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Hepatic Clstn3 Ameliorates Lipid Metabolism Disorders in High Fat Diet-Induced NAFLD through Activation of FXR

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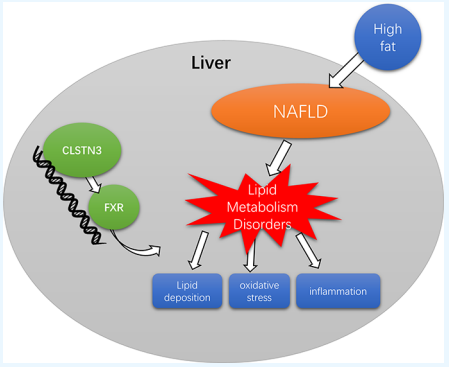
ABSTRACT: Non-alcoholic fatty liver disease (NAFLD) has become serious liver disease all over the world. At present, NAFLD caused by high calorie and fat diet is increasing. Calsyntenin-3 (Clstn3) is a transmembrane protein that has recently been found to participate in lipid energy metabolism. But whether Clstn3 affects NAFLD lipid metabolism has not been analyzed. We stimulate the mice primary hepatocytes (MPHs) with oleic acid and palmitic acid (OA&PA) to establish a cell model. Then, potential targets, including Clstn3 gene, were validated for improving lipid metabolism disorder in NAFLD model mice (HFD and db/db) by silencing and overexpressing hepatic Clstn3. Moreover, the effects of Clstn3 on lipid homeostasis were determined by functional determination, triglyceride (TG) levels, total cholesterol (TC) levels, ELISA, and qRT-PCR detection. Our results displayed that Clstn3 was decreased in the NAFLD mice model. Also, overexpression of Clstn3 improved lipid metabolism disorders, gluconeogenesis, and energy homeostasis and reduced liver injury, inflammation, and oxidative stress injury. However, opposite results were obtained in Clstn3-silencing mice, suggesting that the Clstn3 gene is closely related to lipid metabolism disorder in NAFLD. RNAseq expression demonstrated that Farnesoid X Receptor (FXR) expression was increased after overexpression of Clstn3. Clstn3 supplementation in FXRKO mice can improve the dysfunction caused by insufficient FXR, suggesting that Clstn3 can improve the NAFLD lipid metabolism disorder to some extent through FXR, which may provide a new method for the treatment of NAFLD.



1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) was presented by the Mayo Clinic Ludwig as a set of chronic maladies in which fat accounts for over 5% of liver weight not from overconsumption of alcohol, except for definite liver injury factors. High fat diet (HFD)-associated obesity is greatly common in patients with non-alcoholic fatty liver disease (NAFLD) that is emerging as one of the most universal causes of liver disease worldwide, especially in Western countries.[1] It can be manifested as a series of hepatic diseases.[2] Its etiologies mainly include disorders of lipid metabolism in the liver, lipid peroxidation, insulin resistance, oxidative stress, genetic susceptibility, and other related factors.[3] Growing evidence suggests that oxidative stress plays a role as the critical factor linking obesity with its associated complications, such as NAFLD. Obesity per se can induce systemic oxidative stress through various biochemical mechanisms, further leading to related metabolic diseases.[4] A recent survey showed that the incidence of NAFLD in China even reached 29.2%,[5] while NAFLD is also the frequent etiology of chronic liver disease in Western States, and NAFLD is expected to be a frequent adaptive disease of liver transplantation by 2030.[6] However, there was no reasonable explanation for the pathogenesis of NAFLD, and the currently accepted pathological mechanisms of NASH include the "second strike" and "multiple strike" theories.[7] Reasonable and effective reversal of "strike" factors by means of target gene intervention has an obvious therapeutic effect on NAFLD. For example, the transcription factor KLF16 can improve the abnormal inflammatory response of liver cells by activating Pparα and its target genes, repair the fatty acid oxidation function, and effectively improve the disorder of liver lipid metabolism. Activation of key genes such as SIRT1 in liver cells significantly inhibits the recruitment of liver F4/80 and CD11b-positive cells, thus improving liver inflammation and oxidative stress and playing a role in protecting the liver.[8−10] It is also found that targeting hepatic ZBTB22 can affect oxidative stress, gluconeogenesis, and lipid metabolism and thus play a therapeutic role in diabetes mellitus and liver injury.[11,12] These findings suggest the feasibility of targeting changes in key gene regulatory networks in the liver to reverse lipid metabolism disorders, inflammatory responses, and oxidative stress.

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The calsyntenins (CLSTNs) belong to the superfamily of cell adhesion molecules, including Clstn1, Clstn2, and Clstn3. Clstn3 is a transmembrane protein that is mainly distributed in the postsynaptic membrane, and its extracellular portion contains calmodulin repeats and sex hormone-binding globulin.[13−15] It has recently been found to participate in energy expenditure and obesity and associated metabolic diseases.[16,17] Previous studies on Clstn3 have focused on the brain,[18,19] while studies related to its function in peripheral tissues and organs remain unclear.[20]

For FXR, a key receptor highly expressive in the liver,[21] numerous studies have shown that activation of FXR can regulate cholesterol, glucose, and lipid homeostasis, thereby slowing the progression of NAFLD.[22] FXR agonists were found to reduce plasma cholesterol, TG, and FFA levels in db/db and wild-type mice and also ameliorate glucose homeostasis in ob/ob and db/db mice; nevertheless, no such alterations were observed in FXR knockout mice administered with FXR agonists.[23] In contrast, FXR knockout animal models exhibit mainly reduced insulin sensitivity, hepatic steatosis, hyperlipidemia, hyperglycemia, inflammation, and fibrosis.[24,25] In addition, FXR knockdown mice consume a diet containing 1% cholesterol, resulting in the increase in liver cholesterol and triglycerides.[26] Currently, more and more studies are focusing on FXR, and some FXR agonists such as obeticholic acid, EDP-305, GS-9674, and TERN-101 are already in phase II and III clinical trials,[27] showing that targeting FXR is an effective treatment method for NAFLD.

Like FXR, hepatic Clstn3 also is a critical receptor that is highly expressed in the liver. We hypothesize that Clstn3 supplementation can effectively ameliorate fatty liver degeneration in NAFLD and activate the FXR receptor to further play its role.

2. RESULTS

2.1. Decreased Hepatic Clstn3 Expression in Hepatic Steatosis. To search for a potential target in hepatic steatosis, qRT-PCR was intended for detecting the Clstn3 mRNA levels in HFD, db/db, and ob/ob mice livers, we discovered that in comparison with the contrast group, the Clstn3 expression was greatly reduced in the model groups (Figure 1A). Then, we tested the Clstn3 mRNA in OA&PA-induced MPHs and found that OA&PA significantly depressed the Clstn3 expression (Figure 1B). Immunofluorescence analysis confirmed the reduction of the cellular membrane of Clstn3 in OA&PA-treated MPHs as well (Figure 1C). Overall, these data indicated that Clstn3 may participate in hepatic steatosis.

2.2. Clstn3 Overexpression Suppressed OA&PA-Induced Hepatic Steatosis by Improving Lipid Deposition, Oxidative Stress, and Inflammation in MPHs. To begin with, OA&PA-treated MPHs were used to measure the regulatory function of Clstn3 on hepatic steatosis. Interestingly, we detected that Clstn3 overexpression pronouncedly increased fatty acid oxidation gene expression, particularly peroxisome proliferator-activated receptor-α (Pparα) and associated genes, and suppressed the fatty acid synthesis gene expression (Figure 2A and Figure S1A). Consistent with the above results, lipid TOX staining (Figure 2B) and TG levels (Figure 2C) showed that lipid deposition was well improved in the Clstn3 overexpression group. After that, so as to observe the alterations of oxidative stress, we conducted qRT-PCR, and the expression of removing excess reactive oxygen species (ROS) and sustaining redox homeostasis genes nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase 2 (Sod2), and heme oxygenase 1 (Ho-1) was preeminently improved in comparison with the Ad-Gfp group (Figure 2D). Also, Clstn3 overexpression remarkably reduced the ROS levels of MPHs brought on by OA&PA incubation (Figure 2E). Simultaneously, the inflammation gene expression including tumor necrosis factor α (Tnfα), interleukin 1β (Il-1β), and interleukin 6 (Il-6) in the Ad-Clstn3 group was lower than that in the Ad-Gfp group (Figure 2F). As shown in Figure 2G, Clstn3 overexpression decreased the translocation of Nfκb-p65 from the cytoplasm to the nucleus.

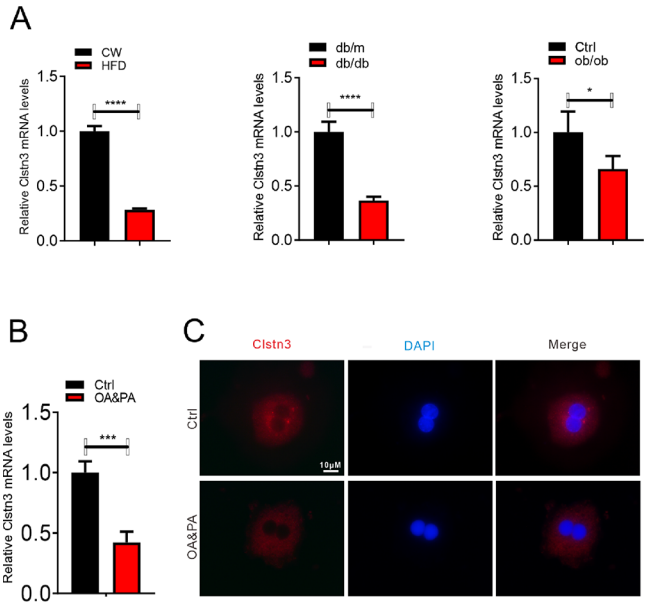

Figure 1. Clstn3 expression in the liver is decreased in hepatic steatosis. (A) Expression of Clstn3 mRNA levels in hepatic tissues of HFD, db/db, and ob/ob mice; (B) Clstn3 mRNA levels in OA&PA-induced MPHs; (C) Clstn3 immunofluorescence analysis (1000×). Data are conveyed as the mean ± SD (n = 4 in groups A and B). *p < 0.05, **p < 0.01, and ****p < 0.0001.

2.3. Deficiency of Hepatic Clstn3 Exacerbates Hepatic Steatosis and Increases Insulin Resistance. First, we established a liver Clstn3 deficiency mice model by tail vein injection of shClstn3 and shGfp adenoviruses. We took samples from mouse livers after virus infection and tested qRT-PCR to ensure that Clstn3 was knocked down (Figure S1B). We observed that Clstn3 deficit dramatically aggravated the fatty acid synthesis gene expression, such as sterol regulatory element binding protein-1c (Srebp1c) and fatty acid synthase (Fas), and inhibited the genes in line with fatty acid oxidation like Pparα. Meanwhile, the expression of acetyl-CoA carboxylase (Acc) gene only slightly increased (Figure 3A). Moreover, Ad-shClstn3-infected mice showed lower fatty acid oxidation rates via the reduction of circulating ketone bodies (Figure 3B). The silencing Clstn3 group intensified TG levels and hepatic lipid deposition (Figure 3C). Concurrently, Clstn3 deficiency significantly worsens liver damage, as shown by ascensions in ALT and AST levels in Figure 3D. In addition, the fasting blood glucose level was impaired due to Clstn3 deficiency (Figure 3E). Furthermore, Clstn3 deficit damaged glucose tolerance, pyruvate tolerance, and insulin sensitivity and was relevant to increased gluconeogenesis gene expression of peroxisome proliferator-activated receptor γ coactivator-1α (Pgc1α), phosphoenolpyruvate carboxykinase (Pepck), and glucose-6-

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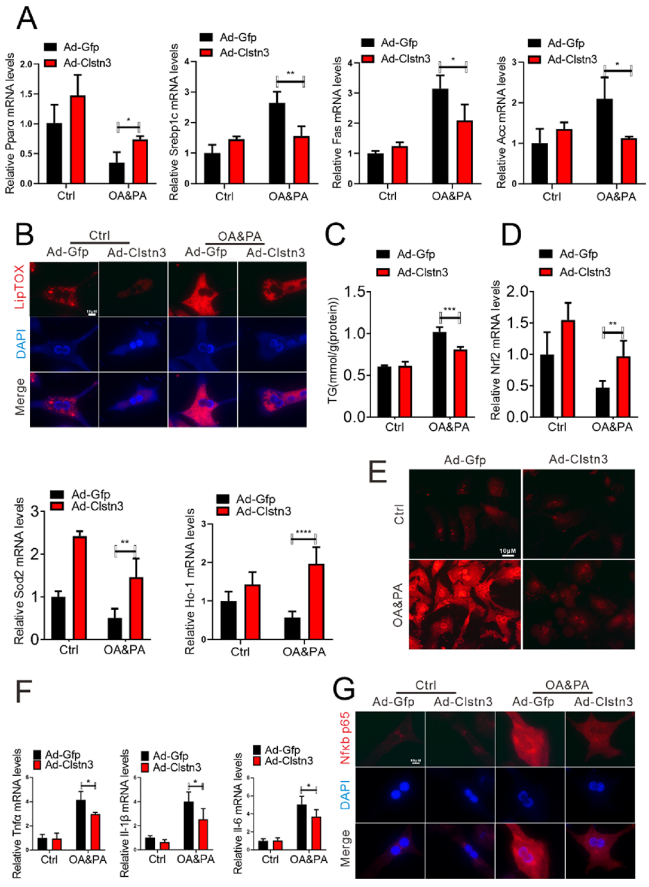

Figure 2. Clstn3 overexpression inhibits hepatic steatosis by ameliorating lipid deposition, oxidative stress, and inflammation in MPHs. (A) Gene expression of lipid metabolism; (B) MPHs stained with TOX lipid (1000×); (C) cellular TG results; (D) oxidative stress gene expression; (E) intracellular ROS levels (400×); (F) inflammation gene expression; (G) Nfκb-p65 immunofluorescence (1000×). Data are conveyed as the mean ± SD ($n = 3–4$/group). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

phosphatase (G6pc) (Figure 3F,H). Interestingly, we found that increased liver/body weight ratio was further supposed to be due to gene silencing, resulting in increased fat deposition (Figure 3G). Immunofluorescence analysis showed that Clstn3 silencing upregulated liver monocyte inflammatory markers F4/80 and CD11b (Figure 3I). H&E and oil red O staining showed that after Clstn3 was knocked down, liver lipid deposition was aggravated and lipid drops increased (Figure S1C).

2.4. Clstn3 Overexpression Alleviates Lipid Metabolism and Insulin Sensitivity. To confirm the effect of Clstn3, we also injected Ad-Clstn3 adenovirus through tail vein to build a liver Clstn3 overexpression mouse model in wild-type mice. Compared to the Ad-Gfp group, the fasting blood glucose level in the Ad-Clstn3 group was obviously improved (Figure 4A). Moreover, the results were consistent with those in Figure 4B; Clstn3 overexpression repaired lipid metabolism and insulin sensitivity as shown by glucose tolerance test, insulin tolerance test, and pyruvate tolerance test. Therefore, we preliminarily guess that Clstn3 has a protective effect on hepatic steatosis.

2.5. Clstn3 Overexpression Conspicuously Mitigated Hepatic Steatosis in db/db and HFD Mice. Furthermore, we established a mouse model of hepatocyte-specific Clstn3 overexpression by injecting Clstn3 into the tail vein to define the role of hepatocellular Clstn3 in hepatic steatosis and lipid metabolic disorders. Injection of Clstn3 adenovirus substantially increased liver-specific overexpression of Clstn3 in db/db mice (Figure S2A), accompanied with the involvement of a coexisting downregulation of adipogenic gene expression (Figure 5A). In addition, mice infected with Ad-Clstn3 showed higher levels of fatty acid oxidation, as confirmed by the increased horizontal of circulating ketone bodies (Figure 5B). It also contributed to a reduction in liver damage and liver lipid deposition in db/db mice, as evidenced by H&E and oil red O staining and a reduction in TG levels. Yet, TC horizontals have no major alteration (Figure 5C,D). In parallel, Ad-Clstn3 alleviated hepatic dysfunction in db/db mice, for example, by lowering ALT and AST levels (Figure 5E). Moreover, Clstn3 overexpression validly reduced the fasting glucose level (Figure 5F) and improved glucose tolerance, pyruvate tolerance, and insulin sensitivity in db/db mice (Figure 5G). Furthermore, overexpression of Clstn3 effectively reduced glucose metabolism and inflammation gene expression in db/db mice, which contributed to reducing hepatic steatosis (Figure 5H,I). Immunofluorescence analysis demonstrated that Ad-Clstn3 downregulated monocyte inflammatory markers F4/80 and CD11b in livers of db/db and HFD mice (Figure 5J). Finally, these above changes may contribute to the reduction of ROS in the liver, as reflected on antioxidant gene expression (Figure S2B).

Similar results were obtained in HFD mice. To estimate the function of Clstn3 in liver energy homeostasis, we monitored the body weight for 15 days and heat production, oxygen consumption (VO_2), and carbon dioxide production (VCO_2) in mice for 24 h (Figure 6A,B). Results displayed the HFD-induced body weight gain and decreases in thermogenesis, and VO_2 and VCO_2 were reversed after Clstn3 overexpression in mice compared to the Ad-Gfp group, indicating that Clstn3 may upregulate and effectively alleviate obesity in HFD mice by increasing energy expenditure. Ad-Clstn3 contraction raised hepatic Clstn3 expression in HFD mice (Figure S2D) and downregulated the expression of genes related to lipogenesis (Figure 6C). This subsequently led to an increase in circulating ketone body levels after Clstn3 overexpression (Figure 6D). Likewise, Ad-Clstn3-contracted HFD mice exhibited an ameliorated NAFLD phenotype as evidenced by decreased liver lipid deposition and TG horizontals (Figure 6E,F). Moreover, overexpression of Clstn3 reduced serum ALT and AST levels, indicating an improved liver function in high-fat mice (Figure 6G). In addition, Ad-Clstn3-infected HFD mice showed reduced fasting glucose levels (Figure 6H). Moreover, overexpression of Clstn3 significantly reversed HFD-induced glucose intolerance, pyruvate intolerance, and insulin resistance (Figure 6I) while inhibiting expression of genes relevant to glucose metabolism (Pgc1α, Pepck, and G6pc) (Figure S2C). Clstn3 overexpression suppressed the markers of inflammatory monocytes and inflammation gene expression in the livers of HFD mice and contributed to the improvement of hepatic steatosis (Figure 5J and Figure S2E). Additionally, Clstn3 overexpression lightly increased antioxidant gene expression and alleviated the overproduction of ROS in the liver (Figure S2F,G).

2.6. Clstn3 Overexpression Promotes the FXR Signal Pathway and Reversed Hepatic Steatosis. To further investigate the effects of Clstn3 overexpression, we assayed RNAseq data from hepatic tissues of HFD mice infected with Gfp or Clstn3 adenovirus. As shown by the heat map data, overexpression of Clstn3 in the tail veins of HFD mice prominently increased genes concerned with antioxidation

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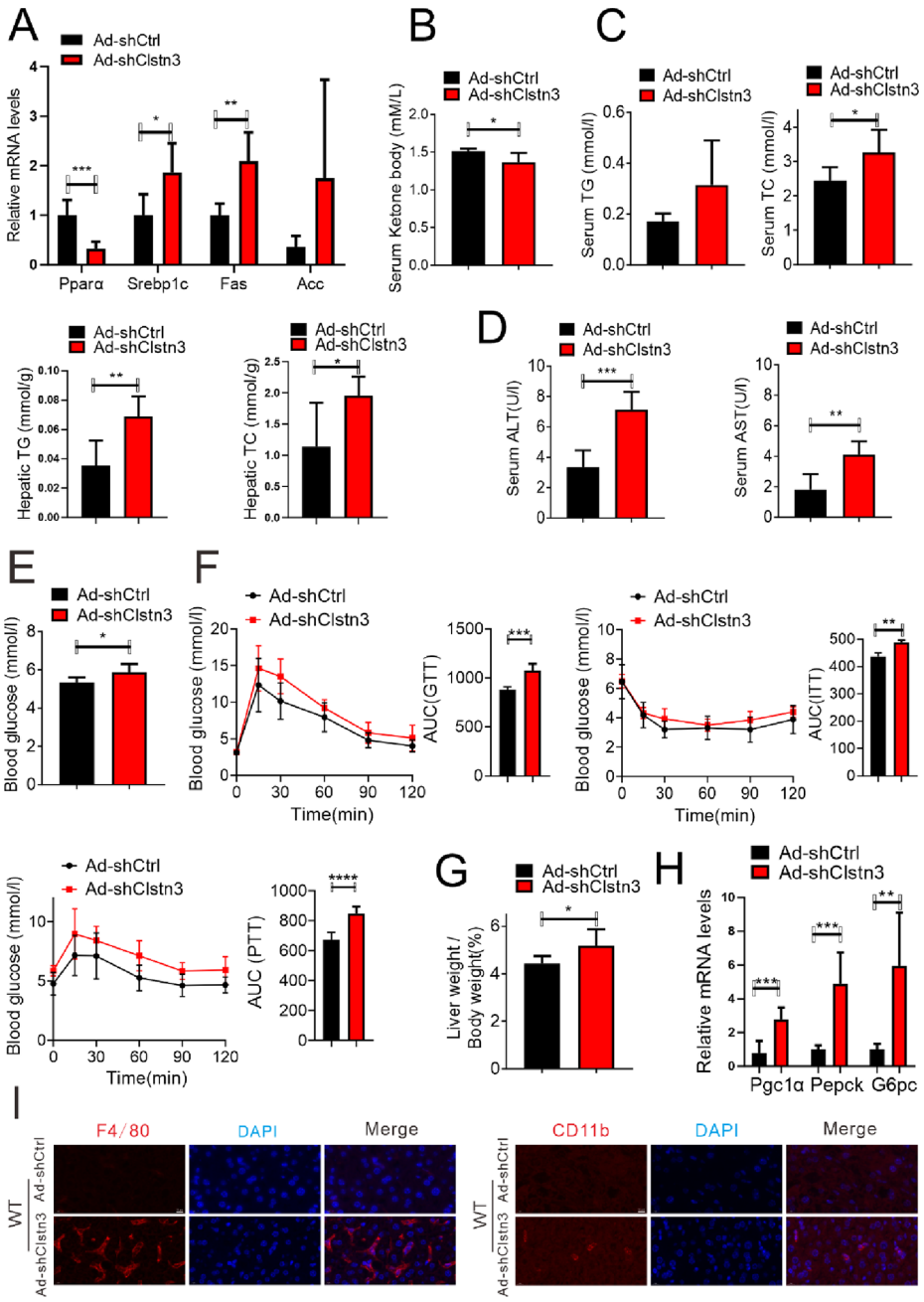

Figure 3. Liver knockdown of Clstn3 impairs lipid metabolism and insulin sensitivity in wild-type mice. (A) Gene expression of lipid metabolism; (B) serum ketone body levels; (C) serum and liver TG and TC results; (D) serum ALT and AST levels; (E) fasting glucose; (F) glucose tolerance test, insulin tolerance test, and pyruvate tolerance test; (G) liver weight-to-body weight ratio; (H) gluconeogenesis gene expression; (I) immunofluorescence analysis of F4/80 and CD11b. Data are conveyed as the mean ± SD (n = 5−8/group). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

activity, for instance, Nrf2, and genes covering β-oxidation, like Pparα, while with the above changes, there was a notable promotion of FXR in mice treated with Ad-Clstn3 (Figure 7A). The increased expression of FXR in vivo was further verified by qRT-PCR (Figure 7B). Therefore, we speculated that FXR was the downstream gene. More importantly, immunofluorescence analysis also validated that Ad-Clstn3 stimulated FXR translocation from the cytoplasm to the nucleus in OA&PA-treated MPHs (Figure 7C). Moreover, overexpression of Clstn3 can validly reduce the expression of genes extended to lipid metabolism in FXRKO mice and TG levels were similarly reduced, while TC levels were just slightly reduced (Figure 7D,E). Clstn3 overexpression efficaciously cut down ALT and AST levels in FXRKO mice, declaring improvement in liver injuries (Figure 7F). The Clstn3 overexpression ameliorated glucose intolerance and pyruvate intolerance and decreased insulin resistance in FXRKO mice fed on chow diet (Figure 7G). Additionally, Clstn3 overexpression significantly increases antioxidant gene expression and diminution in levels of inflammatory genes in the livers of FXRKO mice was found, contributing to the improvement of hepatic steatosis (Figure S3A).

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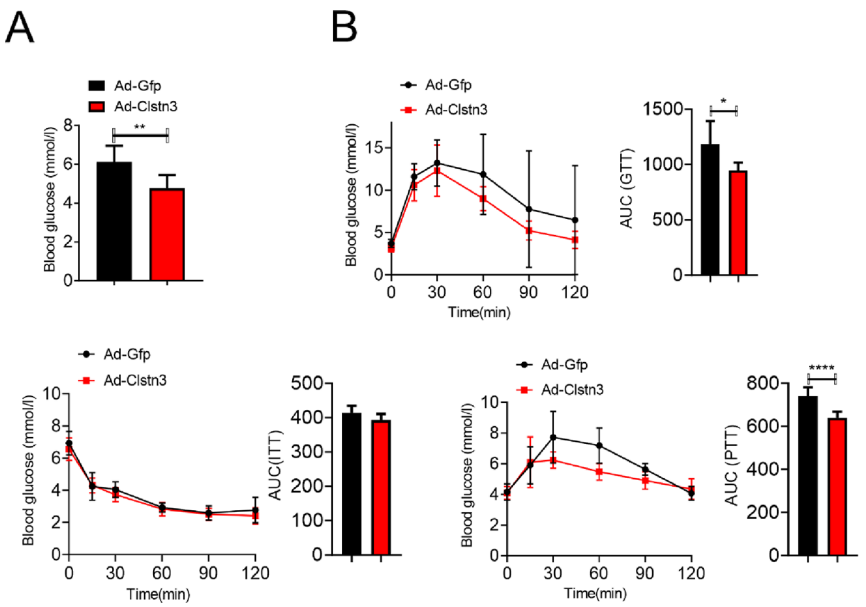

Figure 4. Clstn3 overexpression lipolysis metabolism and insulin sensitivity in wild-type mice. (A) Fasting glucose; (B) glucose tolerance test, insulin tolerance test, and pyruvate tolerance test. Data are conveyed as the mean ± SD (n = 5−8/group). *p < 0.05, **p < 0.01, and ****p < 0.0001.

3. DISCUSSION

Along with the epidemiological tendency of obesity and correlated metabolic syndrome on Earth, the attack rate of NAFLD in China has been growing with each passing year and has proved to be one of the most usual liver diseases.[28] Obesity is strictly associated with NAFLD, but the distribution of excess bodily fat storage may be even more important. In particular, visceral adipose tissue is the most important factor for the development of hepatic steatosis.[29] However, the etiopathogenesis of NAFLD has not been entirely clarified. Patients with NAFLD have impaired lipid metabolism in hepatocytes, leading to lipid deposition in hepatocytes, which results in intrahepatic fatty degeneration and oxidative damage, followed by hepatocellular inflammation and insulin resistance, eventually causing disease progression of liver fibrosis and cirrhosis.[30,31] In NAFLD, liver absorption and fat production were added, which increased due to the fact that the compensation of fatty acid oxidation is not sufficient to normalize fat levels and possibly contributes to cellular damage and progressive disease by eliciting oxidative stress.[32]

Clstn3 is a single-channel transmembrane protein in the cadherin superfamily located on synaptic membranes where interactive α-neurexin motivates excitatory and inhibitory synaptic development in neurons[14,15] and can go through calcium-mediated postsynaptic signal transduction and neurotransmitter release, which can be instrumental in synaptic development.[13] Bai et al. found that Clstn3 provided an optimal approach to the modulation of lipolysis in adipose tissue, besides its fundamental role in the central nervous system.[17] However, functions of Clstn3 in the homeostasis of hepatic lipid metabolism and its effect on NAFLD have not been covered.Clstn3 is highly expressed in many cell systems such as neurons, kidney, intestine, liver, and eye.[33] However, we found a noticeable decrease in Clstn3 expression in hepatic tissues of high-fat, ob/ob, and db/db mice and these can be found in qRT-PCR assays, and second, in liver primary cells after OA&PA culture, we found a cellular membrane decrease in Clstn3 and mRNA expression. These data suggest a reasonable conjecture that Clstn3 is associated with disorders of hepatic lipid metabolism.

The liver is the main site of fatty acid metabolism, and fatty acids accumulate in the liver by way of ingestion from plasma and biosynthesis in hepatocytes and they are eliminated by oxidation in cells or secretion into the plasma of triglyceride-rich VLDL.[34] Birkenfeld and Shulman considered that NAFLD is marked by triglyceride and lipid overdeposition in the liver. It is bound up with obesity, type 2 diabetes, and dyslipidemia and generally takes place in the circumstances of insulin resistance,[35] while the increased flow of fatty acids from adipose tissue and diet to hepatocytes and increased neoadipogenesis (DNL) in hepatocytes are the main factors promoting hepatic TG synthesis.[36] Toxic lipid accumulation in the liver can further trigger major damage in hepatocyte injury and inflammation.[37] Fatty acid oxidation is mainly dominated by Pparα, and activation of Pparα stimulates transcription of many FAO-related genes in mitochondria, further decreasing hepatic lipid deposition.[38] In OA&PA-induced MPHs in wild-type, HFD, and db/db mice, we found that fatty acid oxidation was ameliorated by overexpression of Clstn3, which subsequently alleviated hepatic steatosis in NAFLD. According to our results, Clstn3 overexpression repaired glucose tolerance, pyruvate tolerance, and insulin sensitivity in wild-type, high-fat, and diabetic mice and effectively reduced TG content in cells and the liver. ALT and AST results showed amelioration of liver damage, while expression of gluconeogenesis-related genes G6pc and Pepck and adipogenesis-associated gene Srebp1c was preeminently suppressed. In contrast, Clstn3 silencing resulted in impaired fatty acid oxidation, increased TG content in the liver, increased liver injury, and damaged glucose tolerance, pyruvate tolerance, and insulin sensitivity. Pathological sections likewise showed a significant ameliorative effect of Clstn3 on hepatic lipid accumulation as well as lipid droplet deposition. It was found that hepatic fatty acid oxidation and ketone body synthesis may bring about the amelioration of hepatic steatosis as a disposal mechanism for hepatic free fatty acids.[39] These findings are consistent with the results in the present study.

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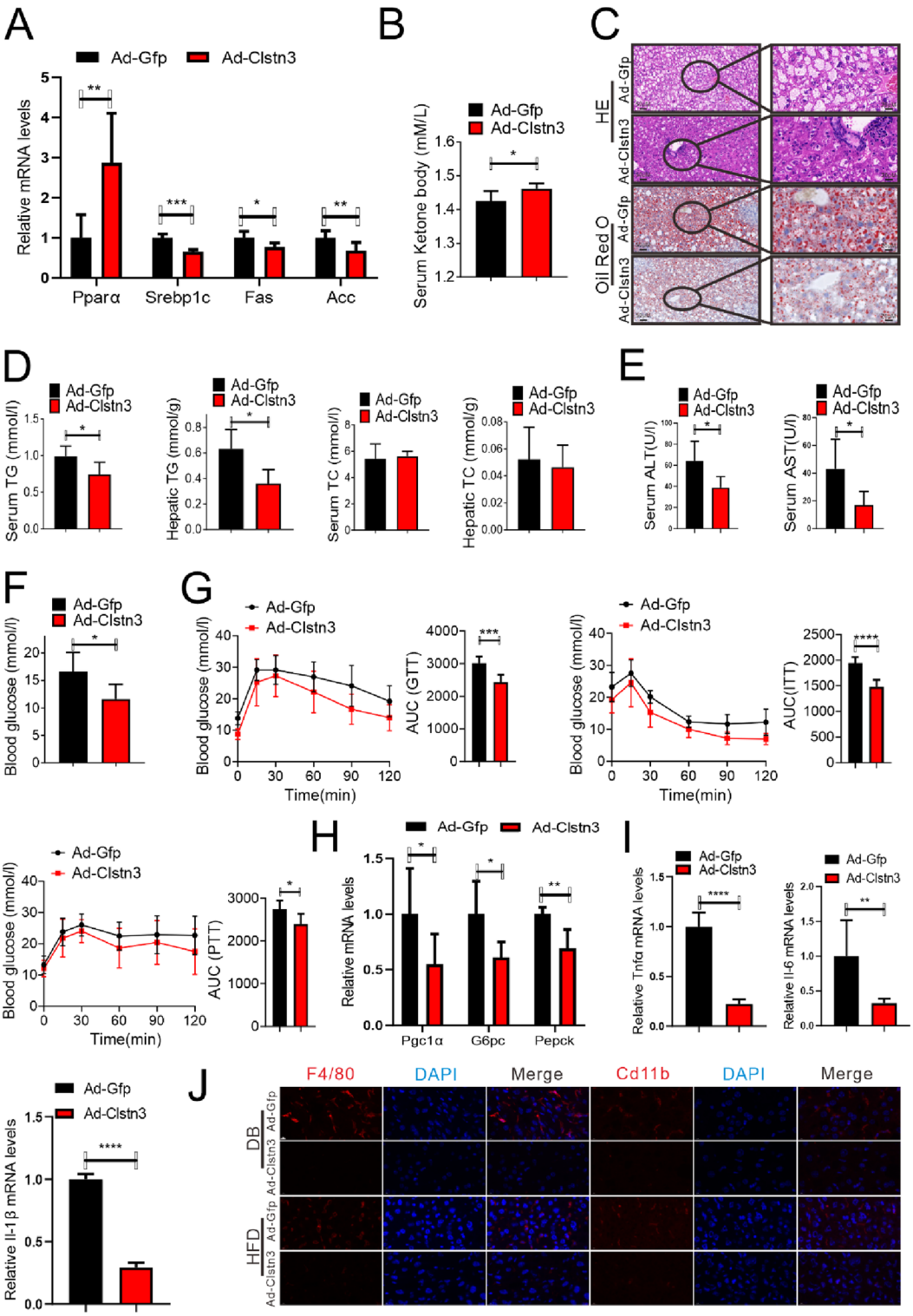

Figure 5. Clstn3 overexpression improves hepatic steatosis in db/db mice. (A) Hepatic lipid metabolism gene expression; (B) serum ketone body levels; (C) H&E and oil red O staining; (D) serum and liver TG and TC levels; (E) serum ALT and AST levels; (F) fasting glucose; (G) glucose tolerance test, insulin tolerance test, and pyruvate tolerance test; (H) gluconeogenesis gene expression; (I) inflammation gene expression; (J) immunofluorescence analysis of F4/80 and CD11b in the liver of db/db and HFD mice (1000×). Data are conveyed as the mean ± SD (n = 5−8/group). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Fat accumulation in adipose tissue and low energy expenditure may lead to obesity.[40] Meanwhile, Clstn3 has the physiological function of measuring energy homeostasis by increasing leptin sensitivity and elevating energy expenditure.[16] The same conclusion was obtained in the livers of the high-fat mouse model we constructed, and our study found that under HFD conditions, Clstn3-overexpressing mice had reduced body weight and increased VO_2, VCO_2, and thermogenesis as a way to promote energy expenditure and thus alleviate hepatic lipid accumulation in NAFLD.

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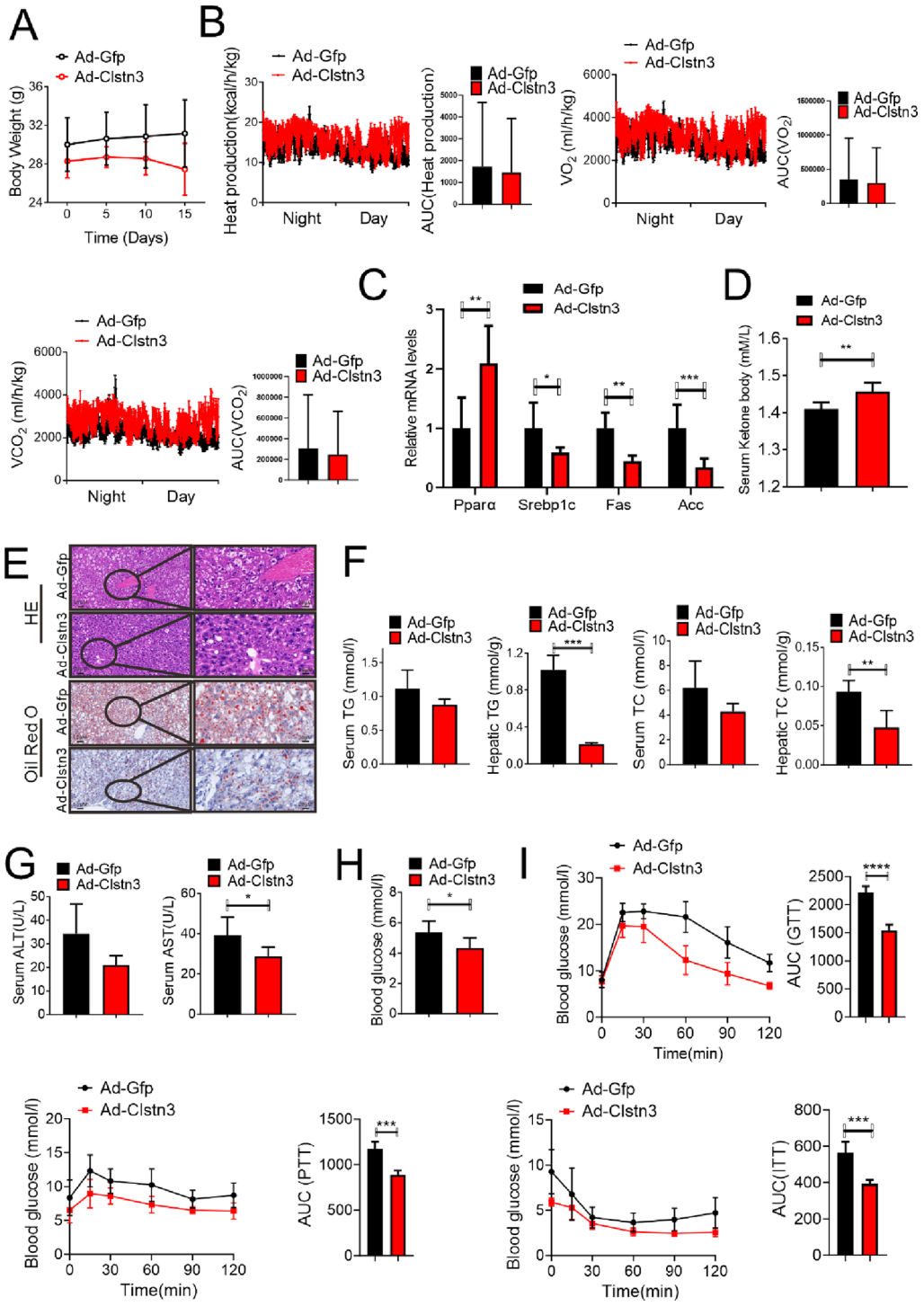

Figure 6. Clstn3 overexpression relieves hepatic steatosis in HFD mice. Mice were laid in the metabolic chamber for more than 24 h, and HFD mice contracted with Clstn3 or Gfp adenovirus were measured for (A) body weight and (B) heat production, oxygen consumption (VO_2), and carbon dioxide release (VCO_2) into the energy expenditure assessment. (C) Gene expression of lipid metabolism; (D) serum ketone body levels; (E) H&E and oil red O staining; (F) serum and liver TG and TC results; (G) serum ALT and AST results; (H) fasting glucose; (I) glucose tolerance test, pyruvate tolerance test, and insulin tolerance test. Data are conveyed as the mean ± SD ($n = 3$ in group B and $n = 5–8$ in other groups). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Clstn3 can act as an auxin and regulator of cellular vitamin C uptake to promote ascorbic acid to reduce oxidative stress and decrease inflammatory factor secretion in Alzheimer's disease.[18] Also, in our study, we speculate that Clstn3 may be involved in hepatic oxidative stress, as revealed by the fact that overexpression of Clstn3 reduces cellular ROS levels and elevates relative mRNA levels of hepatic Nrf2, Sod2, and Ho-1.

Some studies found that during obesity, sustained interactions between adipocytes and stromal cells, including macrophages, cause chronic inflammatory changes in adipose tissue, which further propagates to the liver and leads to insulin resistance,[41]

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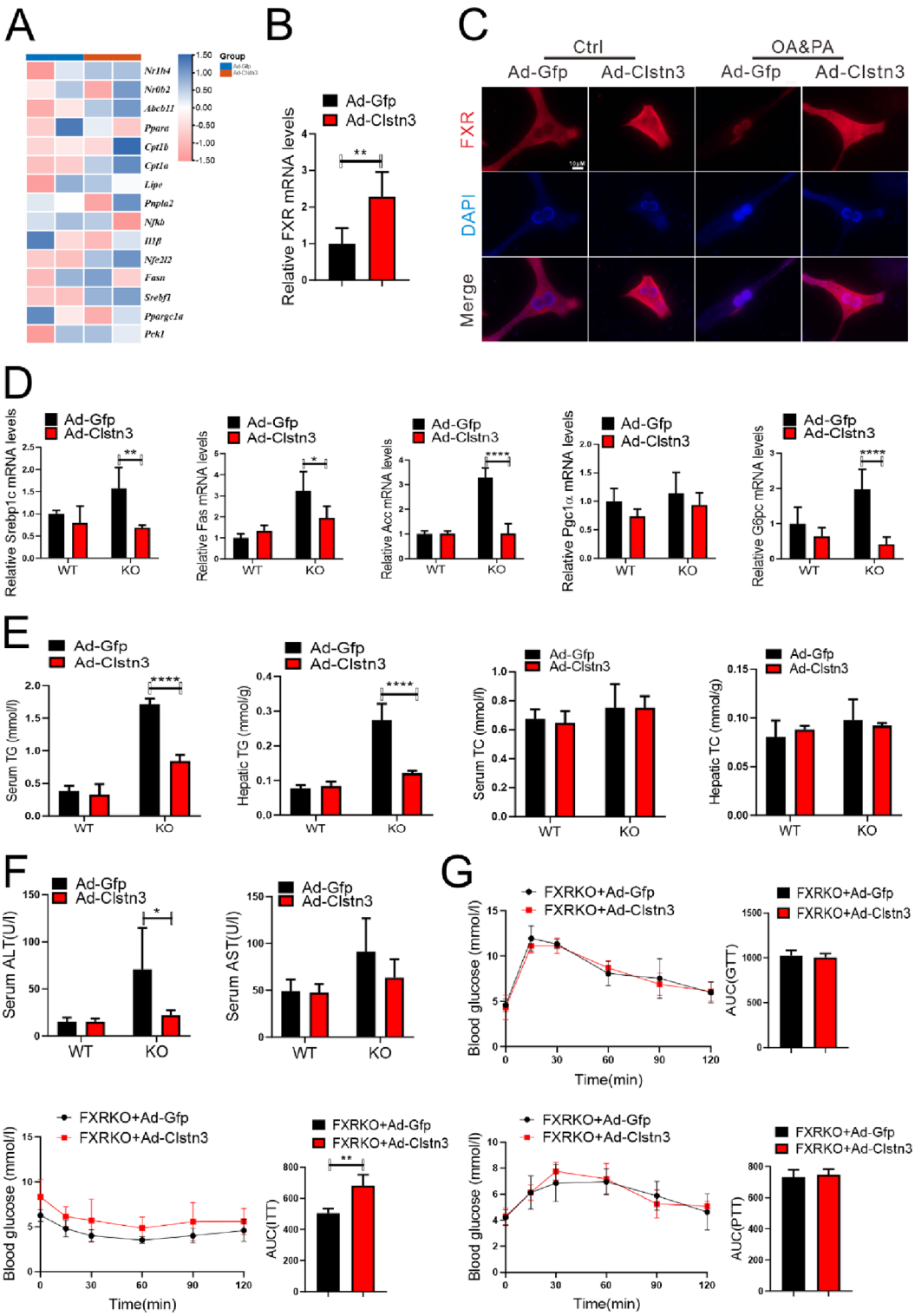

Figure 7. Clstn3 overexpression may promote the FXR signal pathway. (A) Heatmap of RNAseq results from HFD mice infected with Gfp or Clstn3 adenovirus; (B) expression of Clstn3 in vivo; (C) FXR immunofluorescence analysis (1000×); (D) mRNA expression of lipid metabolism and gluconeogenesis genes; (E) serum and liver TG and TC results; (F) serum ALT and AST results; (G) glucose tolerance test, insulin tolerance test, and pyruvate tolerance test. Data are conveyed as the mean ± SD ($n = 2$ in group A, $n = 4$ in group G, and $n = 5–8$ in other groups). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

and we found that Clstn3 suppressed the inflammatory response in the liver, as reflected by the fact that overexpression of Clstn3 alleviated the OA&PA-induced Nfκb-p65 translocation from the cytoplasm to the nucleus, while Tnfα, IL-6, and Il-1β gene expression was debased in livers of NAFLD model mice. This hypothesis was further tested by immunofluorescence. It turned out that Clstn3 probably develops an anti-inflammatory influence by suppressing the expression of inflammation factors in these ways.

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As shown in a large number of studies, the activated bile acid receptor FXR regulates cholesterol, glucose, and lipid homeostasis, thereby slowing the disease progression of NAFLD, while FXR knockout has the opposite effect.[22,42] Insufficient FXR activation leads to a disturbance in bile acid metabolism, resulting in decreased energy expenditure, increased adipogenesis, and increased macrophage activity,[43] by regulating FXR and, thus, glucolipid metabolism, which is a prominent target for the prophylaxis and cure of NAFLD.[44,45] So, we examined this key receptor, and as predicted, FXR mRNA levels and its downstream targets BSEP and SHP were significantly upregulated upon overexpression of Clstn3. Immunofluorescence further showed a correlation between Clstn3 and FXR expression. To further explore the relationship between FXR and Clstn3, we established a mouse model of overexpression of Clstn3 in FXRKO mice, and we found that Clstn3 alleviated glucose tolerance, pyruvate tolerance, and insulin sensitivity caused by FXR knockdown and ameliorated liver damage owing to steatosis and triglyceride accumulation in the liver of FXRKO mice. Relative mRNA assays showed that Clstn3 ameliorated gluconeogenesis, oxidative stress, and inflammatory factor production due to FXR deficiency. Also, in the future, the CLSTN3-FXR pathway may be a prospective target for NAFLD treatment. However, we need more evidence before we reach the conclusion that the activation of FXR by Clstn3 directly ameliorates the disorders of lipid metabolism in NAFLD.

4. CONCLUSIONS

Our study showed that Clstn3 can ameliorate liver lipid metabolism disorders in NAFLD, and Clstn3 may take precautions against NAFLD-induced hepatic steatosis and improve oxidative stress and inflammation by activating the FXR signal pathway and reducing damage formation associated with lipid metabolism and fatty acid oxidation. Also, in the future, the Clstn3-FXR pathway may be a prospective target for NAFLD treatment.

5. MATERIALS AND METHODS

5.1. Animals. We purchased male C57BL/6J mice aged 6 to 8 weeks from Guangzhou University of Chinese Medicine's Experimental Animal Center (CertificateSCXK 2018-0034; Guangzhou, China). Male db/db mice aged 8 weeks were purchased from Guangdong Yaokang Biotechnology Co. (CertificateSYXK 2020-0054; Guangzhou, China). Male ob/ob mice aged 6−8 weeks were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). A set of knockout mice for FXR (FXRKO) was sponsored by Changhui Liu and purchased from the Jackson Laboratory (Bar Harbor, ME, USA).[46]

To establish the NAFLD model, male C57BL/6J mice were fed a high-fat diet (Rodent Diet with 60 kcal% fat) ad libitum for 12 weeks without water restriction. HFD mice were then randomly divided into two groups (n = 6 for each group) to be injected with Ad-Clstn3 and Ad-Gfp adenoviruses for liver-specific overexpression. Male db/db mice were stochastically divided into two groups (n = 6 for each group) and given adenoviruses Ad-Clstn3 and Ad-Gfp by tail vein. For the Clstn3 overexpression model, C57BL/6J wild-type mice were randomly divided into two groups (n = 6 for each group), and Ad-Clstn3 and Ad-Gfp adenoviruses were then injected from the tail vein for overexpression. For the Clstn3 knockdown model, C57BL/6J wild-type mice were randomly divided into two groups (n = 6 for each group), and ShClstn3 and ShGfp adenoviruses were then injected from the tail vein for silencing.

Mice were injected with 10^{11} PFU of Clstn3-expressing adenoviruses to overexpress Clstn3 and 10^{9} PFU of Clstn3-silenting adenoviruses in the liver (OBiO, Shanghai, China). After a week of infection, experiments were conducted. There was no restriction on diet or water for the mice, and mice were maintained on a 12 h cycle (light/dark) with adequate food and water for the duration of the experiment. All animal care and experiments are conducted in accordance with the guidelines approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (20230227011).

5.2. Cell Culture. In accordance with previous reports, primary hepatocytes were cultured from C57BL/6J mice in RPMI-1640 medium.[47] MPHs were infected with Ad-Clstn3 and Ad-Gfp adenoviruses for 24 h, then replaced with fresh medium containing 10 mM oleic acid and 20 mM palmitic acid (OA&PA), and incubated for another 24 h. Cells were then collected for further analysis.

5.3. Measurement of Energy Consumption. Mice were acclimatized to the metabolic chamber for 24 h prior to measurements of metabolic parameters. Body weight was then monitored for 15 days in the animal monitoring system, and oxygen consumption (VO_2), carbon dioxide production, (VCO_2) and heat production were monitored for 24 h.

5.4. Tolerance Test. Mice were injected intraperitoneally (i.p.) with D-glucose (1−2 g/kg) or sodium pyruvate (1−1.5 g/kg) after 16 h of starvation. After 6 h of starvation, insulin (0.5−0.75 U/kg) was injected into mice to determine their tolerance. Blood glucose was surveyed by the caudal vein at 0, 15, 30, 45, 60, 90, and 120 min using a glucose monitor (OneTouch Ultra; On Call EZIV, China).

5.5. Chemical Reagents. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), and total cholesterol levels were measured in serum and cell samples using kits purchased from Nanjing Jiancheng Bioengineering Institute. Serum ketone bodies were measured by ELISA (RUIXIN Co. Fujian, China). The BCA test kit was produced by Beijing Biyuntian Technology Co., Ltd. (Beijing, China).

5.6. ROS Measurement. MPHs were infected with Ad-Gfp and Ad-Clstn3 for 24 h, OA&PA was added in MPHs and incubated for another 24 h, and then 10 μM DCFH-DA (10 mM) was put in and incubated again for half an hour at 37 °C under low light. At last, cells were flushed three times with PBS and the fluorescence emission was observed under a fluorescence microscope to analyze intracellular ROS. For liver ROS measurement, HFD mice were injected to overexpress Ad-Clstn3 and Ad-Gfp viruses through tail vein. Seven days later, DHE (10 mM, 10 μL) was injected by the tail vein for 40 min, and the liver was taken. The liver tissue was fixed with 4% paraformaldehyde solution, further sectioned at 4 μm, and embedded in paraffin. Then, we visualized that under a fluorescence microscope (Nikon 400×).

5.7. Histological Detection and Immunofluorescence Assay. Hepatic tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and separated into parts, which was then used to test liver injury staining with hematoxylin and eosin (H&E). In oil red O staining, hepatic tissue was frozen at optimum cutting temperature, cut into slices, and dyed with oil red O (Sigma) to assess liver lipid droplets.

Liver slices were sealed with BSA (Abclonal, Wuhan, China) for 30 min at ambient temperature and incubated overnight at 4

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°C using F4/80 primary antibody and CD11b (Affinity, USA). These tissues were incubated with a secondary antibody conjugated to goat antiserum (Abclonal, Wuhan, China) for 40 min at ambient temperature and away from light. Last, tissues were transfected with DAPI (Abclonal, Wuhan, China). As for immunofluorescence staining, cells were inoculated on glass and disposed of adenovirus for 24 h or received no treatment and OA&PA for 24 h. Cells were flushed with PBS, then fixed with 4% paraformaldehyde for 30 min at ambient temperature, fixed with BSA closure, incubated with primary antibody Clstn3 (Abclonal, Wuhan, China), Nfκb-p65 (Abclonal, Wuhan, China), and NR1H4 (Proteintech, 25,055-1-AP) as described above, then incubated with secondary antibody (Abclonal, Wuhan, China) for 40 min at ambient temperature, and kept in a dark place. After flushing five times with PBS, images under a fluorescence microscope were obtained (Nikon 1000×).

5.8. Quantitative PCR (qRT-PCR). mRNA was isolated from cells and liver tissues by TRIzol reagent. To obtain better results, we used a high-throughput cDNA reverse transcription kit (Abclonal, Wuhan, China) for reverse transcription. cDNA qPCR analysis was performed using PowerUp SYBR Green Master Mix (Abclonal, Wuhan, China). β-Actin was used to normalize all genes and specific primer sequences as indicated in Table S1.

5.9. Statistical Analysis. All results are conveyed as the mean ± SD. Statistical analysis was performed with GraphPad Prism software (version 8.0). Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess statistical differences between groups. Also, post hoc Tukey's test was performed on the results after comparing multiple groups. $p < 0.05$ was regarded as significant.

■ ASSOCIATED CONTENT

Data Availability Statement

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02347.

Table S1: information on primers for gene amplification; Figure S1: Clstn3 overexpression relieving hepatic steatosis via oxidative stress and fatty acid oxidation in MPHs and Clstn3 knockdown aggravating hepatic steatosis in HFD mice; Figure S2: Clstn3 overexpression attenuating hepatic steatosis in db/db and HFD mice; Figure S3: Clstn3 overexpression that may alleviate liver steatosis caused by FXRKO mice (PDF)

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Author Contributions

All authors participated in the design of the experiments and implementation of data collection and analysis. J.G., S.H., and Q.Y. wrote the draft paper. G.N., Y.G., and L.W. contributed to the study design and revised the paper.

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Notes

The authors declare no competing financial interest.

This study was approved by the Experimental Animal Ethics Committee of Guangzhou University of Traditional Chinese Medicine (20230227011).

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■ ABBREVIATIONS

NAFLD, non-alcoholic fatty liver disease; MPHs, mouse primary hepatocytes; Pparα, peroxisome proliferator-activated receptor-α; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; Ho-1, heme oxygenase 1; Sod2, superoxide dismutase 2; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglycerides; TNFα, tumor necrosis factor α; Il-1β, interleukin 1β; Il-6, interleukin 6; Nfκb, nuclear factor κB; Pgc1α, peroxisome proliferator-activated receptor γ coactivator-1α; Pepck, phosphoenolpyruvate carboxykinase; G6pc, glucose-6-phosphatase; Srebp1c, sterol regulatory element binding protein-1c; Fas, fatty acid synthase; Acc, acetyl-CoA carboxylase

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