

Role of glycoprotein 78 and cidec in hepatic steatosis

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Abstract. Hepatic glycoprotein (gp78), a membrane-anchored E3 ubiquitin ligase, has been reported to be involved in regulating lipid and energy metabolism in animals, and cell death-inducing DFFA-like effector c (cidec) has emerged as an important regulator of metabolism, which has been implicated in the process of fat differentiation. Nonalcoholic fatty liver disease is a metabolic disorder associated with hepatic steatosis. In the present study, to investigate the role of gp78 and cidec in hepatic steatosis, an *in vitro* cell culture model of hepatic steatosis was established, using the AML12 mouse hepatocyte cell line to assess the protein expression of gp78. The results of Oil Red O staining, phase contrast microscopy and triglyceride content detection experiments indicated that the overexpression of gp78 induced lipid accumulation, whereas gp78-knockdown led to a reduction in lipid accumulation in the AML12 cells. The increased expression of gp78 was associated with steatosis. The expression of cidec was consistent with gp78, and the colocalization of gp78 and cidec was observed on the surface of lipid droplets using immunofluorescence analysis. Furthermore, an interaction between gp78 and cidec was detected using coimmunoprecipitation analysis, and this interaction promoted lipid accumulation. Based on these data, it was hypothesized that gp78 is a regulator of hepatic steatosis, and that it may be a putative molecular mediator in metabolic diseases.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common global cause of chronic liver disease and is caused by

fat deposition (steatosis) in hepatocytes, which includes simple steatosis and nonalcoholic steatohepatitis (NASH). Steatosis is defined as the presence of hepatic triglyceride (TG) droplets in >5% of hepatocytes (1). The accumulation of free cholesterol in NAFLD and NASH has also been reported (2). The incidence of NAFLD is rapidly increasing. It has a complex pathophysiology, is closely associated with metabolic syndrome, and is associated with metabolic risk factors, including obesity, type 2 diabetes mellitus and dyslipidemia (3,4). NASH is considered to be a hepatic manifestation of metabolic syndrome (5), which can lead to hepatic injury, fibrosis, cirrhosis and hepatocellular carcinoma (1). It is also associated with metabolic impairment and the dysregulation of endoplasmic reticulum (ER) homeostasis.

Glycoprotein 78 (gp78), also identified as autocrine motility factor receptor (AMFR), is an ER membrane-anchored E3 ligase (6), which may be critical in protecting cultured cells against the disruption of ER homeostasis (7). Several studies have shown that genetic disruption of gp78 in aged mice induces hepatic steatosis fatty liver, inflammation and spontaneous hepatocellular cancer (8-10). However, conflicting results have been reported following the observation that liver-specific gp78-knockout mice were lean, and had lower blood and tissue lipid levels, with evidence to suggest that gp78 ubiquitinates HMG-CoA reductase, an enzyme involved in regulating the rate of cholesterol production, and is a metabolic regulator of genes involved in lipid metabolism (11). Further investigations involving different mouse strains and *in vitro* cell cultures may assist in improving current understanding of the role of gp78 in hepatic steatosis.

Cell death-inducing DFFA-like effector c (cidec), a human homologue of the murine fat-specific protein 27 (FSP27) is an adipocyte lipid droplet protein, which is important in lipid droplet formation (12,13). It is only expressed in mature adipocytes and is associated with adipocyte differentiation (14,15), and loss of cidec can impede adipocyte maturation (16). High expression levels have been identified in fatty liver syndrome, but not in the normal healthy liver (17,18). In addition, obesity caused by a high fat diet can be prevented in FSP27-knockout mice, with these animals exhibiting a lean phenotype (19,20). However, the exact mechanism underlying the effect of cidec in the regulation of adipocyte differentiation remains to be fully elucidated.

In the present study, the role of gp78 and cidec in hepatic steatosis were examined by application of an *in vitro* hepatocyte cell culture model.

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Materials and methods

Plasmid construction. Total RNA was extracted from the AML12 cells using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) and cDNA was synthesized using an RT reagent kit (RR047Q; Takara Biotechnology Co., Ltd.). Using cDNA as the template, the gp78 length of the target fragment was amplified by reverse transcription-quantitative polymerase chain reaction (RT-PCR). The eukaryotic expression plasmid, pCMV5-HA (Biovector, Beijing, China), was digested with *NdeI* and *XbaI*, and the gp78 fragment was inserted into the pCMV5-HA plasmid by overnight incubation with T4 DNA ligase at 16°C. The pCMV5-HA-gp78 construct was extracted using 10 mg/ml agarose gel electrophoresis and transformed into the host bacteria DH5 α (18265017; Invitrogen, Beijing, China). The plasmid constructs were extracted using a Plasmid Midi kit according to the manufacturer's protocol (12843; Qiagen, Inc., Valencia, CA, USA) and enzyme digestion, and sent to Beijing Aoke Biological Technology Co. Ltd. (Beijing, China), where the plasmid construct was sequenced.

Design and verification of the small interfering (si) RNA gp78 interference sequence and control sequence. The following sequences were used for verification of the gp78 interference sequence: siRNA gp78, sense 5'-GCAUGCACACCUUGG CUUUTT-3' and antisense 5'-AAAGCCAAGGUGUGC AUGCTT-3'; scramble RNA of gp78 (negative control), sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'. The interference RNA and negative control RNA were transfected into the AML12 mouse hepatocyte cells, and the cells were collected following incubation at 37°C for 48 h. The total RNA was extracted, and the mRNA expression of gp78 was assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The effect of siRNA on gp78 was also assessed.

Cell culture and transfection. The AML12 cells (CRL-2254; American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM:F-12 medium in 1:1 ratio (DF12) and supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 0.005 mg/ml insulin (Kehao Biological Engineering Co. Ltd., Xi'an, China), 0.005 mg/ml transferrin (Kehao Biological Engineering Co. Ltd.), 0.04 mg/ml hexadecadrol (Kehao Biological Engineering Co. Ltd.), and incubated at 37°C in an atmosphere of 5% CO₂. The cells were transfected with plasmids using Lipofectamine[®] 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

Oil Red O staining. The AML12 cells were stimulated with 400 μ M oleic acid, as previously described (21). Lipid accumulation in the AML12 cells was assessed using Oil Red O staining. In brief, the cells were fixed onto slides at a concentration of 2 \times 10⁶ cells/ml with 4% polyformaldehyde for at least 30 min, washed with phosphate-buffered saline (PBS) and immersed in freshly prepared 2% Oil Red O (Sigma-Aldrich; Merck Millipore) dye at room temperature for 25 min. The slides were then washed in 60% isopropanol (Invitrogen;

Thermo Fisher Scientific, Inc.) followed by distilled water. A phase contrast microscope was used to visualize staining.

Western blot analysis. The AML12 cells were seeded at a concentration of 3 \times 10⁶ cells/ml into 60 mm dishes and, once confluent, were lysed in ice-cold lysis buffer containing 1% NP-40, 50 mM Tris-HCl, 0.1% SDS, 1% sodium deoxycholate and 150 mM NaCl (pH 7.4; Tianjin Chemical Reagent Factory, Tianjin, China) and centrifuged at 12,000 \times g at 4°C for 3 min. The protein content was determined using Quick Start[™] Bradford kit (5000202EDU; Bio-Rad Laboratories, Inc., Hercules, CA, USA), using bovine serum albumin (BSA) as the standard. Subsequently, 15 μ l of the proteins were separated by SDS-PAGE on 12% acrylamide gels, following which proteins were transferred onto a PVDF membrane (EMD Millipore, Bethesda, MA, USA). The membrane was then incubated overnight in a blocking buffer containing appropriate diluent (ab64211; Abcam, Cambridge, UK) of primary antibodies against gp78 (ab54787; 1:1,000, Abcam), cidec (ab77115; 1:1,000, Abcam), PPAR- γ (ab41928; 1:1,000, Abcam) and β -actin (ab8226; 1:1,000, Abcam) at 4°C. The proteins were detected by incubation with horseradish peroxidase-conjugated secondary antibody (ab6789; 1:2,000, Abcam) in diluent (ab64211; Abcam, Cambridge, UK) at room temperature for 1 h. Following extensive washing with Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, the bands were visualized using enhanced chemiluminescence and autoradiography.

Lipid extraction and triglyceride (TG) content determination. To measure the total TG levels, lipids were extracted from the cells using the Folch method, as previously described (22). Briefly, the cells at a concentration of 3 \times 10⁶ cells/ml were washed with PBS, scraped in 1 ml PBS and transferred into a 15-ml tube. Intermixture was added (8 ml of n-hexane: dimethyl carbinol in a 3:2 ratio) and centrifuged (4°C, 12,000 \times g, 5 min). The supernatant was removed into a glass tube, and 0.1 ml 2% Tritonx-100 was added using a dry nitrogen-blowing instrument, followed by shock mixing storage at -20°C. TG content was quantified using Bio-Rad QuantityOne software version 4.62 (Bio-Rad Laboratories, Inc.).

RNA isolation and RT-qPCR analysis. Total RNA was extracted from the AML12 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) Total RNA was converted into complementary DNA using avian myeloblastosis virus reverse transcriptase (Takara Bio, Inc., Otsu, Japan). The primers (forward, 5'-CCTGGCTAGAACAAGACACC-3' and reverse, 5'-ATCCGAGACCCATCGAAA. T-3') were synthesized by Aoke Company (Beijing, China). RT-qPCR was used to quantify the complementary DNA template. Quantitative gene expression analysis was performed using the SYBR[®] Premix Ex Taq[™] GC (RR071Q; Takara Bio, Inc.) and normalized relative to the β -actin mRNA control band. The reaction system included 12.5 μ l of SYBR Premix Ex Taq GC, 0.5 μ l of forward primer, 0.5 μ l of reverse primer, 2 μ l of template and 9.5 μ l of H₂O. The reactions were incubated in 96-well optical plates for initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec then followed by a final extension at 72°C for 10 min.

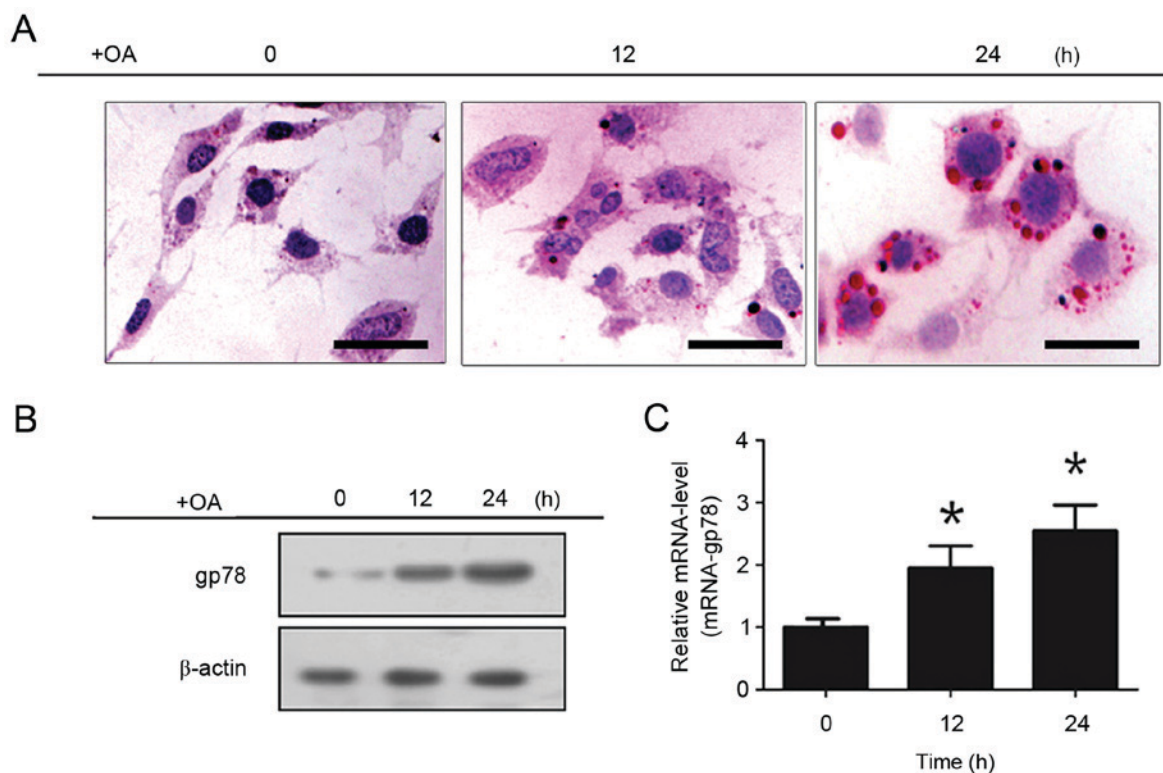


Figure 1. Elevated expression levels of gp78 in the process of hepatic steatosis. (A) Stimulating cells with OA at 0, 12 and 24 h increased lipid accumulation in AML12 cells (scale bar=20 μ m), as observed by Oil Red O staining. (B) Increased protein expression of gp78 above basal levels was observed in hepatic steatosis, as determined using western blot analysis. (C) Reverse transcription-quantitative polymerase chain reaction assessment of mRNA expression levels of gp78, which were upregulated in hepatic steatosis. The relative mRNA level in the control group was designated as 1.0. (* P <0.05, vs. control; n =3). OA, oleic acid; gp78, glycoprotein 78.

Immunofluorescence assay. The AML12 cells were seeded onto sterile coverslips and, following incubation at 37°C for 24 h, the cells were transfected with pCMV-Myc-CIDE-3 and pCMV5-HA-gp78 for 48 h, and in 5% BSA for 30 min. The cells were then incubated with primary antibodies specific for gp78 (ab54787; dilutions 1:1,000, Abcam) or cidec (ab213693; dilutions 1:1,000, Abcam) overnight at 4°C, followed by incubation with secondary antibodies conjugated with fluorescein isothiocyanate for gp78 (ab6785; dilutions 1:3,000, Abcam) and with tetraethyl rhodamine isothiocyanate for cidec (ab6718; 1:3,000, Abcam) for 1 h at 37°C. Prior to imaging, the cells were counterstained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at 37°C to stain the nuclei, and were visualized with an E1000 digital camera (Nikon Corporation, Tokyo, Japan) with SimplePCI software version 65 (Meyer Instruments, Houston, TX, USA).

Coimmunoprecipitation assay. The cells were cultured in 60-mm dishes at a concentration of 3×10^6 cells/ml and were cotransfected with 5 μ g of pCMV-Myc-CIDE-3 and pCMV5-HA-gp78. The cells were lysed with RIPA buffer containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl (pH 7.5; Tianjin Chemical Reagent Factory). The cells were incubated for 10 min on ice and, following brief sonication, the lysate was centrifuged at 12,000 \times g for 10 min at 4°C. An aliquot of the lysates was removed for western blot analysis. Antibody was coupled to Dynabeads® Protein G (Dyna; thermo Fisher Scientific, Inc.) using dimethylpimelimidate coupling according to the

manufacturer's protocol. Equal quantities of cellular protein were incubated for 2 h with the antibody-linked beads at 4°C with continuous agitation. The lysates and coimmunoprecipitates were then separated by SDS-PAGE using 12% gels and transferred onto PVDF membranes for western blot analysis.

Statistical analysis. All data are analyzed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the means \pm standard error of the mean. Two-tailed Student's t test was used to compare the values between two groups. One-way analysis of variance was used to compare values between multiple groups. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Elevated expression levels of gp78 in the process of hepatic steatosis. In the present study, steatosis was induced by oleic acid in AML12, and the cells were collected at 0, 12 and 24 h for Oil Red O staining (Fig. 1A) and phase contrast microscopy (Fig. 1B). Increased lipid accumulation in liver cells was observed with time. The protein and mRNA expression levels of gp78 were confirmed using western blot and RT-qPCR analyses, respectively (Fig. 1C and D). Compared with the control group, the hepatocytes in the steatosis group showed increased expression of gp78 with time.

Effects of the overexpression and knockdown of gp78 in hepatic steatosis. At 48 h post-transfection, the following three

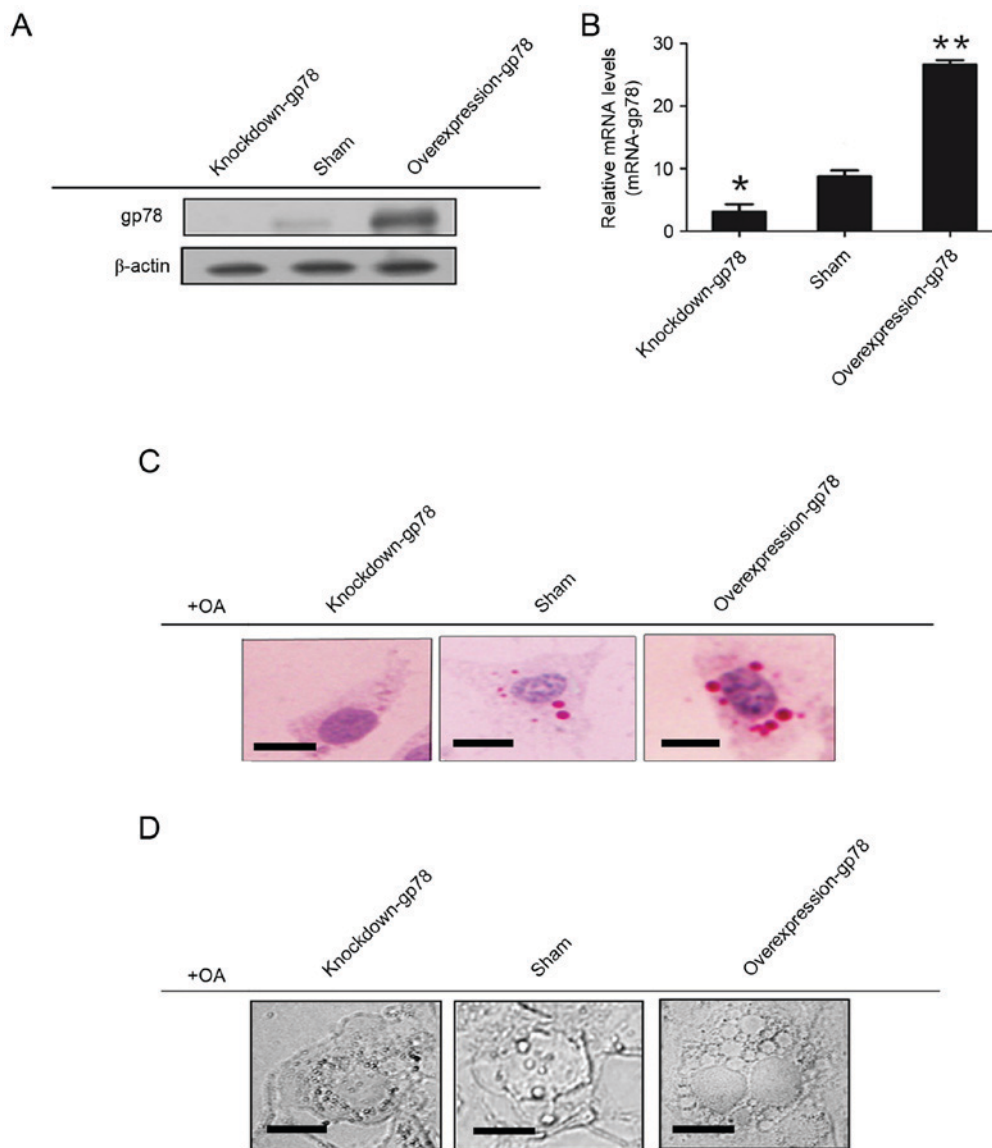


Figure 2. Role of gp78 in hepatic steatosis. (A) Western blot analysis of AML12 cells transfected with the gp78 plasmid (overexpression of gp78) or gp78-siRNA (gp78-knockdown). (B) Reverse transcription-quantitative polymerase chain reaction analysis of AML12 cells transfected with gp78 plasmid or gp78-siRNA. The relative mRNA level in the control group was designated as 1.0. (* $P < 0.05$; ** $P < 0.01$, vs. control; $n = 3$). (C) Oil Red O staining analysis of AML12 cells transfected with the gp78 plasmid or gp78-siRNA to assess lipid accumulation (scale bar = 20 μm). (D) Phase contrast microscopic analysis of AML12 cells transfected with the gp78 plasmid or gp78-siRNA (scale bar = 20 μm). Compared with the control group, an increased number and volume of lipid droplets were observed in AML12 cells overexpressing gp78, whereas a decreased number and volume of lipid droplets were observed in the gp78-knockdown group. OA, oleic acid; gp78, glycoprotein 78.

groups of cells were collected: Control group; gp78-overexpression group; and gp78-knockdown group. Western blot and RT-qPCR analyses were used to confirm the protein and mRNA expression of gp78, respectively (Fig. 2A and B). The lipid accumulation in hepatocytes was observed using Oil Red O staining (Fig. 2C) and phase contrast microscopy (Fig. 2D). Compared with the control group, an increased number and volume of lipid droplets were observed in the gp78-overexpression group, whereas a decreased number and volume of lipid droplets were observed in the gp78-knockdown group.

Roles of gp78 and cidec in hepatic steatosis. The expression levels of cidec and peroxisome proliferator-activated receptor (PPAR)- γ were upregulated, which was observed consistently with the overexpression of gp78 (Fig. 3A). The results suggested

that the interaction between gp78 and cidec promoted lipid accumulation (Fig. 3B and C).

Association between gp78 and cidec in hepatic steatosis. The present study found that the interaction between gp78 and cidec promoted lipid accumulation using coimmunoprecipitation and immunofluorescence confocal microscopy analyses (Fig. 4A and B), which indicated that gp78 and cidec had the same localization in the AML12 cells.

Discussion

Although NAFLD is a commonly occurring liver disorder in industrialized countries (23), the majority of patients present with few or no symptoms. NAFLD is considered to be the

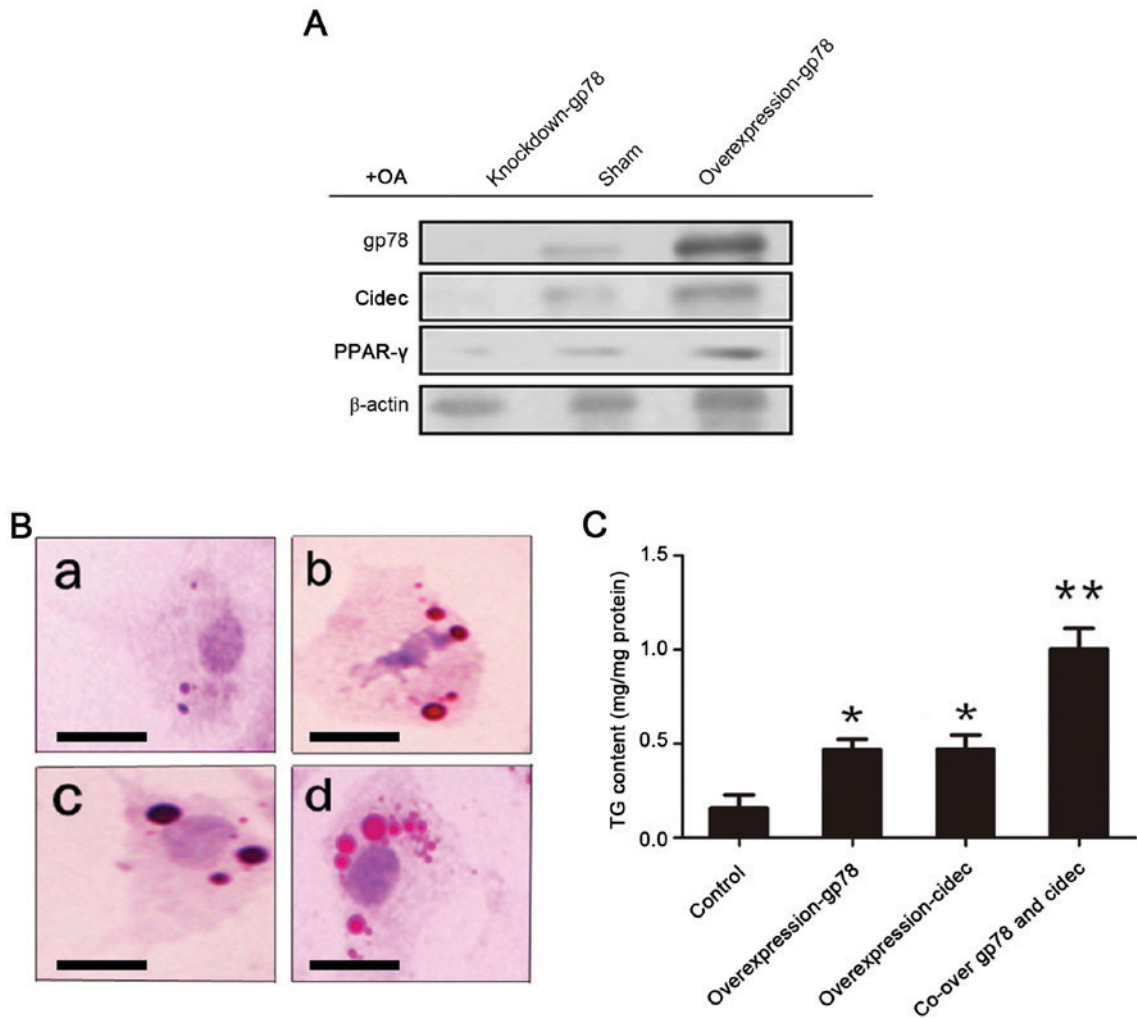


Figure 3. Role of gp78 and cidec in hepatic steatosis. (A) Western blot comparison of alterations in the expression of gp78, cidec and PPAR- γ . (B) Oil Red O analysis of the effect of gp78 and cidec on lipid accumulation (scale bar=20 μ m). (a) Control, (b) overexpression gp78, (c) overexpression cidec and (d) co-overexpression of gp78 and cidec groups. (C) Quantification of TG concentrations to assess the combined effect of gp78 and cidec on lipid accumulation. All data are expressed as the mean \pm standard error of the mean for each group. (*P<0.05 and **P<0.01, vs. control; n=4). gp78, glycoprotein; cidec, cell death-inducing DFFA-like effector c; TG, hepatic triglyceride; PPAR- γ , peroxisome proliferator-activated receptor- γ .

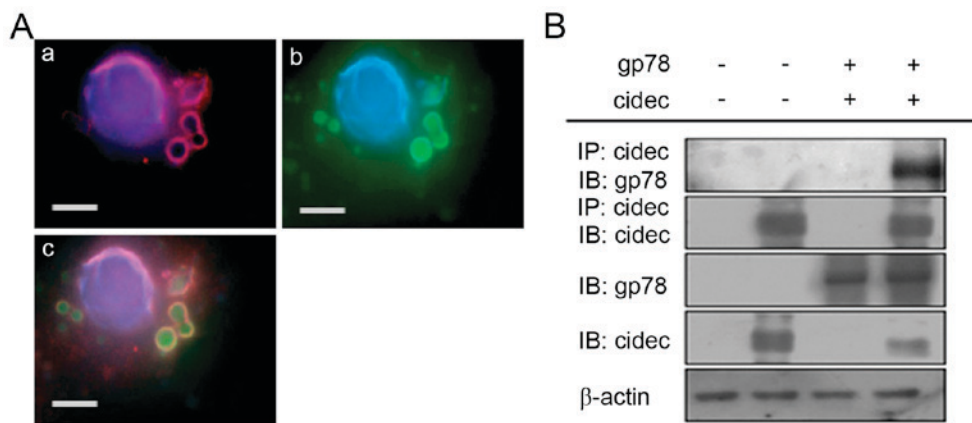


Figure 4. Association between gp78 and cidec in hepatic steatosis. (A) Nuclei were stained with DAPI, (a) gp78 was visualized (fluorescein isothiocyanate conjugated secondary antibody; red staining). (b) Cidec was visualized (tetraethyl rhodamine isothiocyanate conjugated secondary antibody; green staining); (c) Colocalization of gp78 and cidec (yellow) visualized on the surface of lipid droplets using immunofluorescent microscopy and identified by coimmunoprecipitation (scale bar=15 μ m). (B) Gp78 and Cidec direct interaction detected by coimmunoprecipitation assay. gp78, glycoprotein; cidec, cell death-inducing DFFA-like effector c.

most common cause of chronic liver disease, cirrhosis and liver failure (24,25). Furthermore, dysregulated cholesterol

metabolism may contribute to disease severity in NAFLD and NASH (2,26). Cholesterol is synthesized from acetyl-CoA

through a cascade of enzymatic reactions (27), and hepatic gp78 has been reported to be essential in regulating lipid and energy metabolism in animals. However, the exact mechanism by which this regulation takes place remains controversial (10,11,28,29).

In the present study, the expression of gp78 was examined in hepatic steatosis and it was observed that the hepatocytes in the steatosis group showed increased expression of gp78 expression over time. Furthermore, the overexpression of gp78 induced lipid accumulation in hepatocytes, whereas the knockdown of gp78 led to a reduction in lipid accumulation, which indicates its potential role in the biosynthesis of cholesterol and fatty acids in the liver (11). However, further investigations are required to determine the exact mechanism underlying the role of gp78 in hepatic steatosis.

The cell death-inducing DNA fragmentation factor 45-like effector proteins are important in lipid metabolism (30). Cidec is expressed at high levels in white adipose tissue and increases during adipogenesis in mice (12,15). In addition, it has been demonstrated that cidec induces apoptosis in hepatocellular carcinoma (31). PPAR- γ is primarily present in adipose tissue, and it regulates fatty acid storage and glucose metabolism (32). The genes activated by PPAR- γ stimulate lipid uptake and adipogenesis by fat cells, and PPAR- γ -knockout mice fail to generate adipose tissue when fed a high fat diet (32). In the present study, it was found that the overexpression of gp78 upregulates the expression of cidec and PPAR- γ , whereas knocking down gp78 had a suppressive effect. The interaction between gp78 and cidec induced lipid accumulation in hepatocytes.

In conclusion, the present study is the first, to the best of our knowledge, to demonstrate an association between gp78 and cidec, and show their combined effect on hepatic steatosis. However, the involvement of gp78 and cidec in NAFLD requires further experimental investigation.

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