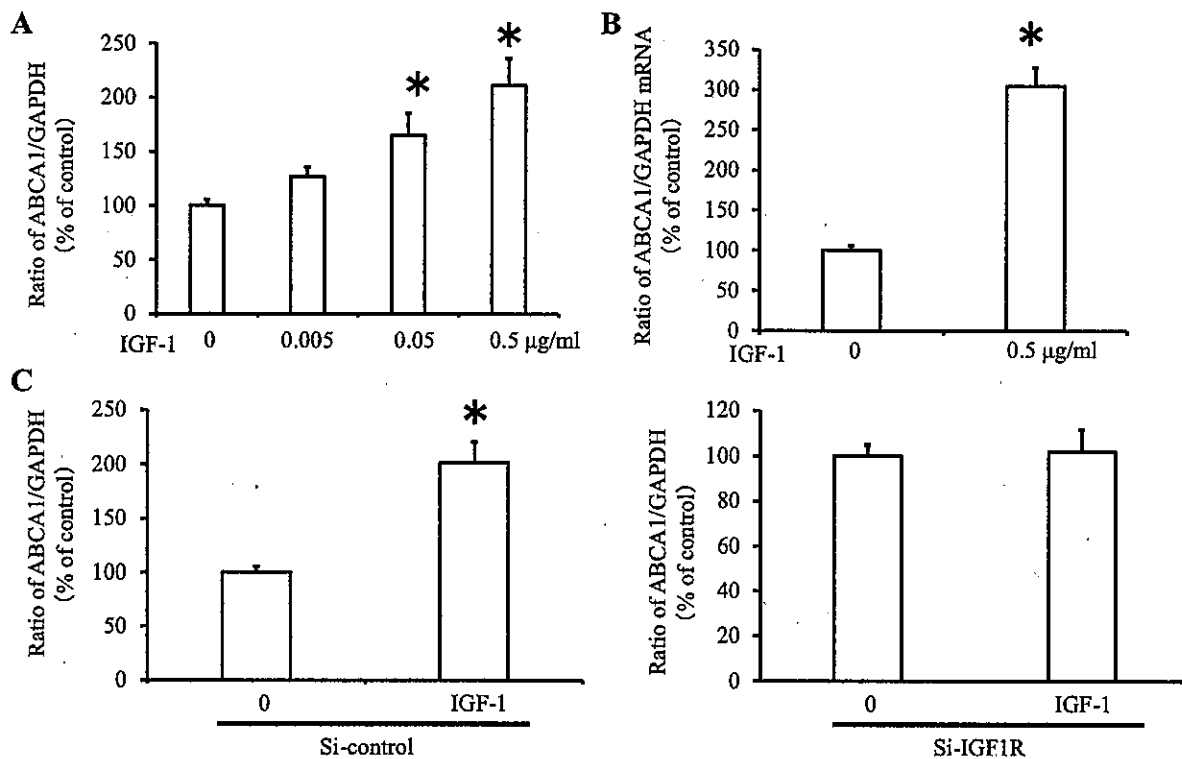
Fig. 1. Effects of IGF1 on ABCA1 expression. IGF1 increases ABCA1 protein expression (A). Whole-cell extracts from HepG2 cells treated with varying concentrations of IGF1 (0, 0.005, 0.05, 0.5 μg/ml) for 24 h were probed for ABCA1 protein using Western blot analysis. GAPDH expression served as a control and the ratio of ABCA1 to GAPDH is shown as a percent of the control. IGF1 increases *ABCA1* mRNA level in HepG2 cells (B). Effect of silencing IGF1R on IGF-1-induced ABCA1 protein expression normalized to GAPDH (C). Data represent the mean ± SE ($n = 3$) of separate experiments for each treatment group. *$P < 0.05$ compared with 0 or control. ABCA1, ATP-binding cassette transporter A1; si-control, scrambled siRNA; si-IGF1R, IGF1R-specific siRNA; siRNA, small interfering RNA.

moter activity, the effect of pharmacological inhibitors on *ABCA1* promoter activity was evaluated. Specifically, the effect of IGF1 (0.5 μg/ml) on p*ABCA1*-LUC-transfected HepG2 cells was checked in the presence of inhibitors against PI3K (10 μM LY294002), p38-MAPK (1 μM SB203585), PKA (1 μM H89), or PKC (1 μM Bisindolylmaleimide I). The results (Fig. 2B) indicated that the stimulatory effect of IGF1 on *ABCA1* promoter activity was sensitive to LY294002, an inhibitor of PI3K but was not sensitive to the inhibitors of p38-MAPK, PKA, and PKC. This result suggests that the effects of IGF1 seem to be mediated by the PI3K signal transduction pathway.

PI3K/Akt regulates ABCA1 promoter activity. Because one of the downstream components of the PI3K cascade is Akt, we hypothesized that it may be involved in the IGF1 induction of ABCA1. To confirm the role of the PI3K/Akt pathway in IGF1 induction of ABCA1 expression in HepG2 cells, we assumed that transfection of the constitutively active Akt (Akt-CA) or p110 (a subunit of PI3K) should induce ABCA1 expression. As predicted, *ABCA1* promoter activity increased without exposure to IGF1 in both Akt-CA and p110 (Fig. 2C). In addition, the effect of IGF1 on *ABCA1* promoter activity was canceled in cells transfected with a dominant-negative form of Akt (Akt-DN) (Fig. 2C). These results support the notion that the PI3K/Akt pathway is needed for IGF1 induction of ABCA1 expression in HepG2 cells.

IGF1 induces FoxO1 phosphorylation, and FoxO1 binds to the ABCA1 promoter. We investigated whether the transcription factor FoxO1 is involved in the pathway by which IGF1 induces ABCA1 expression, as the *ABCA1* promoter contains FRS, which is the FoxO1 binding site. Previous reports indicated that the transcription factor FoxO1 mediated the PI3K/Akt pathway. FoxO1 is one of the members of the FoxO family of transcription factors that contains three putative phosphorylation sites for Akt and a conserved forkhead domain. In this study, it was investigated whether FoxO1 could bind to putative FRS, which is located upstream (−588 bp) from the transcription initiation site within the hepatic *ABCA1* promoter. In a ChIP assay, FoxO1 specifically immunoprecipitated the ABCA1 chromatin containing the FoxO1 binding site (Fig. 3A). No chromatin was pulled down when nonspecific IgGs were used in the ChIP assay. These results suggested that the *ABCA1* promoter region contains an FRS in HepG2 cells. Perhaps IGF1 activates Akt activity and results in the phosphorylation of FoxO1. As a result, there is a possibility that the amount of FoxO1 in the nucleus decreases, and the suppression of *ABCA1* promoter activity is released. To confirm this idea, we measured the kinetics of FoxO1 phosphorylation and indicated that IGF1 stimulated the phosphorylation of FoxO1 (Fig. 3B). Then we examined the amount of FoxO1 in the nucleus by Western blotting. The FoxO1 protein was found to decrease from the nucleus with phosphorylation of FoxO1 (Fig. 3B).
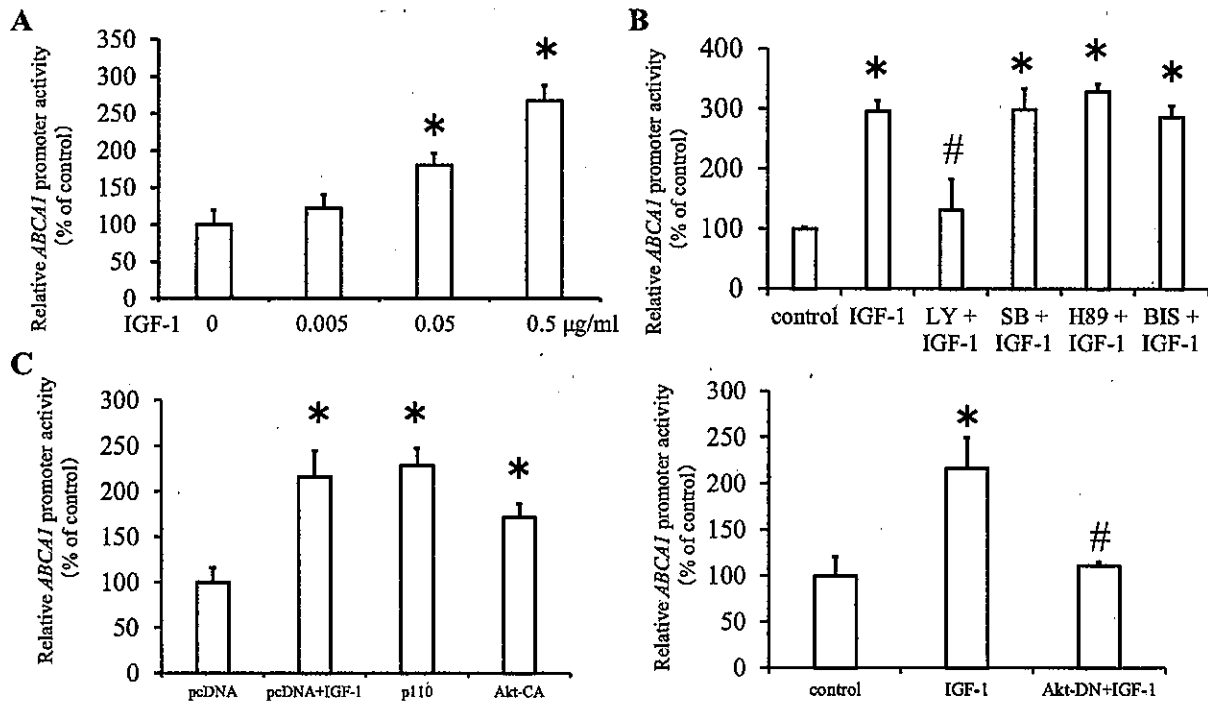
Fig. 2. Effect of IGF1 on *ABCA1* promoter activity in HepG2 cells. IGF1 increases *ABCA1* promoter activity in a dose-dependent manner (*A*). Effects of a PI3K inhibitor [LY294002 (LY)], a p38-MAPK inhibitor [SB203580 (SB)], a PKA inhibitor (H-89), or a PKC inhibitor [bisindolylmaleimide I (BIS)] on IGF1-induced *ABCA1* promoter activity (*B*). Role of the PI3K/Akt signal transduction pathway on *ABCA1* promoter activity induced by IGF1 (*C*). pcDNA was used as the empty vector control. Promoter activity was relative to the control or pcDNA. Data represent the mean ± SE (*n* = 3) of separate transfections for each treatment group. *P < 0.05 compared with control or pcDNA, #*P* < 0.05 compared with IGF1 or pcDNA plus IGF1. ABCA1, ATP-binding cassette transporter A1; Akt-CA, constitutively active Akt; Akt-DN, dominant-negative mutant of Akt; P110, p110 catalytic subunit of PI3K; phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C.

Furthermore, the transcriptional activity of the *ABCA1* promoter was inhibited by overexpressing FoxO1, and the inducing effect of IGF1 on *ABCA1* promoter activity decreased (Fig. 3*C*). To analyze further the importance of FoxO1 for the activity of the *ABCA1* promoter, we created a reporter gene construct with a mutation within the FRS motif and found it canceled the effect of FoxO1 overexpression (Fig. 3*D*). Akt-CA and p110 expression also could not induce the activity of the mutant *ABCA1* promoter. Moreover, mutation of FRS dramatically reduced the ability of IGF1 to stimulate *ABCA1* promoter function (Fig. 3*E*), whereas IGF1 stimulated the activity of the wild-type promoter (Fig. 2*A*). These results suggest that IGF1-mediated induction of *ABCA1* promoter activity requires an intact FRS motif.

Effect of IGF1 on cholesterol accumulation. We stained HepG2 cells using Oil Red O and measured the cholesterol content. Figure 4*A* shows that lipid droplets are fewer and smaller in cells treated with IGF1 than in cells without IGF1 treatment in Oil Red O staining of HepG2 cells. Oil Red O stain is used for staining neutral lipids, including TG and cholesteryl esters. Specifically, we conducted experiments on cholesterol content; we have assessed cholesterol accumulation by measuring cholesterol content. Figure 4*B* indicates that IGF1 decreased the cholesterol content in HepG2 cells.

Effect of IGF1 on ABCA1 expression in vivo. Although in vitro experiments revealed that IGF1 stimulates ABCA1 expression, it is unknown whether IGF1 has the same effect in an in vivo model. At first, we have examined the expression of IGF1R in the mice treated with both MF or HFD by Western

blot and immunohistochemistry. As shown in Fig. 5, *A* and *B*, the expression of IGF1R was observed in the liver treated with HFD, however, not in the liver with MF. To reduce the IGF1 levels in mice, we used PEG, a GH receptor inhibitor. A previous report indicated that serum IGF1 levels, hepatic IGF1 protein levels, and hepatic *IGF1* mRNA levels are reduced by PEG administrations in a dose-dependent manner (26). We also confirmed that serum IGF1 level was decreased by administration of PEG (Fig. 6*A*). In Western blot (Fig. 6*B*) or real-time PCR (Fig. 6*C*), the ABCA1 expression in the liver was decreased in the PEG group, and the expression of ABCA1 significantly improved in the PEG + IGF1 group in comparison with that in the PEG without IGF1 group. In the PEG + IGF1 group compared with the PEG group, although the serum HDL-cholesterol level was significantly increased (Fig. 6*D*), the alanine aminotransferase (Fig. 6*E*) and TG concentrations (Fig. 6*G*) were significantly decreased. The cholesterol accumulation in the liver was increased in PEG group, and the change was improved in the PEG + IGF1 group (Fig. 6*H*). In histological analysis, H&E staining revealed that fatty change of hepatocytes was observed in the PEG group, and its change was restored in the PEG + IGF1 group (Fig. 6*I*). These results suggest that IGF1 improved fatty change of hepatocytes induced by PEG.

DISCUSSION

In this report, we found that IGF1 improved GH deficiency-induced fatty liver by regulating ABCA1 gene expression. In
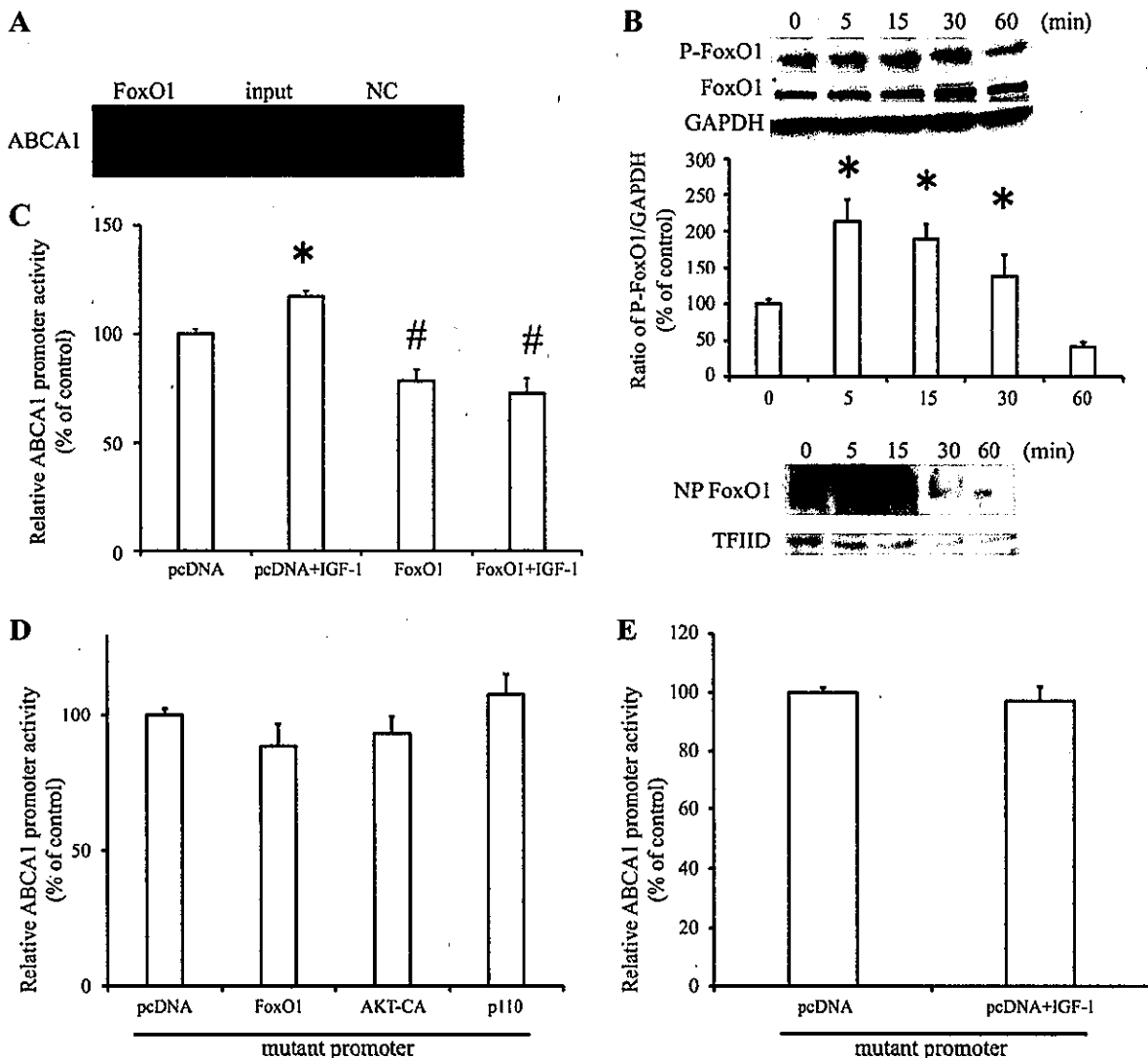
Fig. 3. Role of FoxO1 in *ABCA1* promoter activity in HepG2 cells. Chromatin immunoprecipitation (ChIP) assay using a FoxO1 antibody immunoprecipitates the *ABCA1*-containing chromatin, which contains the FoxO1-binding site (*A*). No chromatin was immunoprecipitated when the ChIP assay was performed with nonspecific immunoglobulins (IgGs); this was used as the negative control (NC). IGF1 stimulates the phosphorylation of FoxO1 (*B*). Cultured HepG2 cells were exposed to 0.5 μg/ml IGF1 for the indicated time, and FoxO1 phosphorylation was determined by Western blot analysis with a phospho-specific FoxO1 antibody (p-FoxO1) and total FoxO1 antibody. An identical experiment independently performed gave similar results. Each data point shows the mean ± SE ($n = 3$) of separate experiments. *$P < 0.05$. Nuclear extracts of the cells treated with IGF1 were subjected to Western blot analysis to examine the FoxO1 expression. Analysis of TFIID as a control is shown on the bottom of each lane. An identical experiment independently performed gave similar results. These HepG2 cells were treated with 0.5 μg/ml IGF-I for the indicated time. Effect of FoxO1 on *ABCA1* promoter activity (*C*). Data represent the mean ± SE ($n = 3$) of separate transfections for each treatment group. *$P < 0.05$ compared with pcDNA or 0, #$P < 0.05$ compared with pcDNA plus IGF1. The site-directed mutagenesis of the FRS by altering 4 base pairs (AACA to GGAG) abrogated the response to FoxO1, Akt-CA, P110, or IGF1 (*D* and *E*). pcDNA is an empty vector as control. Akt-CA, constitutively active Akt; FoxO1, overexpression of FoxO1 gene; P110, p110 catalytic subunit of PI3K; PI3K, phosphatidylinositol 3-kinase.

addition, we identified the signaling pathways in which IGF1 action promotes ABCA1 expression in HepG2 cells. As a specific inhibitor of PI3K (LY294002) prevents IGF1 promotion of the ABCA1 expression, this effect is suggested to be mediated by PI3K. Furthermore, the constitutively active form of the PI3K catalytic subunit p110 increases *ABCA1* transcription activity. To clarify the downstream effectors of PI3K, we evaluated both effects of Akt-CA and of Akt-DN on *ABCA1* promoter activity. Consistent with our hypothesis, Akt-CA mimics the effect of IGF1 on *ABCA1* promoter activity, and this effect was blocked with the dominant-negative mutant. In this study, the decreased amount of FoxO1 in the nucleus may

reduce the restraint of *ABCA1* promoter activity. Finally, we performed in vivo experiments to inject PEG into mice to reduce the action of GH, which induced fatty liver disease and decreased ABCA1 expression in the liver. IGF1 treatment of these mice improved fatty liver disease by increasing ABCA1 expression.

Frequently, patients with hypothalamic and pituitary dysfunction develop NAFLD (2). There are several reports that suggest that such hepatic impairment may be particularly related to GH deficiency (GHD). AGHD is characterized by decreased serum IGF1 levels, increased visceral adiposity, and an abnormal lipid profile, including an elevated TG (42). A
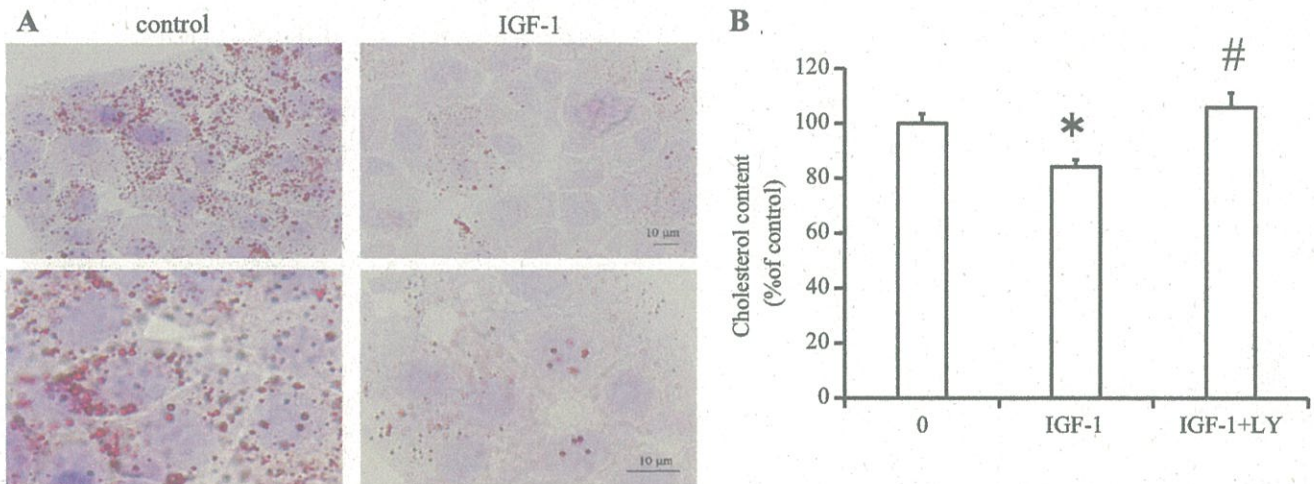
Fig. 4. Effect of IGF1 on cholesterol content in HepG2 cells. HepG2 cells were treated by IGF1 at 0.5 μg/ml for 24 h and then stained with Oil Red O (*A*). Intracellular cholesterol ester concentration was measured (*B*). Data represent the mean ± SE ($n = 3$) of separate experiments for each treatment group. *$P < 0.05$ compared with control, #$P < 0.05$ compared with IGF1. LY, LY294002.

previous report showed that hepatic steatosis was more frequently observed in patients with hypopituitarism with GHD than in patients without GHD (17). The liver is important as a metabolic organ that is regulated by various hormones. Indeed, the important roles of GH and IGF1 in the liver are recently drawing attention. IGF1 production is regulated mainly by GH, and both GH and IGF1 have anabolism in skeletal muscle and bone. It has been suggested that hepatocytes scarcely express IGF1 receptor under normal conditions and that IGF1 does not directly affect the function of hepatocytes (8). However, recent studies have shown that the expression of IGF1R increases under pathological conditions. In NAFLD/NASH, chronic hepatitis B, and chronic hepatitis C (19), IGF1R expression in hepatocytes was obviously detected compared with the expression in the normal liver (40), suggesting that the signaling of IGF1 may play a role under these pathological conditions (3, 8).

One of the aims of this study was to investigate the signal transduction pathways activated by IGF1 in hepatocytes in more detail. These contain two substrates: not only FoxO1 but also Akt. As a downstream target of Akt, there is the FoxO subfamily of forkhead transcription factors. FoxO1, FoxO3a (FoxO1L-1), and FoxO4 (AFX) constitute this subfamily, and Akt inactivates all of them (4, 6). Phosphorylation of FoxO isoforms by Akt results in their nuclear exclusion and leads to the inhibition of the forkhead transcriptional program. FoxO transcription factors are involved in the regulation of diverse cellular functions, including metabolism, differentiation, proliferation, and survival (1, 38). It is known that the PI3K-activated signaling pathway regulates the activity of the FoxO1 family (5). The PI3K pathway was initially implicated when cultured cells were treated with growth factors, including IGF1, which are known to activate the PI3K pathway, and FoxO1 was shown to be excluded from the nucleus. There are numerous consensus-binding motifs for transcription factors in the *ABCA1* promoter (14). Our results show that the PI3K/Akt pathway regulates the expression of ABCA1, and its effect is mediated via the FoxO transcription factors. Reporter gene studies prove that FoxO proteins inhibit *ABCA1* promoter

activity through this site and that this FoxO site is indispensable for the effect of IGF1 and Akt on promoter function. The mutation of this site on ABCA1 negated the effect of IGF1, raising the possibility that the PI3K/Akt/FoxO1 pathway might mediate the effect of IGF1 on the *ABCA1* gene. Although it is speculated that IGF1 exerts its effect on hepatocytes by the IGF1R, it is also necessary to consider that direct and/or indirect effects of other pathways such as insulin receptor. Previously, we demonstrated that FoxO1 is phosphorylated after exposing the rat insulinoma cell line to IGF1 and silencing of IGF1R cancels the effect of IGF1 on FoxO1 phosphorylation, indicating that rapid phosphorylation of FoxO1 is induced by IGF1 via IGF1R (49, 50). A previous report shows that insulin itself decreases ABCA1 protein expression in a dose-dependent manner via the insulin receptor (31). We also show that IGF1R knockdown in the cells cancelled the effects of IGF1 on ABCA1 expression in this study. These results suggest that IGF1 mainly exerts its effect through IGF1R in HepG2 cells. A previous report indicated that HepG2 cells expresses IGF1R more than normal hepatocytes (36); furthermore, several reports also confirm the expression of IGF1R in primary rodent hepatocytes (9, 12, 16). There are reports describing tumorigenic transformation or partial hepatectomy may induce IGF1R expression in liver parenchymal cells (13, 36); however, IGF1R expression by lipid accumulation has not been established yet. Although we have shown one of the mechanisms by which IGF1 stimulates ABCA1 expression mediated by IGF1R, we could not exclude the possibility that nonparenchymal cells express IGF1R, mediating these changes on lipid metabolism. Further studies will be necessary to decide the precise mechanism for the regulation of the *ABCA1* gene.

Previously, GH deficiency, in which IGF1 production in the liver is impaired, has been reported to increase the prevalence of NASH in rats and humans (28, 47). GH or IGF1 treatment improved NASH in an animal model, showing that IGF1 plays an essential role in a GH-independent manner (29). These results don't apply to everything because there are differences in the lipid metabolism between the mouse and human, but our
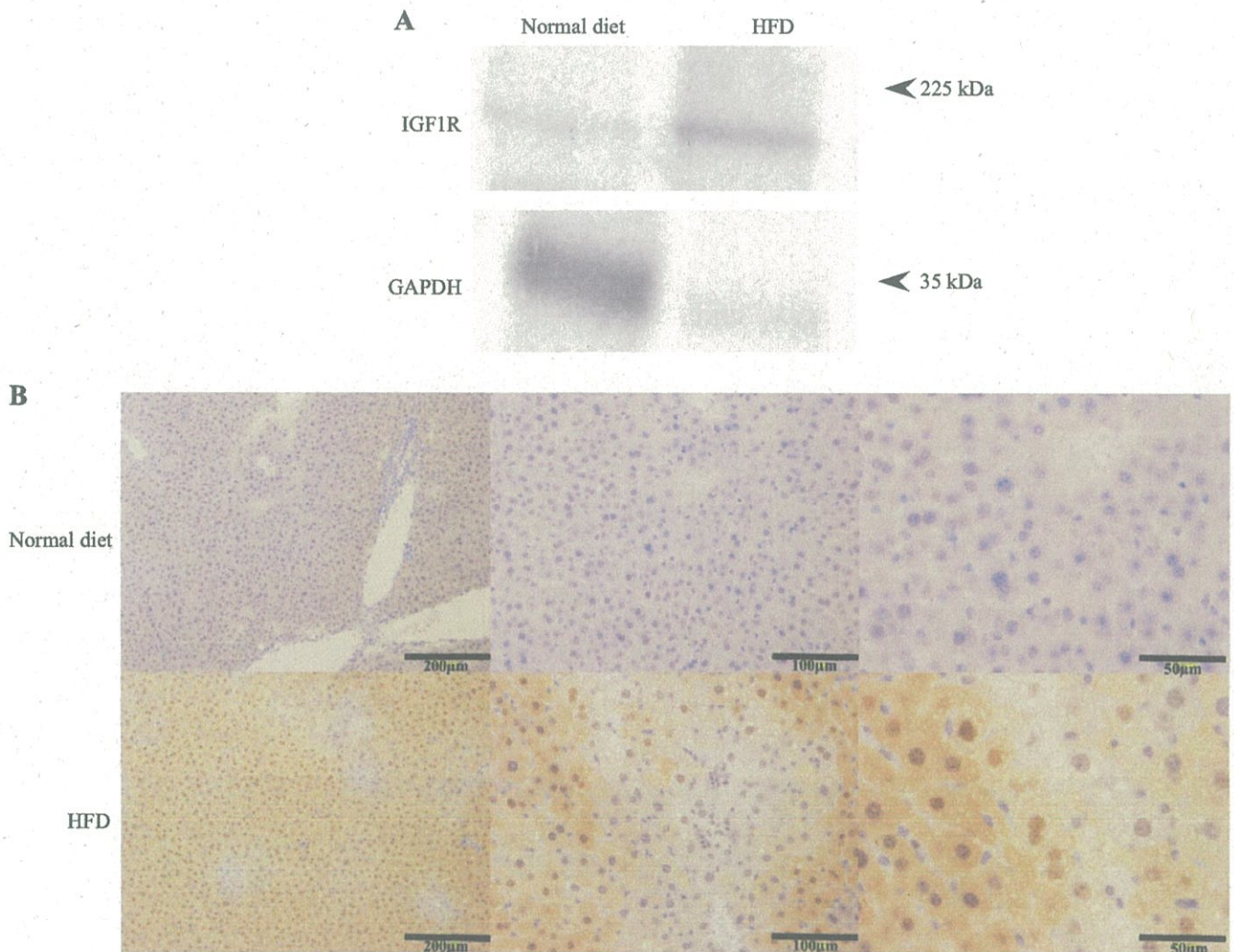
A

B

Fig. 5. Expression of IGF1R in mouse liver. IGF1R expression in the liver treated with normal diet (MF) or HFD mice using Western blot (*A*). Histological analysis of the liver with the normal diet (MF) mouse or HFD mouse by the immunostaining (*B*). The liver of HFD mouse was positive for IGF1R. HFD, high-fat diet.

results might suggest one possibility that IGF1 has the positive effects on human liver. Although we have shown one of the mechanisms by which ABCA1 expression, mediated by IGF1, is related to lipid metabolism and fatty liver disease, another mechanism (e.g., elevated metabolism of cholesterol and bile excretion, insulin resistance, oxidative stress, the inflammatory cascade, etc.) should also be considered. In the muscle, adipose tissues, and liver, IGF1 improves insulin sensitivity. Deficiency of the IGF1 gene in the liver leads to insulin resistance (47), indicating that systemic insulin sensitivity is regulated by hepatic IGF1. The effects of IGF1 in improving insulin sensitivity in vivo are well known. Abdominal adiposity, inducing insulin resistance, plays an essential role in the development of fatty liver, NAFLD, and NASH. Insulin resistance and excessive obesity increase lipid influx into the liver, and this results in increased lipogenesis in the liver and promotes hepatic TG accumulation (39). Otherwise, specific elimination of IGF1 results in reduced insulin sensitivity in muscle, liver, and adipose tissue (47). Based on IGF1 reports, IGF1 may partially ameliorate fatty changes in the liver by improving insulin

sensitivity. On the other hand, disorders of mitochondrial function and excessive lipid accumulation promote enhanced oxidative stress. Previous reports indicated that IGF1 administration improved mitochondrial function in aging rats (34) and improved fibrosis and hepatic dysfunction in the rat of cirrhosis model (33).

NAFLD is typically characterized by TG accumulation in the liver, but cholesterol homeostasis has been involved in more advanced NAFLD stages (fibrosis and NASH) (35, 44). Cholesterol efflux plays an important role not only in normal liver physiology and lipid metabolism but also in several pathological processes such as NAFLD (22, 25). A previous report showed that the protein levels of ABCA1 were significantly reduced in NASH subjects (45). There are few studies analyzing the expression of ABCA1 in NAFLD, and the results are inconsistent. These results are inconsistent with observations in the murine model, where steatosis was related to decreased expression of ABCA1 protein and increased hepatic lipid accumulation, whereas overexpression of ABCA1 resulted in increased cholesterol efflux and decreased lipid con-
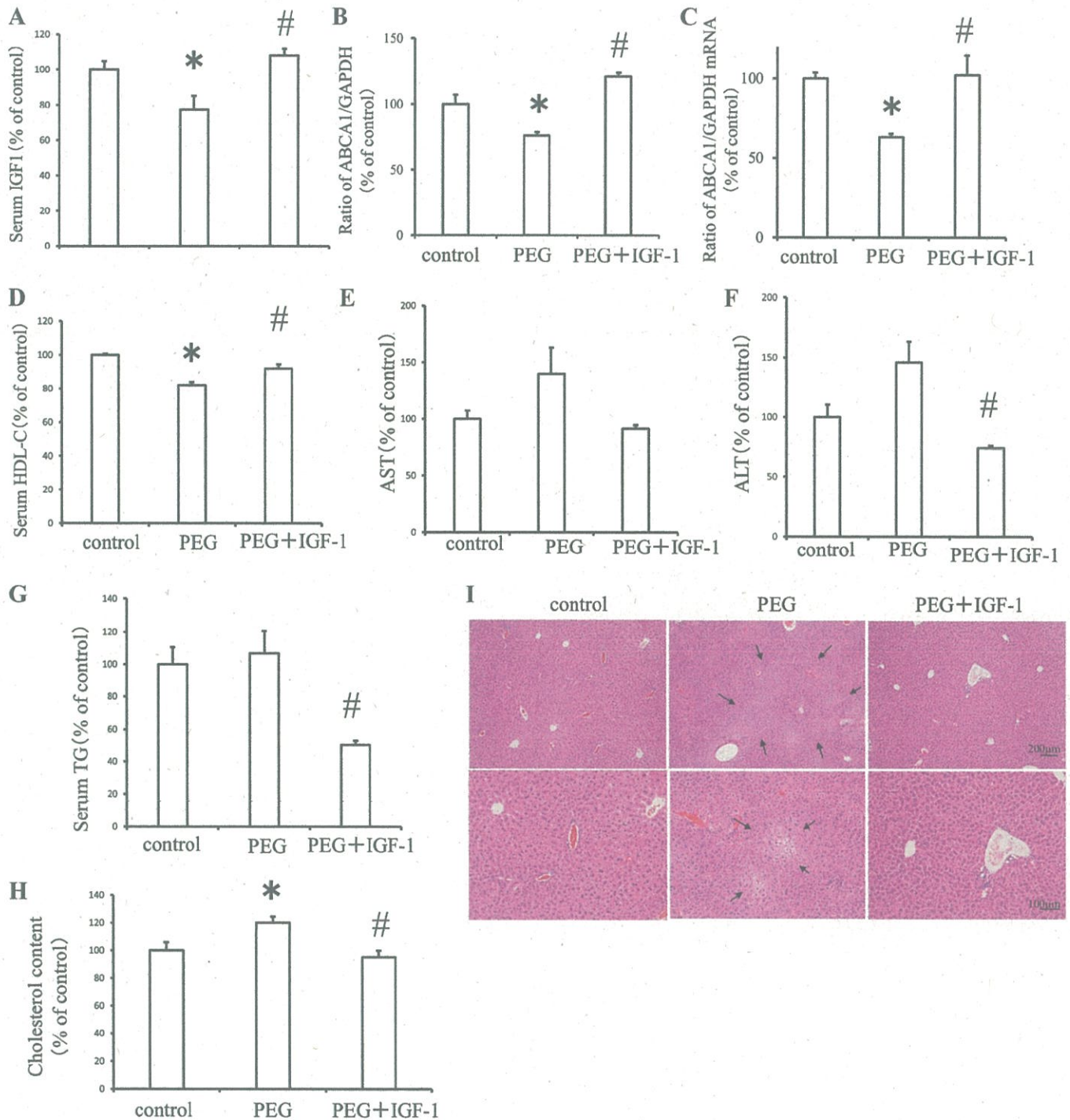
Fig. 6. Expression of ABCA1 and the effect of IGF1 on cholesterol content in mice liver. Serum concentration of IGF1 (*A*). Protein was extracted and purified from the liver of mice, and Western blot analysis was performed to measure ABCA1 expression (*B*). *ABCA1* mRNA expression in mouse liver (*C*). *ABCA1* mRNA was purified from mice livers and quantified by quantitative real-time PCR. Results are expressed as the mean ± SE ($n = 3$) of separate experiments for each treatment group. Serum concentration of HDL cholesterol (*D*), AST (*E*)/ALT (*F*), and TG (*G*). Data from each group ($n = 3$) are presented as mean ± SE *$P < 0.05$ compared with control. Cholesterol ester concentration in mouse liver was measured (*H*). Data represent the mean ± SE ($n = 3$) of separate experiments for each treatment group. #$P < 0.05$ as compared with the PEG group. Histological analysis of the liver in the PEG group and the PEG + IGF1 group with the H-E stain (*I*). ABCA1, ATP-binding cassette transporter A1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin-eosin; PEG, pegvisomant; TG, triglyceride.

tent in hepatocytes (10, 11). In liver-specific ABCA1-deficient mice, blood HDL was reduced by 80%, and liver ABCA1 was found to have the most important role in HDL production (43). In addition, attention is also paid to the relationship between ABCA1 and the synthesis of TG and fatty liver disease. Patients with Tangier disease have severe fatty liver and raised plasma TG concentrations in addition to very low plasma HDL concentrations (30). The deficiency of hepatic ABCA1 in mice almost replicates the lipid phenotype of Tangier disease such as fatty liver and increased plasma TG, suggesting that hepatic ABCA1 expression plays an essential role in altered plasma lipid phenotype expression in these individuals (43). TG-enriched VLDL overproduction is positively correlated with hepatic fat content (20). Liu et al. (21) suggested a mechanism that is responsible for the elevated plasma TG concentrations in ABCA1-deficient states. They claim that hepatic overproduction of TG is caused by dysfunction of ABCA1 in hepatocytes. Because the liver plays a major role in the production and catabolism of VLDL, LDL, and HDL, hepatic ABCA1 plays a pivotal role in the metabolism of all three major plasma lipoprotein species. When ABCA1 function is diminished or absent, large nascent HDL assembly decreases, leading to decrease PI3K activation, increased lipid mobilization into maturing VLDL particles, and secretion of larger, TG-enriched VLDL particles. Clinically, it is concerning that liver cirrhosis develops because of the progress of fatty liver/NAFLD/NASH, and it is important to clarify the role of IGF1 on the mechanism of fatty liver formation associated with AGHD (25, 35). According to the present data, IGF1 within the physiological range, which is not in the pharmacological range, improved fatty liver; however, elevated serum IGF1 levels were involved with an increased risk of breast, colon, and prostate cancer even in the physiological range (10). Regarding the development of hepatocellular carcinoma, it has been reported that the IGF1 signaling pathway plays an essential role in cellular and animal models (46). It is necessary to observe carefully for the development of hepatocellular carcinoma, especially when IGF1 is used for fatty liver disease, although the serum IGF1 level is under the physiological range in patients with AGHD.

In summary, our results show that IGF1 prevents cholesterol accumulation in the liver by increasing the expression of ABCA1 via the PI3K/Akt/FoxO1 signaling pathway. From the above, there is a possibility that the treatment using IGF1 may be effective against diseases such as fatty liver associated with AGHD.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.F., H.I., J.L., T.D., S.S., T.I., T.K., T.Y., K.Y., T.M., and K.M. conceived and designed research; K.F., J.L., T.D., and T.M. performed experiments; K.F., H.I., J.L., T.D., S.S., T.I., T.K., T.Y., K.Y., T.M., and K.M. analyzed data; K.F. interpreted results of experiments; K.F. prepared figures; K.F. drafted manuscript; K.F. and K.M. edited and revised manuscript; K.F. and K.M. approved final version of manuscript.

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