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Pcsk9 knockout exacerbates diet-induced non-alcoholic steatohepatitis, fibrosis and liver injury in mice

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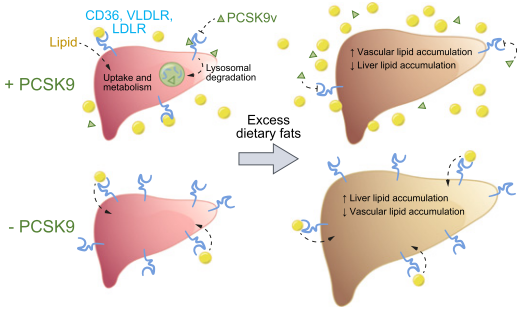
Graphical abstract



Highlights
- PCSK9 reduces the expression of hepatic CD36 in mice.
- CD36 is a known driver of liver steatosis and injury.
- PCSK9 protects from palmitate-induced ER stress and ROS in cultured hepatocytes.
- High-fat diet causes severe hepatic steatosis, ER stress, inflammation and insulin resistance in *Pcsk9*$^{-/-}$ mice.
- PCSK9 expression protects mice from diet-induced liver injury.

https://doi.org/10.1016/j.jhepr.2019.10.009

Lay summary
The proprotein convertase subtilisin/kexin type 9 (PCSK9) is a circulating protein known to reduce the abundance of receptors on the surface of liver cells charged with the task of lipid uptake from the circulation. Although PCSK9 deficiency is known to cause lipid accumulation in mice and in cultured cells, the toxicological implications of this observation have not yet been reported. In this study, we demonstrate that PCSK9 can protect against cytotoxicity in cultured liver cells treated with a saturated fatty acid and we also show that Pcsk9 knockout mice develop increased liver injury in response to a high-fat diet.

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Pcsk9 knockout exacerbates diet-induced non-alcoholic steatohepatitis, fibrosis and liver injury in mice

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JHEP Reports **2019.** https://doi.org/10.1016/j.jhepr.2019.10.009

Background & Aims: The fatty acid translocase, also known as CD36, is a well-established scavenger receptor for fatty acid (FA) uptake and is abundantly expressed in many metabolically active tissues. In the liver, CD36 is known to contribute to the progression of non-alcoholic fatty liver disease and to the more severe non-alcoholic steatohepatitis, by promoting triglyceride accumulation and subsequent lipid-induced endoplasmic reticulum (ER) stress. Given the recent discovery that the hepatocyte-secreted proprotein convertase subtilisin/kexin type 9 (PCSK9) blocks CD36 expression, we sought to investigate the role of PCSK9 in liver fat accumulation and injury in response to saturated FAs and in a mouse model of diet-induced hepatic steatosis.

Methods: In this study, we investigated the role of PCSK9 on the uptake and accumulation of FAs, as well as FA-induced toxicity, in a variety of cultured hepatocytes. Diet-induced hepatic steatosis and liver injury were also assessed in *Pcsk9*$^{-/-}$ mice.

Results: Our results indicate that PCSK9 deficiency in cultured hepatocytes increased the uptake and accumulation of saturated and unsaturated FAs. In the presence of saturated FAs, PCSK9 also protected cultured hepatocytes from ER stress and cytotoxicity. In line with these findings, a metabolic challenge using a high-fat diet caused severe hepatic steatosis, ER stress inflammation and fibrosis in the livers of *Pcsk9*$^{-/-}$ mice compared to controls. Given that inhibition of CD36 ablated the observed accumulation of lipid *in vitro* and *in vivo*, our findings also highlight CD36 as a strong contributor to steatosis and liver injury in the context of PCSK9 deficiency.

Conclusions: Collectively, our findings demonstrate that PCSK9 regulates hepatic triglyceride content in a manner dependent on CD36. In the presence of excess dietary fats, PCSK9 can also protect against hepatic steatosis and liver injury.

Introduction

Given that the liver plays a central role in glucose and lipid metabolism, it is no surprise that non-alcoholic fatty liver disease (NAFLD) has emerged as a contributor to other chronic diseases, such as type 2 diabetes and cardiovascular disease (CVD).[1,2] Severity ranges from simple steatosis to progressive stages of non-alcoholic steatohepatitis (NASH), depending on the presence or absence of inflammation, fibrosis and hepatocyte injury. Excessive consumption of dietary saturated fats and sugars is widely accepted as the primary driver of NAFLD and its prevalence is currently on the rise; affecting 30 to 46% of individuals in Western societies.[3]

Although the exact mechanisms by which NAFLD progresses to NASH are not well understood, an accumulation of events including oxidative stress, mitochondrial dysfunction, adipokine alteration, lipid peroxidation and Kupffer cell activation are all believed to contribute to the currently accepted *multiple hit* hypothesis.[4] Chief among these *hits* is the process by which misfolded polypeptides accumulate in the ER and cause ER stress. Paradoxically, this cellular stress pathway is also known to promote oxidative stress, mitochondrial dysfunction and Kupffer cell-mediated inflammation.[5,6] In a manner similar to other secretory cells, hepatocytes are rich in ER and the signaling cascades associated with its state of stress have been shown to promote apoptotic cell death, lipotoxicity, inflammation and insulin resistance; all of which are commonly observed in patients with obesity, NAFLD and NASH.[4]

In response to ER stress, the unfolded protein response (UPR) is activated via 3 signaling cascades that include (a) the highly conserved inositol-requiring 1α (IRE1α)-X-box-binding protein 1 (XBP1) pathway required for hepatic lipid regulation during conditions of ER stress,[7] (b) the PKR-like ER kinase (PERK)-activating transcription factor (ATF)4 pathway known to modulate *de novo* lipogenesis through fatty acid synthase (FAS) and the sterol regulatory element-binding protein-1C (SREBP1-C),[8] and (c) ATF6, which in its nuclear active form interacts directly with nuclear SREBP-2,

Key words: ER stress; CD36; HFD; NAFLD; Liver disease; Cardiometabolic disease; CVD.
Received 13 April 2019; received in revised form 16 October 2019; accepted 24 October 2019; available online 5 November 2019

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thereby attenuating the expression of lipid regulatory genes.[9] Overall, canonical UPR activation increases the folding capacity of the ER and blocks global protein synthesis in order to reduce ER burden. In a manner similar to NAFLD, ER stress and ER stress-induced apoptosis are also well-established contributors to CVD.[10]

In recent years, CVD has been considered the leading cause of mortality in the USA, accounting for 34% of total deaths in individuals <75 years of age.[11] The discovery of PCSK9[12] and its ability to induce the degradation of the low-density lipoprotein (LDL) receptor (LDLR) once secreted from the liver, firmly positioned PCSK9 as a target for the management of CVD.[13] These seminal discoveries have since led to the development of human anti-PCSK9 monoclonal antibodies capable of reducing circulating LDL levels by 60% in patients at high risk of CVD.[14] In addition to its ability to induce the degradation of cell-surface LDLR, secreted PCSK9 was recently shown to promote the degradation of several other receptors known to be involved in the uptake of lipid from the circulation into the liver, such as the very low-density lipoprotein receptor (VLDLR),[15,16] LDLR-related protein-1,[17] the apolipoprotein E (ApoE) receptor-2[15] and CD36.[18]

Based on these studies, circulating PCSK9 may impact the levels of these receptors on the cell surface of hepatocytes, thereby increasing liver burden via enhanced hepatic lipid uptake and accumulation. The purpose of this study was to determine whether the previously reported increase in hepatic lipid content observed in *Pcsk9*$^{-/-}$ mice[18] correlated with the hallmark features of NASH, such as ER stress, apoptosis, inflammation and fibrosis.

Materials and methods

Cell culture and free FA treatments

HuH7 and HepG2 immortalized human hepatocytes were cultured at 37°C with 5% CO_2 in complete Dulbecco's Modified Eagles Medium (Gibco, Thermorfisher Scientific) containing 10% fetal bovine serum (Sigma-Aldrich) and 50 U/ml of penicillin and streptomycin (Sigma-Aldrich). HepatoSure 100-donor primary human hepatocytes were purchased from Xenotech and cultured in complete William's E medium as per manufacturer's instructions. FAs used for cell treatment such as oleate (OA; Sigma-Aldrich), linoleate (LA; Sigma-Aldrich), stearate (SA; Sigma-Aldrich) and palmitate (PA; Sigma-Aldrich) were conjugated to FA-free bovine serum albumin (BSA; Sigma-Aldrich), as previously described,[19] to a stock concentration of 1 mM. Cells were also treated with LDL (Lee Biosolutions), high-density lipoprotein (HDL; Alpha Aesar) and oxidized (ox)LDL (Alpha Aesar). Additional treatments used in cell culture studies included recombinant human PCSK9 (Cayman Chemical), DiI-LDL (Alpha Aesar) and sulfo-N-succinimidyl oleate (SSO; Cayman Chemical). All cell treatments were carried out for 24 h, unless specified otherwise. Small interfering RNA (siRNA) targeted against *PCSK9* (si*PCSK9*) and *CD36* (si*CD36*) were purchased from siGenome, Dharmacon, and transfections were carried out as per manufacturer's instructions using RNAiMAX (ThermoFisher Scientific). The overexpression of human wild-type *PCSK9*^{WT} and the secretion-deficient *PCSK9*^{Q152H}, as well as human *CD36* (Addgene, # 52025) was achieved using X-tremeGENE transfection reagent as per manufacturer's instructions.

Animal studies

Hepatic lipid accumulation was first examined in 12-week-old male *Pcsk9*$^{-/-}$ mice (n = 10) on a C57BL/6J background and age-matched C57BL/6J controls (*Pcsk9*$^{+/+}$; n = 10) fed normal control diet (NCD). Experiments were then repeated in a second cohort of *Pcsk9*$^{-/-}$ mice (n = 5) on a C57BL/6J background and age-matched *Pcsk9*$^{+/+}$ controls (n = 5), which were fed either NCD or a high-fat diet (HFD) (60% fat/kcal, ENVIGO #TD.06414), starting at 6 weeks of age for an additional 12 weeks prior to sacrifice. A final cohort of 12-week-old male *Pcsk9*$^{-/-}$ mice on a C57BL/6J background (n = 6) were treated with SSO (10 mg/kg; intraperitoneal injection) and 1 h later with OA (1 g/kg, intraperitoneal injection) for an additional 2 h prior to study endpoint. All animals were housed in a vented rack system, had access to food and water *ad libitum* and were exposed to 12 h light:dark cycles. Animal experiments were performed in strict accordance with the McMaster University animal care guidelines.

Statistical analysis

All data are presented as the mean and error bars as SD. Statistical differences between 2 groups were determined using the unpaired *t* test. For analysis of experiments involving multiple groups, the one-way ANOVA was performed. All comparisons were considered statistically significant when $p < 0.05$. For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

PCSK9 reduces lipid accumulation in cultured hepatocytes treated with FAs and lipoproteins

The effect of PCSK9 on cellular lipid accumulation was first examined in cultured HepG2 hepatocytes stably transfected with short hairpin RNA (shRNA) targeted against *PCSK9* or control shRNA. Knockdown of PCSK9 in these cells was first confirmed via ELISA for secreted PCSK9 and immunoblotting of PCSK9-regulated receptors, LDLR and CD36 (Fig. 1A and B). Consistent with previous studies, PCSK9 expression was inversely correlated with LDLR and CD36 expression.[13,18] Increased uptake of fluorescently labelled DiI-LDL cholesterol was also observed in *PCSK9* shRNA knockdown cells compared to shRNA controls (Fig. S1A and S1B). Staining with Oil red O (ORO), as well as triglyceride extraction and quantification, revealed a marked increase in triglyceride accumulation in *PCSK9* shRNA cells treated with dietary saturated FAs (PA and SA), unsaturated FAs (OA and LA) and lipoproteins (LDL and HDL) (Fig. 1C and D). The dose-dependent effect of exogenously added extracellular PCSK9 on intracellular lipid accumulation was also examined in OA-treated HepG2 cells using recombinant human PCSK9 (Fig. 1E). Modest but significant reductions in CD36 expression and in OA-induced lipid accumulation were also observed in HepG2 cells and primary mouse hepatocytes transfected with *PCSK9*^{WT} compared to those transfected with a loss-of-function *PCSK9*^{Q152H} variant that fails to be secreted (Fig. S1C to S1F).[20] Transfection efficiency in these cells was confirmed via immunoblotting for PCSK9 and GFP encoded by the bicistronic plasmid utilized in these studies (Fig. S1G). Lipid uptake and accumulation was also assessed in primary human hepatocytes that were exposed to medium harvested from HuH7 cells transfected with either *PCSK9*^{WT} or the secretion-deficient *PCSK9*^{Q152H} variant (Fig. 1F). Consistent with cultured HepG2 cells, secreted *PCSK9*^{WT} significantly reduced OA-induced lipid accumulation in primary human hepatocytes.

The CD36 inhibitor SSO reduces OA-induced lipid droplet accumulation in PCSK9 knockdown hepatocytes

Our next aim was to assess the recently described inhibitory role of PCSK9 on CD36 (using a pharmacologic antagonist of CD36,

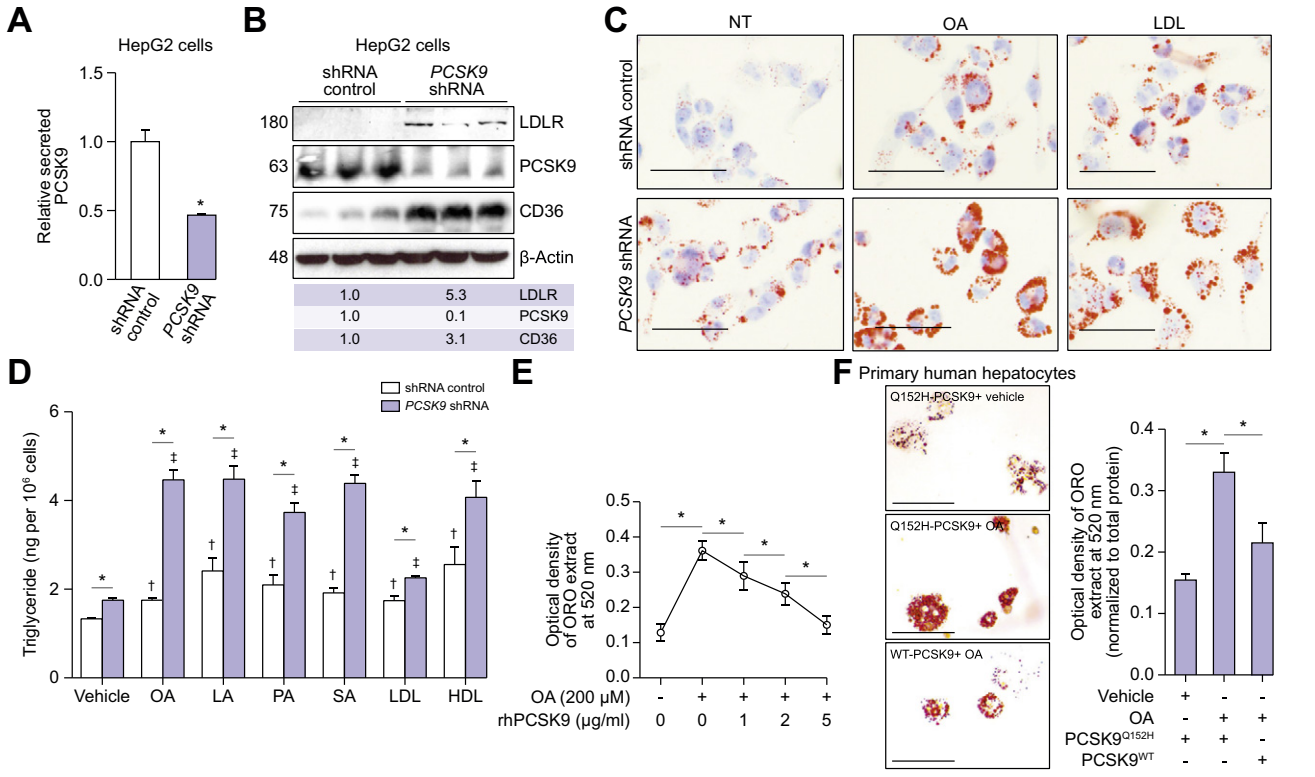

Fig. 1. PCSK9 reduces the triglyceride content of HepG2 cells and primary human hepatocytes. (A,B) PCSK9 knockdown in *PCSK9* shRNA HepG2 cells was confirmed via ELISA for secreted PCSK9 (*p <0.05) and immunoblots for PCSK9-regulated receptors LDLR and CD36. (C) ORO lipid droplet staining of HepG2 control cells (shRNA control) and PCSK9 knockdown cells (*PCSK9* shRNA) in the presence or absence of OA (200 μm) and LDL (50 μg/ml). (D) Intracellular triglyceride content was also quantified in cells treated with either OA (200 μM), LA (200 μM), PA (200 μM), SA (200 μM) and lipoproteins LDL (50 μg/ml) and HDL (50 μg/ml) for 24 h (*p <0.05; †p<0.05 *vs.* vehicle-shRNA control; ‡p<0.05 *vs.* vehicle-*PCSK9* shRNA). (E) The effect of recombinant human PCSK9 on OA uptake was examined in HepG2 cells via quantification of the density of ORO extracts (*p <0.05). (F) ORO staining was carried out in primary human hepatocytes that were exposed to medium harvested from HuH7 cells transfected with either secreted *PCSK9*^{WT} or a *PCSK9* variant that fails to exit the cell,[20] *PCSK9*^{Q152H}, and treated with OA (200 μM) for 24 h. ORO extracts from primary human hepatocytes were also quantified (*p <0.05). Results are shown as the mean and error bars as SD. Differences between groups were determined via Student's *t* test or one-way ANOVA. Scale bars, 50 μm. HDL, high-density lipoprotein; LA, linoleate; LDL, low-density lipoprotein; OA, oleate; ORO, Oil red O; PA, palmitate; SA, stearate; SSO, sulfo-N-succinimidyl oleate.

SSO) as this is the mechanism by which PCSK9 is thought to regulate hepatic lipid levels.[21] Consistent with *PCSK9* shRNA knockdown HepG2 cells, the knockdown of *PCSK9* in HepG2 and HuH7 cells using siRNA increased intracellular lipid content; a result that was further exacerbated by incubating cells in OA (Fig. 2A and B). No such increase in lipid accumulation, however, was observed in cells pre-treated with SSO. Consistently, SSO pre-treatment also attenuated hepatic lipid accumulation in *Pcsk9*$^{-/-}$ mice exposed to a bolus injection of OA (Fig. 2C). The knockdown of CD36 using siRNA (si*CD36*) also reduced lipid accumulation in HepG2 cells challenged with OA (Fig. 2D and E). Lastly, to confirm the role of CD36 as a driver of lipid accumulation in the context of PCSK9 deficiency, experiments were repeated in cells co-transfected with si*PCSK9* and si*CD36*. ORO extract and quantification from these cells demonstrates that ablation of CD36 expression markedly reduced lipid accumulation occurring as a result of PCSK9 knockdown in the presence or absence of OA (Fig. 2F). Similar findings were observed in HuH7 cells (Fig. S2A). The difference in lipid accumulation between control and *PCSK9* shRNA cells was also lost in cells overexpressing CD36 (Fig. 2G). Effective knockdown of PCSK9 and CD36, as well as overexpression of CD36 in HepG2 cells, was confirmed via immunoblotting (Fig. S2B to S2D). Collectively, these data suggest that increased expression of CD36 strongly contributes to the heightened levels of intracellular lipids observed in hepatocyte cell lines with reduced PCSK9 expression.

PCSK9 protects cultured hepatocytes from palmitate-induced ER stress and cytotoxicity

To substantiate our findings, we next investigated whether PCSK9 would protect from the well-established ER stress-associated effects of increased CD36 expression, such as ER Ca^{2+} depletion, reactive oxygen species (ROS) production and cytotoxicity.[22] First, increased expression levels of the ER stress markers IRE1α and glucose-regulated protein of 78-kDa (*GRP78*), and the pro-apoptotic markers CCAAT/enhancer-binding protein homologous protein (*CHOP*), *ATF6* and s*XBP1* were observed in *PCSK9* shRNA compared to control shRNA HepG2 cells treated with PA, via immunoblotting and quantitative real-time PCR (Fig. 3A and B). Similarly, primary human hepatocytes exposed to medium harvested from HuH7 cells transfected with *PCSK9*^{Q152H} also yielded increased PA-induced expression of ER stress markers compared to those exposed to medium containing *PCSK9*^{WT} (Fig. 3C). Consistent with previous studies,[19] treatment with OA and lipoproteins did not cause ER stress in HepG2 cells (Fig. S3A, S3B and S3C).

PCSK9 shRNA HepG2 cells also exhibited increased cytotoxicity in response to PA relative to controls (Fig. 3D and S3D). Interestingly, although SSO-mediated CD36 inhibition failed to reduce

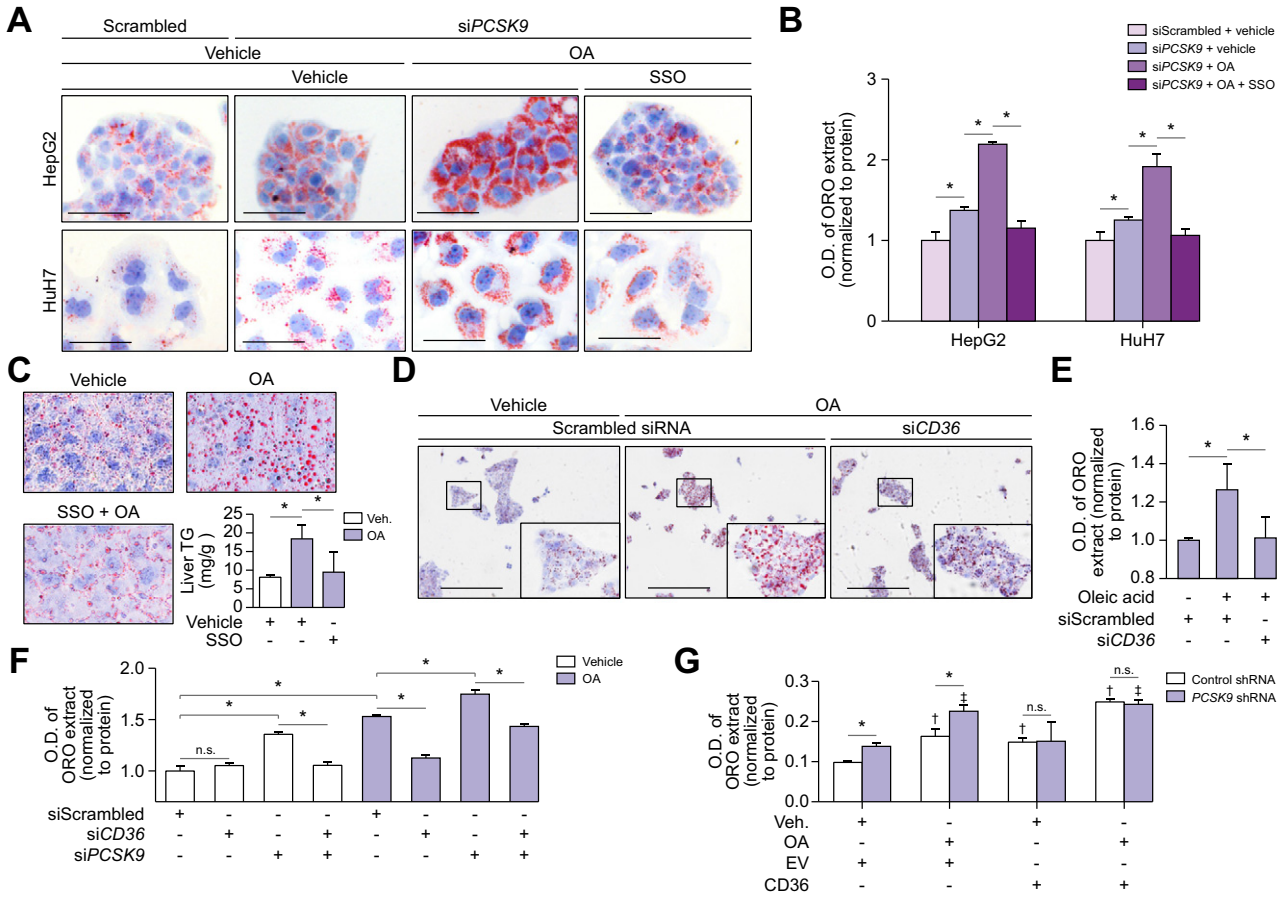

Fig. 2. PCSK9 regulates FA uptake in immortalized hepatocytes through CD36. (A,B) HepG2 and HuH7 cells were transfected with si*PCSK9* or scrambled control siRNA. Cells were subsequently treated with OA and or SSO (10 μM), for an additional 24 h (**p* <0.05). (C) 12-week-old male C57BL/6J mice were pre-treated with SSO (10 mg/kg) for 1 h and subsequently administered with OA for an additional 2 h. Hepatic lipid accumulation was visually examined via ORO staining and quantified using a triglyceride assay. (D,E) The effect of CD36 inhibition on OA uptake was also assessed using si*CD36* (**p* <0.05). Finally, intracellular lipid accumulation was examined in HepG2 cells co-transfected with si*PCSK9* and si*CD36*, via ORO extract and quantification using a spectrophotometer (**p* <0.05). (G) The effect of CD36 overexpression on lipid accumulation was examined in HepG2 cells via quantification of ORO extract (**p* <0.05; †*p*<0.05 *vs.* control shRNA; ‡*p*<0.05 *vs. PCSK9* shRNA). Results are shown as the mean and error bars as SD. Differences between groups were determined via Student's *t* test or one-way ANOVA. Scale bars, (A) 50 μm, (D) 200 μm. FA, fatty acid; OA, oleate; ORO, Oil red O; SSO, sulfo-N-succinimidyl oleate.

PA-induced cytotoxicity in shRNA control cells, the CD36 inhibitor attenuated cytotoxicity in *PCSK9* shRNA knockdown cells (Fig. 3D). Given the role of ER stress in the production of ROS, the ability of PCSK9 to protect from PA-induced ROS was also examined in HepG2 cells (Fig. 3E). We observed that PA treatment led to a significant increase in ROS production in *PCSK9* shRNA HepG2 cells compared to shRNA controls during the course of the experiment.

Next, we assessed ER Ca^{2+} depletion, which represents another established outcome of ER stress in cells treated with PA.[23] The fluorometric cytosolic Ca^{2+} indicator utilized in these studies, Fura-2-AM, increases in fluorescence intensity as Ca^{2+} exits the ER into the cytosol.[24] To measure relative ER Ca^{2+} content, HepG2 cells pre-treated with PA (5 h; 500 μM) were exposed to a potent sarco/endoplasmic reticulum ATPase (SERCA) pump inhibitor, cyclopiazonic acid (CPA; 50 μM; Fig. 3F). In response to CPA, we observed that PA-treated shRNA control cells exhibited ER Ca^{2+} loss to a similar extent as untreated controls. In contrast to this result, *PCSK9* shRNA knockdown cells pre-treated with PA failed to exhibit loss of ER Ca^{2+} in response to CPA, suggesting that PA induced ER Ca^{2+} depletion during pre-treatment in these cells. In support of these results, a time-course study in which ER Ca^{2+} depletion was monitored over a 210 min period yielded similar findings (Fig. S3E). Increased ER expansion, another hallmark feature of ER stress, was also observed in HepG2 cells transfected with a splice-switching oligomer that causes ER retention of PCSK9 in response to PA[25] (Fig. S4A and S4B). Appropriate splice-switching and ER retention of PCSK9 in these cells was confirmed by immunoblotting (Fig. S4C).

Pcsk9$^{-/-}$ mice exhibit compensatory changes in biliary cholesterol excretion and in the expression of hepatic lipid regulatory proteins

Given the observed increase in lipid droplet content in cultured hepatocytes, PCSK9-dependent changes in hepatic lipid content were also examined *in vivo*. The livers of NCD-fed 12-week-old *Pcsk9*$^{-/-}$ mice exhibited increased ORO staining of lipid droplets and protein levels of the lipid droplet marker perilipin (Fig. 4A) compared to controls. Consistent with previous reports, immunohistochemical staining also demonstrated a marked increase in cell-surface levels of LDLR, VLDLR and CD36 expression in the livers of *Pcsk9*$^{-/-}$ mice compared to controls.[13,16,18] We also report the novel finding that *Pcsk9*$^{-/-}$ mice exhibit increased levels of the cholesterol efflux transporter ABCA1 compared to controls (Fig. 4A and S5A). In contrast to the LDLR, CD36 and the VLDLR, changes in hepatic ABCA1 expression in *Pcsk9*$^{-/-}$ mice likely occurred as a result of

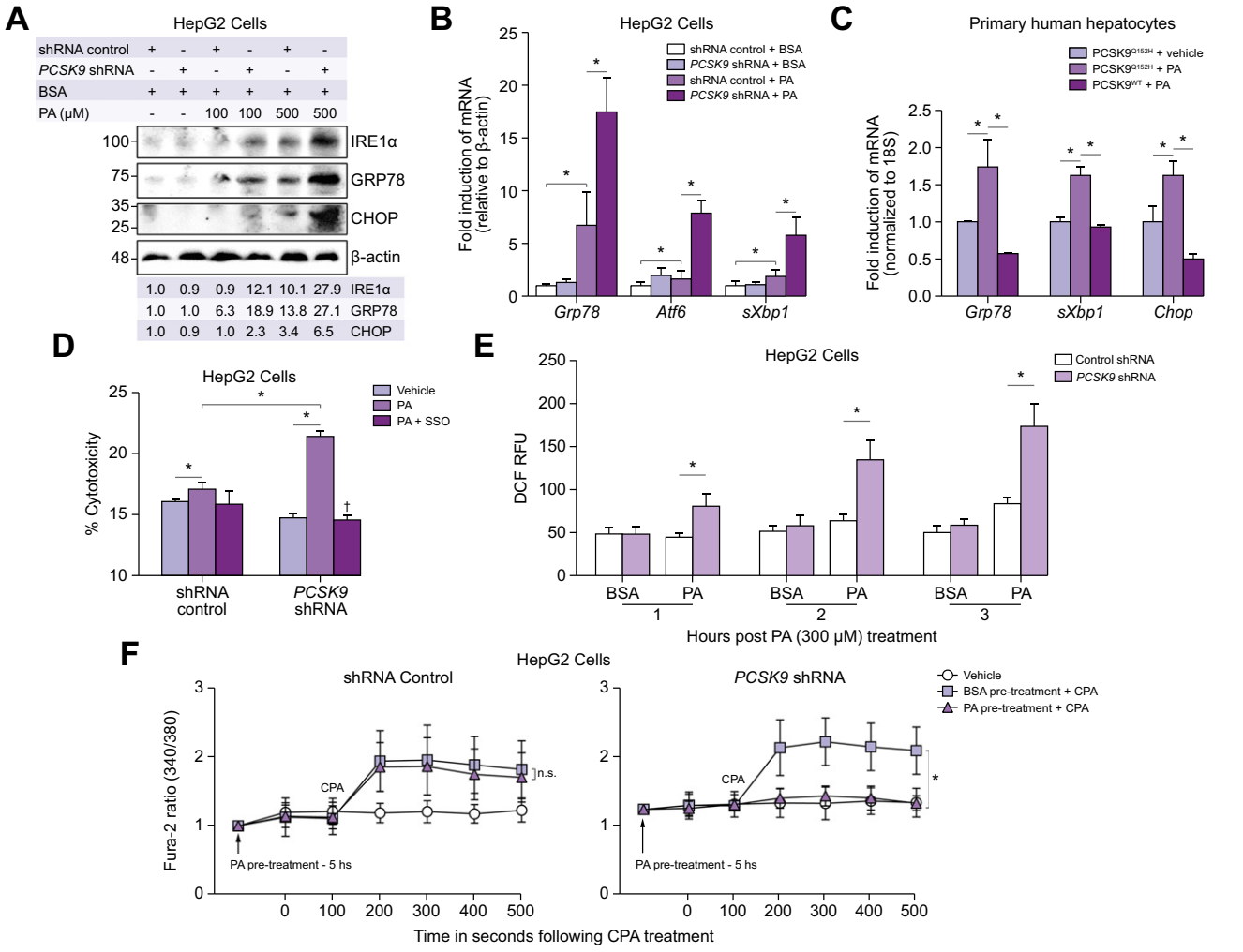

Fig. 3. PCSK9 blocks palmitate-induced ER stress in HepG2 cells. (A,B), Immunoblot and real-time PCR analysis of ER stress marker expression in shRNA control and *PCSK9* shRNA HepG2 cells treated with vehicle (BSA) or PA (100 or 500 μM) for 24 h (**p* <0.05). (C) Primary human hepatocytes were exposed to medium harvested from HuH7 cells transfected with either $PCSK9^{WT}$ or a $PCSK9^{Q152H}$ variant that fails to be secreted. Hepatocytes were then treated with PA (500 μM) for 24 h and assessed for ER stress marker expression via real-time PCR (**p* <0.05). (D) Cytotoxicity of PA-treated HepG2 cells was also examined using a lactate dehydrogenase assay in the presence or absence of CD36 inhibitor, SSO (10 μM; **p* <0.05; †*p* <0.05 *vs.* PA). (E) ROS production was assessed by pre-loading cells with DCF, a ROS-sensitive fluorogenic dye, at 1, 2 and 3 h in HepG2 cells following PA treatment (**p* <0.05). (F) The Fura-2-AM Ca^{2+} indicator was used to examine PA-induced ER Ca^{2+} release. Following the incubation of HepG2 cells with PA for 5 h (500 μM), the SERCA blocker CPA (50 μM) was used to promote the spontaneous release of ER Ca^{2+} to examine total ER Ca^{2+} content (**p* <0.05). Results are shown as the mean and error bars as SD. Differences between groups were determined by Student's *t* test or one-way ANOVA. CPA, cyclopiazonic acid ER, endoplasmic reticulum; PA, palmitate; SSO, sulfo-N-succinimidyl oleate.

increased mRNA transcript expression (Fig. S5B). Elevated expression of hepatic PCSK9-regulated receptors, LDLR, VLDLR and CD36 was also confirmed using immunoblots (Fig. 4B). Given that hepatic biliary secretion or transintestinal cholesterol excretion are established mechanisms of cholesterol clearance,[26,27] fecal cholesterol content was also assessed (Fig. 4C). The feces of *Pcsk9*$^{-/-}$ mice contained increased levels of cholesterol compared to controls. The positive correlation between PCSK9 and circulating cholesterol and triglycerides was also confirmed via colorimetric assays (Fig. 4D). Consistent with our hypothesis that elevated hepatic lipid content in *Pcsk9*$^{-/-}$ mice occurs as a result of increased lipid uptake, no difference was observed in the FA oxidation marker, β-hydroxybutyrate (Fig. 4E). Furthermore, we observed that the livers of *Pcsk9*$^{-/-}$ mice exhibited (a) a significant reduction in peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC1α), a protein commonly associated with *de novo* cholesterol synthesis and PPARγ-mediated NAFLD; (b) a modest but significant increase in mRNA levels of the lipolysis markers ACOX1 and APOC2; and (c) no change in the expression of the FA oxidation marker PPARα (Fig. 4F). Consistent with immunohistochemistry data, a significant increase of perilipin at the mRNA level was also observed in *Pcsk9*$^{-/-}$ mice relative to controls (Fig. 4F). Although additional studies are required to confirm that *de novo* lipogenesis and lipid oxidation do not contribute to the observed phenotype, our data suggest that lipid accumulation in the livers of *Pcsk9*$^{-/-}$ mice occurs largely as a result of increased lipid uptake at the cell surface.

Pcsk9 knockout exacerbates diet-induced hepatic steatosis in mice

Although we did not observe profound differences in gross liver morphology or injury in NCD-fed *Pcsk9*$^{-/-}$ mice compared to *Pcsk9*$^{+/+}$ counterparts, this diet is not lipid-rich or used for the study of hepatic steatosis. Therefore, our next aim was to assess the effect of a HFD on liver function and injury in the context of PCSK9 deficiency and increased hepatic CD36 expression.

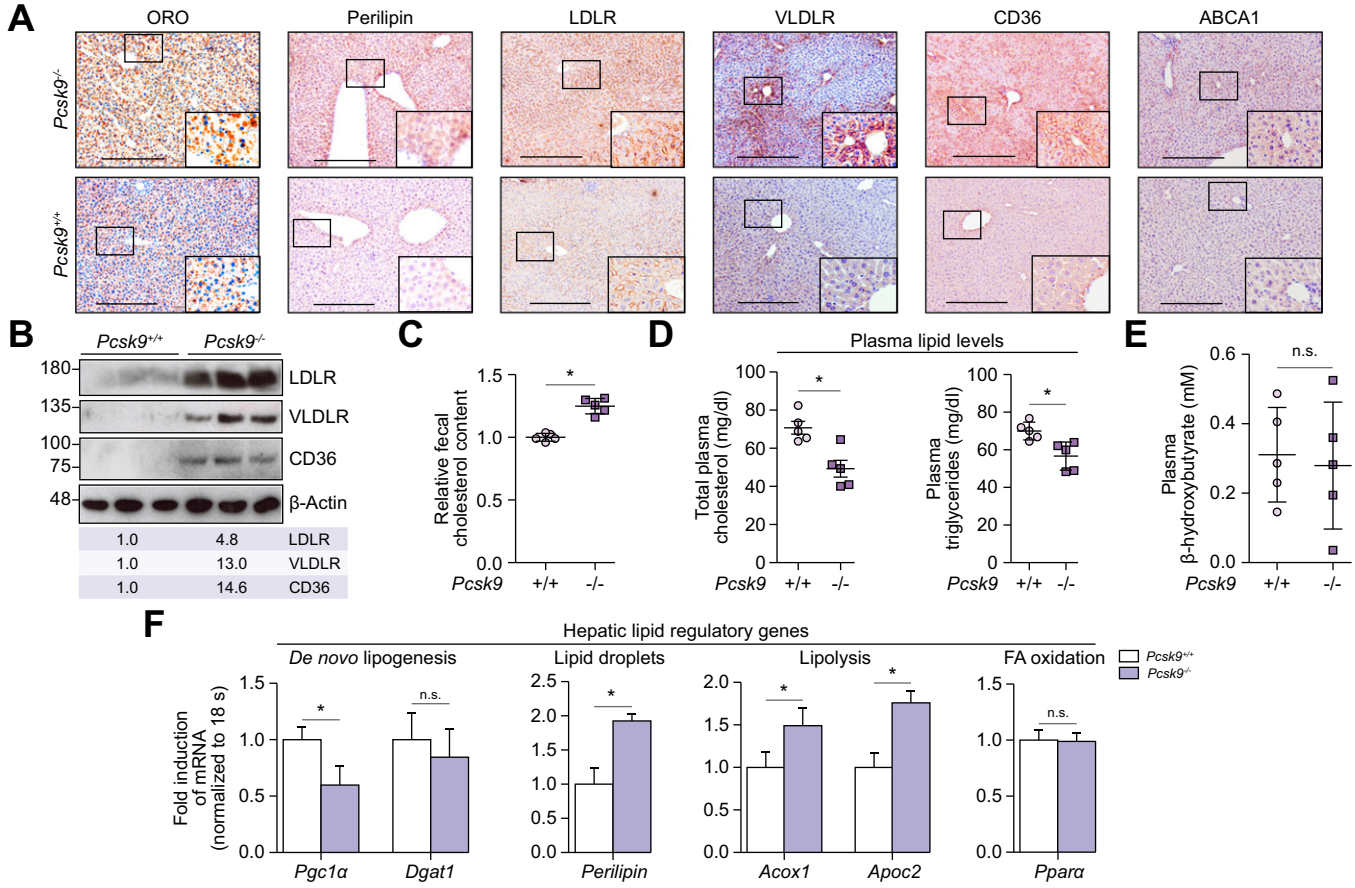

Fig. 4. *Pcsk9*$^{-/-}$ mice exhibit compensatory changes in the expression of lipid regulatory proteins. (A) Representative ORO staining of hepatic lipid droplets in *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ mice (n = 10; C57BL/6J). Protein levels of the lipid droplet marker, perilipin, as well as LDLR, VLDLR, CD36 and the cholesterol efflux transporter, ABCA1, were also examined by immunohistochemistry. (B) Hepatic expression of LDLR, VLDLR and CD36 was also examined using immunoblots. (C, D,E) Fecal cholesterol, total plasma cholesterol and triglycerides, as well as a circulating marker of FA oxidation, β-hydroxybutyrate, were measured using colorimetric assays (n = 5). (F) mRNA levels of genes known to play a role in FA metabolism (n = 5). Results are shown as the mean and error bars as SD. *p <0.05 by Student's t test or one-way ANOVA. Scale bars, 200 μm. FA, fatty acid; ORO, Oil red O.

Accordingly, 6-week-old *Pcsk9*$^{-/-}$ mice and *Pcsk9*$^{+/+}$ controls were fed either NCD or HFD for 12 weeks. Upon removal of the liver from these animals during sacrifice, an apparent increase in size and change in color were observed in the HFD-fed *Pcsk9*$^{-/-}$ mice compared to controls (Fig. 5A). Assessment of hepatic lipid droplets via ORO and H&E staining also demonstrated an increase in lipid droplets in the livers of HFD-fed *Pcsk9*$^{-/-}$ mice compared to HFD-fed controls (Fig. 5B). Although total body weight was not affected by PCSK9 (Fig. 5C) in these experiments, significant increases in liver weight (Fig. 5D) and liver triglyceride and cholesterol content (Fig. 5E) were observed in HFD-fed *Pcsk9*$^{-/-}$ mice compared to controls. A trend was also apparent for elevated gonadal, inguinal and brown adipose tissue weights in the *Pcsk9*$^{-/-}$ mice on HFD, but the differences between genotypes were not statistically significant in this experiment (Fig. 5F).

Pcsk9 knockout mice exhibit increased diet-induced liver injury and insulin resistance

We next examined hepatic ER stress in these mice; a process well known to contribute to hepatic steatosis.[4–6] Increased expression levels of ER stress markers, pro-apoptotic markers, and pro-fibrotic markers were observed in the livers of HFD-fed *Pcsk9*$^{-/-}$ mice compared to HFD-fed *Pcsk9*$^{+/+}$ control mice (Fig. 6A, B). Increased staining of F4/80, a marker of pro-inflammatory Kupffer cells, and increased hepatic mRNA expression of pro-inflammatory markers were also observed in the livers of HFD-fed *Pcsk9*$^{-/-}$ mice compared to controls (Fig. 6A,C). A modest increase in fibrosis was also observed in these mice via Mason's trichrome staining of collagen (blue), as well as immunohistochemical staining of fibronectin. Assessment of plasma alanine aminotransferase (ALT) activity, as well as apoptosis using a TUNEL assay, also revealed that *Pcsk9*$^{-/-}$ mice were more prone to diet-induced hepatic injury compared to controls (Fig. 6D and E). Similarly, an increase in protein aggregate accumulation was observed in HFD-fed *Pcsk9*$^{-/-}$ mice compared to controls using thioflavin-T (Fig. S6)

Hepatic steatosis is also an established driver of insulin resistance and diabetes as it tends to promote chronically elevated circulating glucose levels resulting from constitutively activated gluconeogenic pathways.[28] For this reason, our last aim was to examine the effect of *Pcsk9* knockout on markers of hepatic insulin resistance in HFD-fed mice. First, however, we examined glucose production in vehicle- and PA-treated primary hepatocytes isolated from *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ littermate control mice. Glucose production was also examined in PA-treated primary hepatocytes isolated from C57BL/6J mice in the presence or absence of recombinant PCSK9 (1 μg) for 24 h. In both cases, the presence of PCSK9 attenuated PA-induced glucose production. Assessment in HepG2 cells also revealed that PA treatment induced glucose

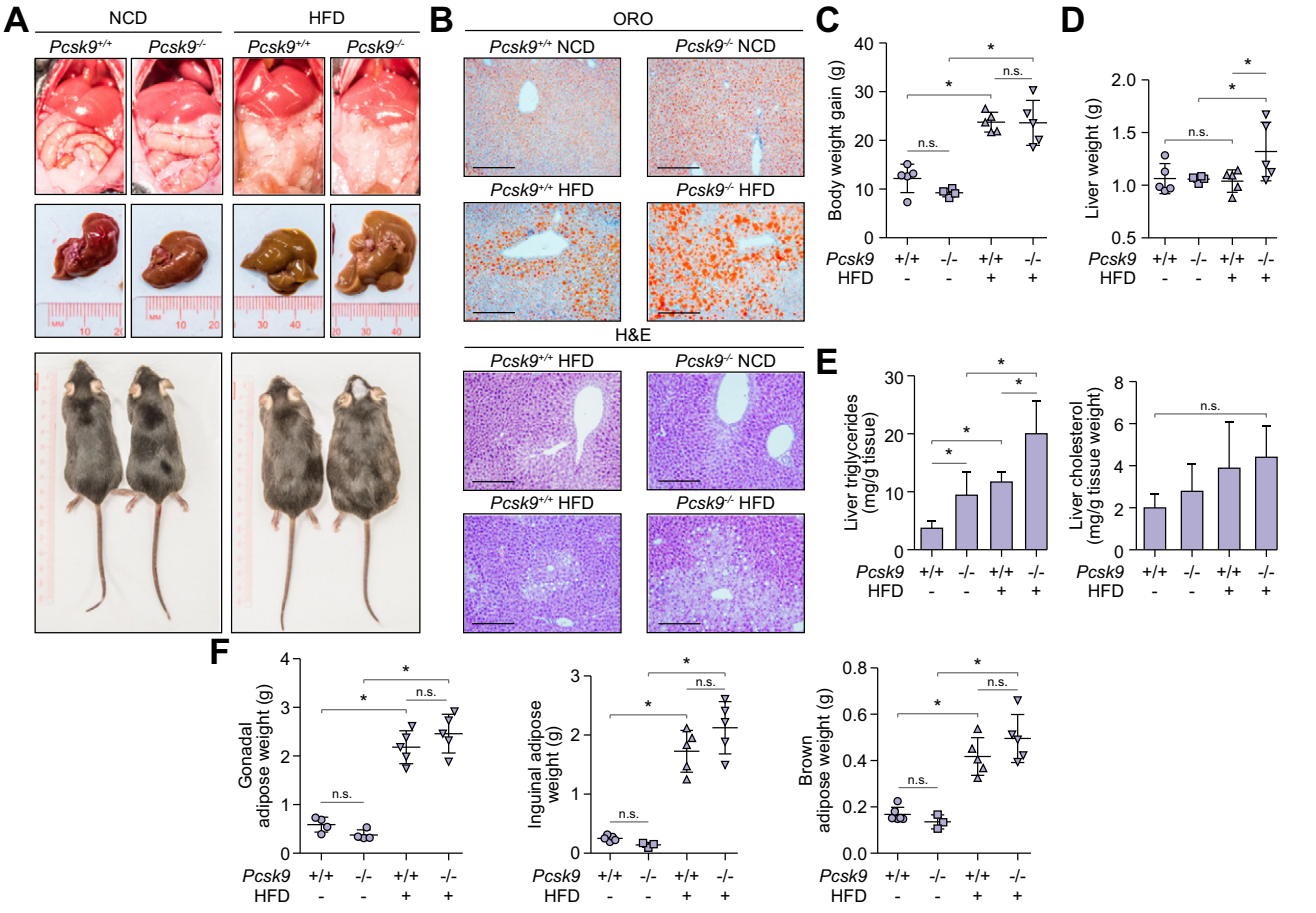

Fig. 5. PCSK9 reduces ER stress-induced hepatic lipid accumulation. (A) Changes in liver size and gross morphology were first visualized and imaged following the sacrifice of NCD- and HFD-fed *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ mice (n = 5). (B) Hepatic lipid accumulation was then examined via ORO and H&E. Changes in body and liver weight (C and D), as well as liver triglycerides and cholesterol levels (E) and adipose weights (F) were also examined. Results are shown as the mean and error bars as SD. $^{*}p$ <0.05 by Student's *t* test or one-way ANOVA. Scale bars, 200 μm. ER, endoplasmic reticulum; HFD, high-fat diet; NCD, normal control diet, ORO, Oil red O.

production to a greater extent in PCSK9 knockdown cells than in control cells (Fig. S7A). Further, exposure of naive HepG2 cells to medium harvested from HuH7 cells transfected with either *PCSK9*^{WT} or the secretion-defective *PCSK9*^{Q152H} also demonstrated that PCSK9 protected against PA-induced gluconeogenesis (Fig. S7B).

In mice, glucose and insulin tolerance tests (GTT and ITT, respectively) revealed that HFD-fed *Pcsk9*$^{-/-}$ mice exhibited a reduction in glucose uptake in response to a bolus injection of glucose or insulin (Fig. 7C), indicative of resistance to insulin.[29,30] A significant increase in resting glucose levels, which represents another characteristic of insulin resistance, was also observed in HFD-fed *Pcsk9*$^{-/-}$ mice compared to HFD-fed controls (Fig. 7D). In contrast to other models of diet-induced insulin resistance,[31] plasma insulin levels were reduced in *Pcsk9*$^{-/-}$ mice compared to controls, but this likely involved pancreatic islet abnormalities, as reported previously (Fig. 7E).[29,30] Mice were also injected with insulin 1 h prior to sacrifice in order to examine the phosphorylation/activation status of protein kinase B (AKT); a central regulator and strong promoter of hepatic glucose uptake and metabolism (Fig. 7F).[31] As expected, insulin treatment led to the phosphorylation of AKT in the livers of NCD-fed mice, but no difference was observed between *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ mice. In HFD-fed mice, however, the ability of insulin to activate hepatic AKT was reduced to a greater extent in *Pcsk9*$^{-/-}$ mice than in the *Pcsk9*$^{+/+}$ control mice. Consistent with other studies, hepatic steatosis severity in the mice was positively correlated with the mRNA abundance of hepatic gluconeogenic markers, including signal transducer and activator of transcription 3 (*Stat3*), glucose 6 phosphatase (*G6p*), stearoyl-CoA desaturase-1 (*Scd1*), pyruvate dehydrogenase kinase 4 (*Pdk4*) and the dimethylaniline monooxygenase [N-oxide-forming] 2 (*Fmo2*) (Fig. 7G).[32] In line with the other endpoints examined in these mice, increased gluconeogenic marker expression was observed in the livers of *Pcsk9*$^{-/-}$ compared to *Pcsk9*$^{+/+}$ mice. Collectively, these data suggest that PCSK9 can reduce liver lipid accumulation and protect against ER stress, hepatic insulin resistance and glucose production in response to a HFD.

Discussion

Previous studies have demonstrated that the equilibrium of hepatic lipid levels can favor hepatic steatosis during conditions of (a) increased *de novo* lipogenesis, (b) heightened lipid uptake (c) reduced lipid efflux and (d) reduced lipid oxidation.[32] In the case of *Pcsk9*$^{-/-}$ mice, our studies as well as those of others,[18] suggest that PCSK9 prevents the uptake of lipid into the liver by downregulating the expression of CD36. Consistent with our observations, previous studies have also shown that increased expression of CD36 can promote fatty liver disease and contribute

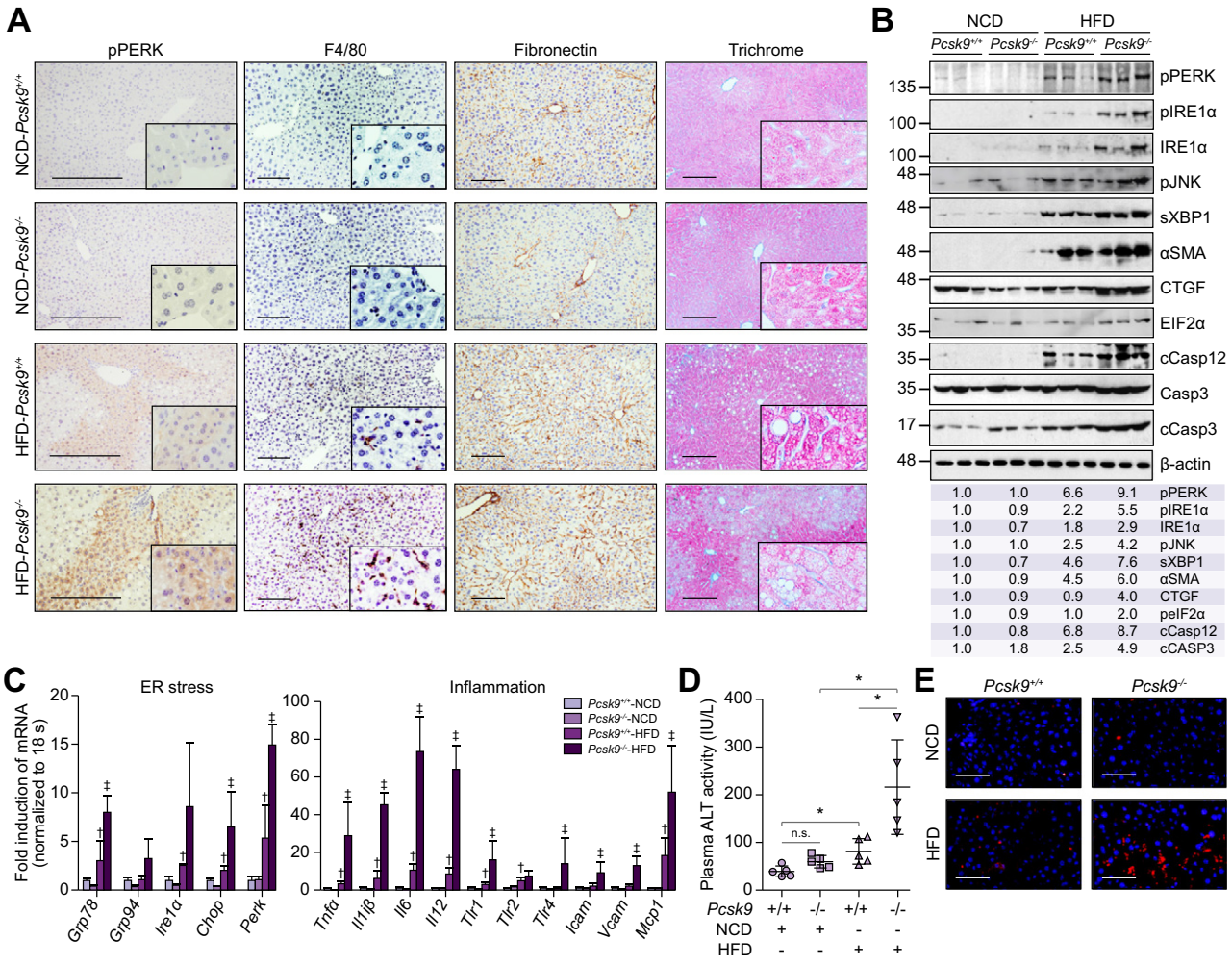

Fig. 6. *Pcsk9* knockout exacerbates diet-induced ER stress and inflammation in the livers of mice. (A, B and C) Markers of ER stress (pPERK, pIRE1α, IRE1α, pJNK, sXBP1, peIF2α and *Chop*), inflammation (F4/80, *Tnfα, Il1β, Il6, Il12, Tlr1, Tlr2, Tlr4, Icam, Vcam* and *Mcp1*), fibrosis (fibronectin, αSMA, CTGF) and apoptosis (cCasp12 and Casp12) were examined in the livers of NCD- and HFD-fed mice via immunohistochemical staining, immunoblotting and real-time PCR. Hepatic collagen deposition (blue) was also examined via Masson's Trichrome. (D,E) Liver injury was examined via assessment of plasma ALT activity, as well as liver TUNEL staining of apoptotic cells (DAPI staining shown as blue and TUNEL-positive apoptotic cells shown as red). Results are shown as the mean and error bars as SD. *p <0.05 by Student's t test or one-way ANOVA. Scale bars, 100 μm. ALT, alanine aminotransferase; ER, endoplasmic reticulum; HFD, high-fat diet; NCD, normal control diet.

to hepatic insulin resistance.[33] Furthermore, CD36-mediated FA uptake is known to promote ER stress and lipotoxicity in cell types with high capacity for FA metabolism including adipocytes, cardiomyocytes, hepatocytes, endothelial cells, macrophages, pancreatic β cells, podocytes and neurons.[22] Here, we demonstrate that PCSK9 prevents CD36-mediated FA uptake and accumulation in cultured hepatocytes. We also show that under normal dietary conditions, several compensatory mechanisms in the livers of *Pcsk9*$^{-/-}$ mice may act in concert to suppress hepatic steatosis. During a HFD metabolic challenge, however, we observed a significant increase in the expression of markers of ER stress, fibrosis, inflammation and apoptosis, as well as plasma levels of ALT in *Pcsk9*$^{-/-}$ mice compared to *Pcsk9*$^{+/+}$ controls.

The current landscape of studies examining the role of PCSK9 in hepatic steatosis in humans remains controversial. However, pre-clinical data have been largely consistent, demonstrating that circulating PCSK9 can prevent hepatic lipid uptake and accumulation in mice. Demers and coworkers were the first to demonstrate that PCSK9 negatively regulates the expression of CD36 and also reported a 3- to 4-fold increase in hepatic triglyceride levels in NCD-fed *Pcsk9*$^{-/-}$ mice.[18] Recent studies have also identified *E2f1* as a major regulator of hepatic lipid homeostasis in a manner dependent on PCSK9.[34] Lai and colleagues reported increased hepatic lipid content in *E2f1*$^{-/-}$ mice and also discovered that these mice exhibit significantly reduced circulating PCSK9 levels. Upon re-expression of PCSK9 in these mice, the fatty liver phenotype was lost. Consistent with this growing body of literature, a recent network analysis done in primary human hepatocytes identified PCSK9 as a gene linked to liver fat content and NAFLD.[35]

Two independent clinical studies have now demonstrated consistent findings with regards to the association between liver fat content and circulating PCSK9 levels. The Dallas heart study reported a modest but significant positive correlation between liver fat content and circulating PCSK9 levels (n = 2,027; p <0.0001).[36] Ruscica and colleagues have now also reported a positive correlation between circulating PCSK9 and liver steatosis grade (p = 0.0011) as well as necroinflammation, ballooning and

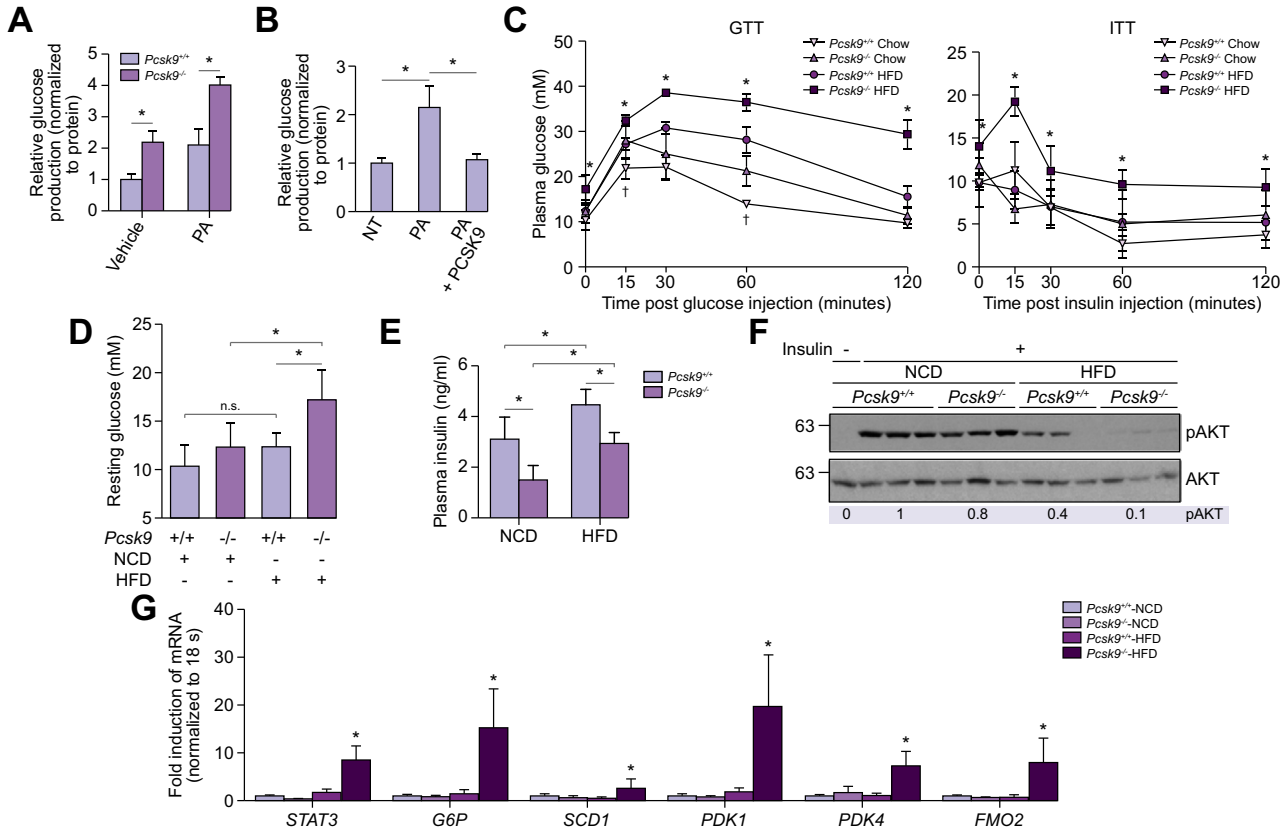

Fig. 7. *Pcsk9* knockout contributes to gluconeogenesis and diet-induced insulin resistance. (A) Gluconeogenesis was first examined in primary hepatocytes isolated from *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ littermate control mice (n = 5) treated with PA. (B) Gluconeogenesis was also assessed in primary hepatocytes isolated from male C57BL/6J mice (n = 4) treated with PA in the presence or absence of recombinant human PCSK9 (1 μg; p <0.05). (C) One week prior to sacrifice, mice were injected with either insulin (1 IU/kg) or glucose (0.8 g/kg) (ITT and GTT, respectively) to assess insulin resistance in NCD- and HFD-fed *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ mice (*p <0.05 *vs. Pcsk9*$^{+/+}$). (D) Resting glucose levels were also assessed prior to injections. (E) Resting plasma insulin levels in these mice were examined using an ELISA. (F) Mice were injected with insulin 1 h prior to sacrifice to assess hepatic insulin resistance via phosphorylation of AKT by immunoblotting. (G) The expression of pro-gluconeogenic markers (*Stat3, G6p, Scd1, Pdk1, Pdk4 and Fmo2*) was examined in the livers of HFD-fed mice by real-time PCR (*p <0.05. *vs.* HFD-fed *Pcsk9*$^{+/+}$). Results are shown as the mean and error bars as SD. *p <0.05. Differences between groups were determined via Student's *t* test or one-way ANOVA. GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; NCD, normal control diet; PA, palmitate.

fibrosis stage (n = 201).[37] Consistent with these findings our research group has observed that diet-induced steatosis promotes *de novo* hepatic PCSK9 expression and increases circulating PCSK9 levels in mice.[38] In contrast to these data, Wargny and colleagues failed to observe a significant correlation between circulating PCSK9 levels and plasma aminotransferases, liver fat content, histological liver lesions, steatosis severity, NASH activity score, lobular and/or portal inflammation or ballooning in 3 patient cohorts with advanced stages of NASH (n = 478).[39] Given the severity of steatosis in the patients from this study, however, the authors highlight that variations between studies suggest that in humans, PCSK9 likely plays a role in the early development of NAFLD and not in the late stages of NASH.

Beyond liver fat content, Mbikay and colleagues have reported abnormalities in pancreatic islets of *Pcsk9*$^{-/-}$ mice, which were shown to contribute to insulin resistance.[29] Consistent with these findings, we also report the novel finding that diet-induced hepatic steatosis attenuates insulin-induced phosphorylation and activation of AKT in the livers of *Pcsk9*$^{-/-}$ mice to a significantly greater extent than in controls. We have consistently shown that HFD-fed *Pcsk9*$^{-/-}$ mice exhibit reduced rates of glucose uptake and insulin sensitivity, all of which represent hallmark features of hepatic insulin resistance. In line with these findings, a recent study also identified a positive correlation between loss-of-function *PCSK9* mutations and increased fasting glucose, body weight, waist-to-hip ratio and odds ratio of type 2 diabetes.[40]

Accumulating evidence demonstrates that PCSK9 regulates the uptake and accumulation of lipid in the livers of mice. Given the current inconsistencies in clinical data sets, additional studies are required before strong conclusions can be made in the context of human disease. Interestingly, recent studies have demonstrated that FDA-approved monoclonal antibodies targeted against the LDLR-binding domain of circulating PCSK9 increase its concentrations 7-fold as a result of antagonizing LDLR-mediated clearance.[41] Given that it is likely that the domain utilized by PCSK9 to interact with CD36 differs from its LDLR-binding domain,[18] it remains possible and even likely that such antibodies may also reduce CD36 expression as a byproduct of increasing the circulating pool of PCSK9. Additional studies, however, are required to answer this important question and to determine whether anti-PCSK9 antibodies could be utilized to protect against CD36-driven diseases like NAFLD and NASH.

Overall, the abundance of data characterizing PCSK9 as a modulator of circulating cholesterol strongly suggest that its influence on the latter exceeds that of its effect on circulating and peripheral triglyceride levels. Nonetheless, our findings as well as those discussed herein,

suggest that hepatic PCSK9 expression is upregulated during conditions of steatosis to prevent further lipid uptake and accumulation in the liver, thus acting as a classical feedback modulator of hepatic lipid levels. Moreover, our data demonstrate that in mice, PCSK9 can protect against ER stress, fibrosis and injury of the liver in response to conditions of excessive fat consumption.

Abbreviations

ABCA1, ATP-binding cassette subfamily A member 1; ACOX1, acyl-coA Oxidase 1; AKT, protein kinase B; ALT, alanine aminotransferase; APOC2, apolipoprotein C2; ApoE, apolipoprotein E; ATF, activating transcription factor; cCasp, cleaved-caspase; CHOP, CCAAT/enhancer-binding protein homologous protein; CPA, cyclopiazonic acid; CTGF, connective tissue growth factor, CVD, cardiovascular disease; eIF2, eukaryotic initiation factor 2; ER, endoplasmic reticulum; FA, fatty acid; FAS, fatty acid synthase; FMO2, flavin containing monooxygenase 2; GRP78, 78-kDa glucose regulated protein; GRP94, 94-kDa glucose regulated protein; GTT, glucose tolerance tests; G6P, glucose 6-phosphate; HDL, high-density lipoprotein; HFD, high-fat diet; IL, interleukin; IRE1α, Inositol-requiring enzyme-1 alpha; ITT, insulin tolerance tests; JNK, c-Jun N-terminal kinase; LA, linoleate; LDL, low-density lipoprotein; LDLR, LDL receptor; MCP-1, monocyte chemoattractant protein-1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NCD, normal control diet; NS, non-significant; OA, oleate; ORO, Oil Red O; oxLDL, oxidized LDL; PA, palmitate; PCSK9, proprotein convertase subtilisin/kexin type 9; PDK1, pyruvate dehydrogenase kinase 1; PDK4, pyruvate dehydrogenase kinase 4; PERK, Protein kinase RNA-like endoplasmic reticulum kinase; PGC1α, PPAR gamma coactivator 1-alpha; PPAR, peroxisome proliferator-activated receptors; PPARγ, peroxisome proliferator-activated receptor gamma; PSR, Picrosirius Red; ROS, reactive oxygen species; SA, stearate; SCD1, stearoyl-coA desaturase-1; SERCA, sarco/endoplasmic reticulum ATPase; shRNA, short hairpin RNA; siRNA, small interfering RNA; αSMA, alpha smooth muscle actin; SREBP, sterol regulatory element-binding protein; SSO, sulfosuccinimidyl oleate; STAT3, signal transducer and activator of transcription 3; TLR, toll-like receptor; TM, tunicamycin; TNFα, tumor necrosis factor alpha; UPR, unfolded protein response; VCAM, vascular cell adhesion protein 1; VLDLR, very low-density lipoprotein receptor; WT, wild-type; XBP1, X-box-binding protein.

Financial support

This work was supported in part by research grants to Richard C. Austin from the Heart and Stroke Foundation of Canada (G-13-0003064 and G-15-0009389), the Canadian Institutes of Health Research (74477), to Nabil G. Seidah and Annik Prat from the Leducq Foundation (13 CVD 03), CIHR Foundation grant (148363) and Canada Research Chair (216684). Kenneth N. Maclean gratefully acknowledges financial support from the William R. Hummel Homocystinuria Research Fund and holds the Ehst-Hummel-Kaufmann Family Endowed Chair in Inherited Metabolic Disease. Financial support from the Research Institute of St. Joseph's Healthcare Hamilton is acknowledged. Richard C. Austin is a Career Investigator of the Heart and Stroke Foundation of Ontario and holds the Amgen Canada Research Chair in the Division of Nephrology at St. Joseph's Healthcare Hamilton and McMaster University.

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

PL, NGS and RCA conceived the studies. PL, JHB, KP, AAA, SL, and MEM performed all of the *in vitro* and *in vivo* studies. The manuscript was written by PL and RCA and revised by AMB, AP, SAI, KNM, BT and NGS.

Acknowledgements

The mammalian expression plasmid encoding His-CD36 was a gift from Gavin Wright (Addgene plasmid # 52025).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhepr.2019.10.009.

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Author names in bold designate shared co-first authorship

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