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RESEARCH PAPER

DJ-1 Deficiency Alleviates Steatosis in Cultured Hepatocytes

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Abstract Imbalance in lipid metabolism within hepatocytes can lead to hepatosteatosis, which is a cause of numerous hepatic dysfunctions. Previous studies have demonstrated the roles of DJ-1 in Parkinson's disease, diet-induced oxidative stress, obesity, and diabetes. Although recent studies have shown that DJ-1 is involved in metabolic complications, the roles of DJ-1 in steatosis are largely unknown. Therefore, the aim of the current study was to elucidate the potential roles of DJ-1 in hepatosteatosis *in vitro*. Normal rat liver cells Clone 9 (C9) were treated with 1 mM oleic acid (OA) for 24 h for establishment of a steatosis model, after which various biochemical parameters, including triglyceride, total cholesterol, and free fatty acid contents, were determined after knockdown with *Dj1*-specific siRNA. Silencing of *Dj1* prevented hepatic steatosis by suppressing expression of hepatic lipogenic markers, which was confirmed by immunoblotting and real-time PCR analysis. Up-regulation of mitochondrial and β-oxidation-associated proteins showed potential to reduce fat accumulation in the liver. Silencing of *Dj1* resulted in alleviation of steatosis by reducing lipogenesis and improving mitochondrial biogenesis. Further *in vivo* studies will be required to understand the molecular mechanism behind the role of DJ-1 in steatosis.

Keywords: C9, DJ-1, lipolysis, mitochondria, steatosis

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1. Introduction

An imbalance in lipid metabolism along with abnormal accumulation of lipids is known as steatosis, which is associated with various metabolic complications such as adiposity, dyslipidemia, insulin resistance, and hepatocarcinoma [1,2]. Non-alcoholic fatty liver disease (NAFLD) causes hepatic manifestation of metabolic syndrome and insulin resistance [3,4]. A high proportion of the Western population is stricken by NAFLD at an estimated rate of 15 ~ 20%, and this number is increasing rapidly [5,6]. Obesity is also a cause of steatosis, and the incidence of steatosis in patients with obesity is about 75% [7]. It was reported that uncontrolled steatosis increases the risk of hepatocellular carcinoma [8,9]. However, several studies have been carried out to address this issue using different approaches such as gene silencing [10,11], microRNA silencing [12,13], and use of inhibitors [14,15].

Parkinson's disease (PD)-associated protein DJ-1 is expressed in multiple tissues and is a highly conserved protein in mammals [16]. A growing body of evidence has indicated that DJ-1 is an antioxidant protein that protects against oxidative stress and cell death [17,18]. In our previous studies, we showed that DJ-1 plays potential roles in diet-induced oxidative stress [19] and STZ-induced diabetes [20]. DJ-1 is also known to participate in the regulation and protection of pancreatic β-cells both *in vitro* and *in vivo* [21]. Moreover, an increased level of DJ-1 has been observed in C57BL6/J male mice fed a high fat diet (HFD) [22], whereas a suppressed level of DJ-1 has been observed in the islets of elderly diabetic patients in a gender-dependent manner [23]. However, the physiological function of DJ-1 in metabolic syndrome, specifically steatosis, remains elusive. Therefore, the current study was designed to investigate whether silencing of hepatic *Dj1* can improve steatosis in cultured hepatocytes.

2. Materials and Methods

2.1. Cell culture

C9 cells (normal liver cell line derived from rats, ATCC®-CRL-1439™) were cultured in complete F12 medium (Gibco, Carlsbad, CA, USA). Cells were cultured in a cell culture dish and incubated at 37°C in a humidified chamber with 5% CO_2 for 24 h. Steatosis cell model was created by treatment with oleic acid, which is a monounsaturated fatty acid (MUFA) and serves as a source of non-arachidonic acid [24]. To this end, oleic acid was dissolved in ethanol and diluted in complete culture medium containing 0.01% (w/v) BSA to a final concentration of 1 mM. Fatty acids were added to the medium, which was sonicated at 40°C for 45 min, cooled to 4°C, and then sterilized by using a 0.22 μm filter. This homogeneous mixture was supplemented to cells for 24 h.

2.2. Silencing of *Dj1*

For silencing of *Dj1*, a commercially available Silencer® Select siRNA (Invitrogen, Carlsbad, CA, USA) and X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) were used. Post-confluent cells in a 6-well culture dish were washed twice with transfection medium and overlaid with a previously made mixture of siRNA and transfection reagent. The transfection process was carried out for 5 ~ 7 h depending on cell conditions, after which cells were maintained in complete medium for 24 h.

2.3. Quantitative real-time RT-PCR

To isolate total RNA from each group of cells, a total RNA isolation kit (RNA-spin, iNtRON Biotechnology, Seongnam, Korea) was used. Briefly, 1 μg of RNA was converted into cDNA using Maxime RT premix (iNtRON Biotechnology). Transcriptional levels of each gene were quantitatively analyzed by using FastStart Universal SYBR Green master (Rox), (Roche Diagnostics) with real-time RT-PCR (Stratagene 246 mx 3000p QPCR System, Agilent Technologies, Santa Clara, CA, USA). Primers used in this study are presented in Table 1.

Table 1. Sequences of primers for real-time RT-PCR used in this study

Genes	Orientation	Primer sequence (5' → 3')
Acaca	Forward	TGCAGGTATCCCCACTCTTC
	Reverse	TTCTGATTCCCTTCCCTCCT
Cebpb	Forward	CAAGCTGAGCGACGAGTACA
	Reverse	CAGCTGCTCCACCTTCTTCT
Cox8b	Forward	ACATTCAGGGTGCCTCTTTG
	Reverse	CATGGCAGAAGTGGGAGATT
Cpt1b	Forward	GCAAACTGGACCGAGAAGAG
	Reverse	CCTTGAAGAAGCGACCTTTG
Cycs	Forward	TCAATGATGCTGCCTTTCAC
	Reverse	ACTCCCAATCAGGCATGAAC
Dj1	Forward	GCTCACGAAGTAGGCTTTGG
	Reverse	AGGACTTTTCTTCCCCCAGA
Fabp4	Forward	AGA AGT GGG AGT TGG CTT CG
	Reverse	ACT CTC TGA CCG GAT GAC GA
Hsl	Forward	TCCCTGTACCACAGCAATCA
	Reverse	AGCTGGAGGTGGTTCTGCT
L-Acbp	Forward	TGTGGAAAAGGTAGAAGAGC
	Reverse	AAGGAAGGAGGAGCAGTAAT
L-Fabp	Forward	AAACTCACCATCACCTAT
	Reverse	TTGTCACCCTCCATCTTA
Lpl	Forward	TATGGCACAGTGGCTGAAAG
	Reverse	CTGACCAGCGGAAGTAGGAG
Ppara	Forward	TCACACAATGCAATCCGTTT
	Reverse	GGCCTTGACCTTGTTCATGT
Prkaa1	Forward	ATCCAAGAGCCGAGTTGCTC
	Reverse	GTCCGTTCTATGCGCTGGAT
Srebpf1	Forward	CATGGACGAGCTACCCTTCG
	Reverse	CTGTCTCACCCCCAGCATAG
β-Actin	Forward	AGCCATGTACGTAGCCATCC
	Reverse	CTCTCAGCTGTGGTGGTGAA

2.4. Immunoblot analysis

Cell lysates were prepared with RIPA buffer (Sigma-Aldrich), vigorously vortexed, and centrifuged at 12,000 × *g* for 15 min. Lysates were mixed with 2X sample buffer (50 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) and heated for 5 min at 95°C, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an 8, 10, or 12% (w/v) polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics) and then blocked for 1 h in 5% skim milk or 5% bovine serum albumin (BSA) prepared in TBS-T (Tris-buffered saline with Tween-20) buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membrane was rinsed three times consecutively with TBS-T buffer, followed by incubation for 1 h or overnight with 1:1,000 dilutions of primary monoclonal anti-β-actin, polyclonal anti-AMPK, anti-CPT1C, anti-C/EBPβ, anti-DJ-1, anti-FAS, anti-FABP, anti-HSL, anti-LPL, anti-pAMPK, anti-PPARγ, anti-PGC-1α, anti-SREBP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-COX 4, and anti-HSP90 (Cell Signaling Technology, Beverly, MA, USA) antibodies in TBS-T buffer containing 1% skim milk. After three washes, the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG secondary antibody (1:1000, AbFrontier, Seoul, Korea) in TBS-T buffer containing 1% skim milk. Membranes were then developed using enhanced chemiluminescence (Westzol, iNtRON Biotechnology). Chemiluminescence signal detection

was performed using the FUSION SOLO chemiluminescence and fluorescence imaging system (Vilber-Lourmat, Eberhardzell, Germany), and data were analyzed using Kodak image analysis software (KODAK 1D, Estman Kodak, Rochester, NY, USA) with normalization using β-actin.

2.5. Immunofluorescence analysis

For immunofluorescence, cells were cultured on sterile coverslips placed on a 6-well plate following the culture protocol described in the cell culture section. Cells were fixed with 4% *p*-formaldehyde in PBS of pH 7.4 for 20 min at room temperature. Cells were permeabilized by incubation with PBS containing 0.25% Triton X-100 for 10 min. Cells were then washed with PBS three times, blocked with 5% BSA for 1 h, incubated with polyclonal anti-DJ-1 antibody (1:200 dilution) (Santa Cruz Biotechnology) overnight at 4°C, washed three times with PBS, and incubated again with rhodamine-conjugated anti-rabbit secondary antibody (1:1,000) for 1 h. Cells were counterstained with DAPI (Sigma-Aldrich) and mounted using commercially available mounting medium (Dako North America Inc., Carpinteria, CA, USA). Fluorescence images were captured using a confocal laser scanning microscope LSM700 (Carl Zeiss, Oberkochen, Germany). Analysis of images was performed by ZEN 2009 Light Edition (Carl Zeiss).

2.6. Oil Red O staining

Control (CON), oleic acid-treated (OA), and knockdown cells supplemented with OA (KD+OA) were cultured for 24 h, followed by washing with PBS, fixation with 10% formalin for 1 h at room temperature, and washing three times with deionized water. A mixture of Oil Red O solution (0.6% Oil Red O dye in isopropanol) and water at a 6:4 ratio was filtered through a syringe filter and layered on cells for 20 min, followed by washing four times with deionized water. Images were captured using an Olympus IX51 inverted microscope (Olympus Co., Tokyo, Japan).

2.7. Biochemical parameters

Cultured cells were washed twice with PBS and harvested in order to prepare cell lysate using RIPA buffer (Sigma-Aldrich). TG content was measured according to the manufacturer's instructions using a TG test kit (Asan Pharm. Co., Yeongcheon, Korea). TG content was normalized to protein content as determined by the Bradford method. Similarly, cells were harvested to determine total cholesterol and free fatty acid contents. Both assays were performed according to the manufacturer's instructions by using a Cholesterol/cholesteryl Ester Quantitation kit (BioVision Inc., Mountain View, CA, USA) and Free Fatty Acid Quantification kit (BioVision Inc.), where absorbance was measured at 550 nm.

2.8. Statistical analysis

Experimental results are presented as mean ± S.D. Student's *t*-test was used to compare significance between control (CON) and OA-treated cells (OA) as well as significance between OA-treated control (OA) and knockdown cells treated with OA (KD+OA).

3. Results

3.1. Knockdown of *Dj1*

Gene-specific knockdown of *Dj1* was carried out using a commercially available siRNA transfection system. Knockdown efficiency was measured by immunoblotting, real-time PCR, and immunofluorescence analysis based on expression levels of DJ-1 in hepatocytes. Knockdown resulted in around 77 and 74% reduction of DJ-1 expression at both the mRNA and protein levels, respectively (Fig. 1).

3.2. Biochemical parameters

We investigated whether DJ-1 regulates OA-induced lipid accumulation in hepatocytes *in vitro*. We observed increased lipid droplet accumulation in OA-treated group, whereas knockdown of *Dj1* significantly reduced accumulation of oil droplets in hepatocytes (Fig. 2A). Silencing of *Dj1* markedly reduced hepatic triglyceride (27%), total cholesterol (30%), and free fatty acid contents (18%) as compared to OA-treated control cells (Fig. 2B).

3.3. Silencing of *Dj1* regulates lipid metabolism

As an essential enzyme for cellular energy metabolism, we confirmed reduced expression of AMPK and its encoding gene as well as elevated expression of pAMPK by immunoblotting and real-time PCR (Figs. 3 and 4). As SREBP1, C/EBPβ, PPARγ, and HSP90 are key regulators of the progression of hepatic steatosis, we examined their expression patterns by immunoblot analysis and/or real-time PCR analysis. After silencing of *Dj1*, we observed markedly reduced expression of these mediators as compared to OA-treated control cells (Figs. 3 and 4). In addition, we investigated expression patterns of key lipogenic and lipolytic markers at both the protein (Fig. 3) and mRNA levels (Fig. 4). We observed elevated levels of lipogenic markers after treatment with OA, and their levels were remarkably reduced after silencing of *Dj1*.

3.4. Silencing of *Dj1* enhances mitochondrial biogenesis

As β-oxidation is initiated in mitochondria, we evaluated whether silencing of *Dj1* resulted in increased mitochondrial biogenesis. As shown in Fig. 5, most mitochondria-specific markers were significantly elevated at both the protein and mRNA levels after knockdown of *Dj1*. Hence, knockdown

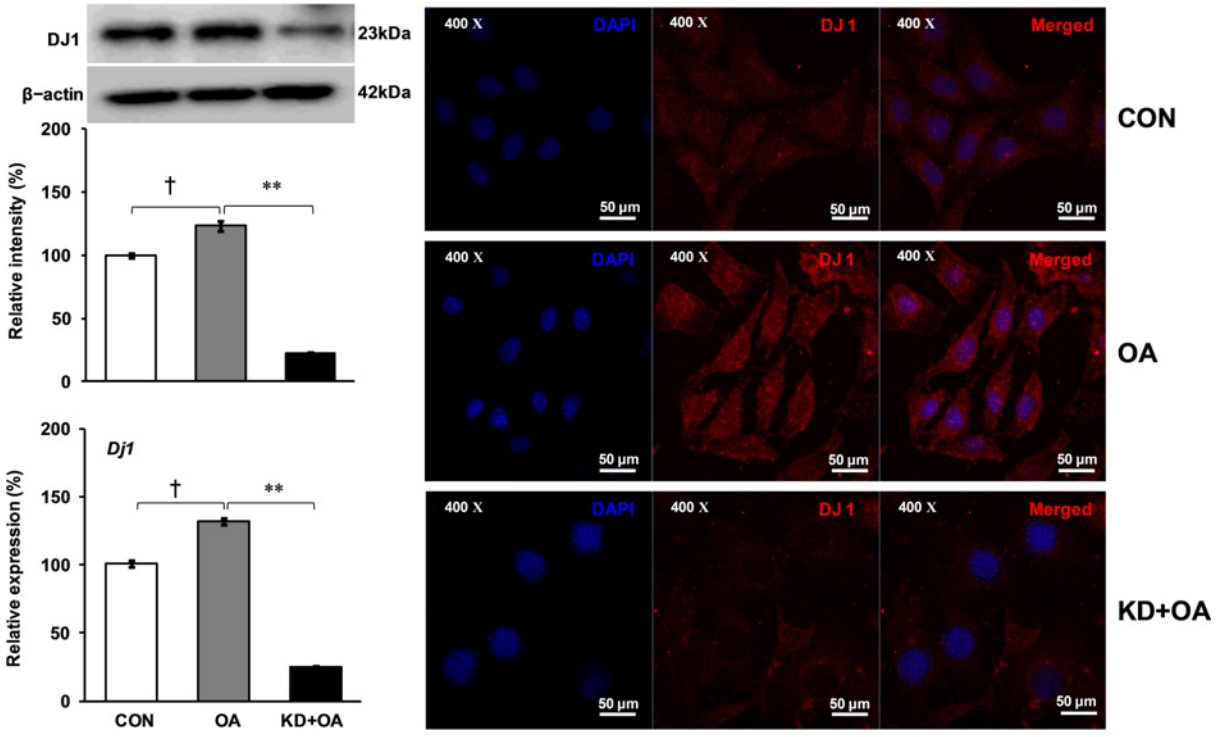

Fig. 1. Expression of DJ-1 after silencing of *Dj1* at protein and mRNA levels as well as confocal microscopic observation in C9 cells. Band densities were calculated using ImageMaster 2D software version 4.95, and relative intensity (%) values of protein and mRNA were normalized to β-actin. Statistical significance between each group was calculated by Student's *t*-test. Significance between control and oleic acid (OA)-treated cells was set at either $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. Significance between OA-treated control and OA-treated knockdown cells was set at either $^{*}p < 0.05$ or $^{**}p < 0.01$. Scale bars = 50 μm, magnification = 400 X.

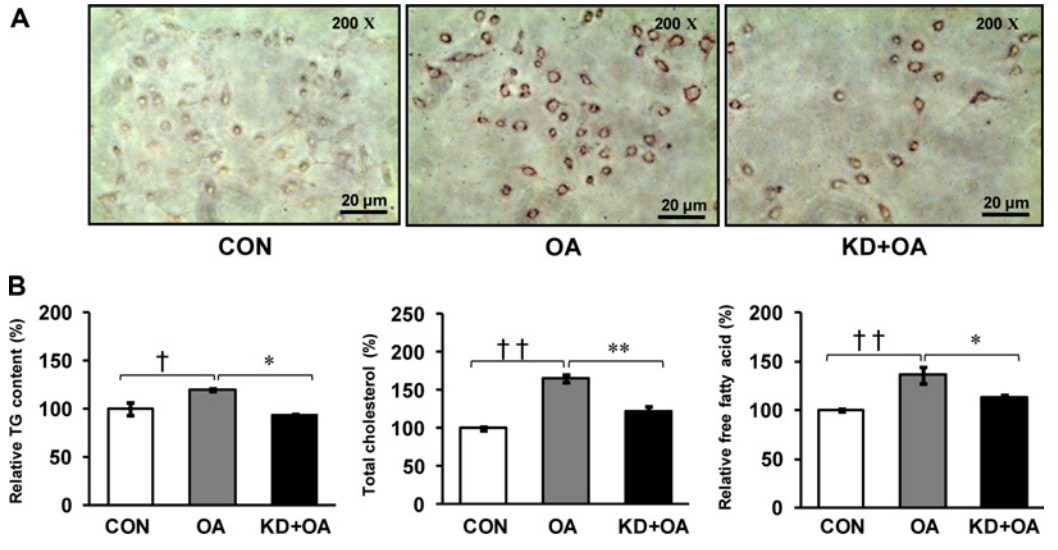

Fig. 2. Accumulation of lipid droplets (A) as well as total cholesterol, triglyceride, and free fatty acid (B) contents measured in C9 cells before and after *Dj1* silencing. Statistical significance between each group was calculated by Student's *t*-test. Significance between control and oleic acid (OA)-treated cells was set at either $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. Significance between OA-treated control and OA-treated knockdown cells was set at either $^{*}p < 0.05$ or $^{**}p < 0.01$. Scale bars = 20 μm, magnification = 200 X.

of *Dj1* improved mitochondrial biogenesis to enhance fatty acid catabolism, thereby alleviating steatosis. Fig. 6 depicts the proposed mechanism underlying DJ-1-dependent steatosis in hepatocytes.

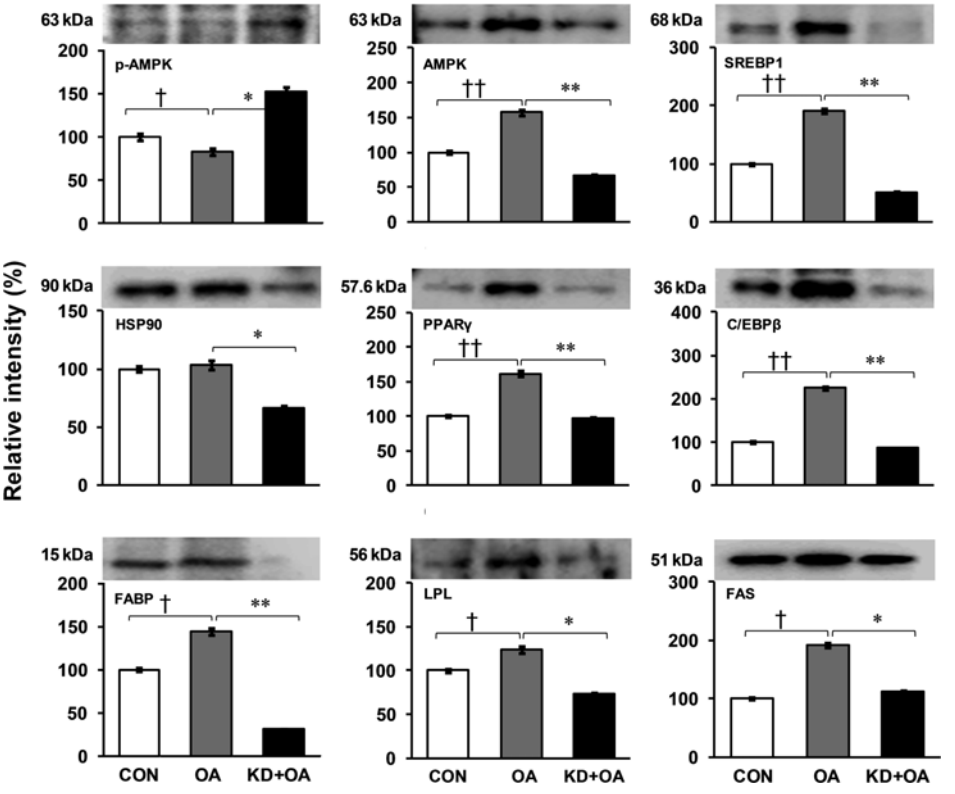

Fig. 3. Knockdown of *Dj1* down-regulates steatotic markers at the protein level. Band densities were calculated using ImageMaster 2D software version 4.95, and relative intensity (%) values of protein were normalized to β-actin. Statistical significance between each group was calculated by Student's *t*-test. Significance between control and oleic acid (OA)-treated cells was set at either $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. Significance between OA-treated control and OA-treated knockdown cells was set at either $^{*}p < 0.05$ or $^{**}p < 0.01$.

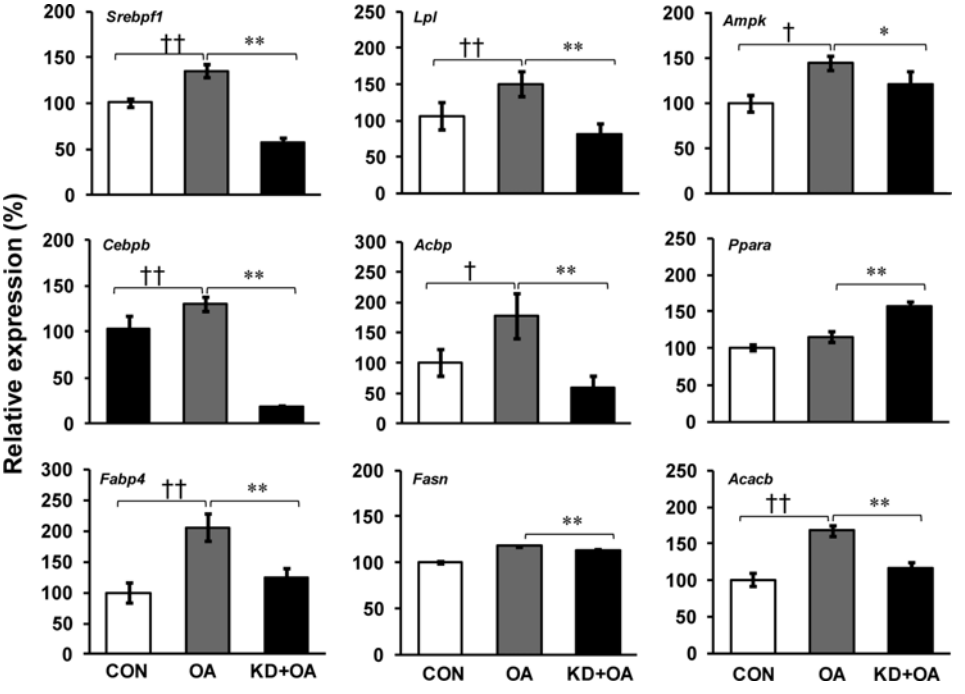

Fig. 4. Knockdown of *Dj1* down-regulates steatotic markers at the mRNA level. Relative expression (%) values of mRNA were normalized to *Actb*. Statistical significance between each group was calculated by Student's *t*-test. Significance between control and oleic acid (OA)-treated cells was set at either $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. Significance between OA-treated control and OA-treated knockdown cells was set at either $^{*}p < 0.05$ or $^{**}p < 0.01$.

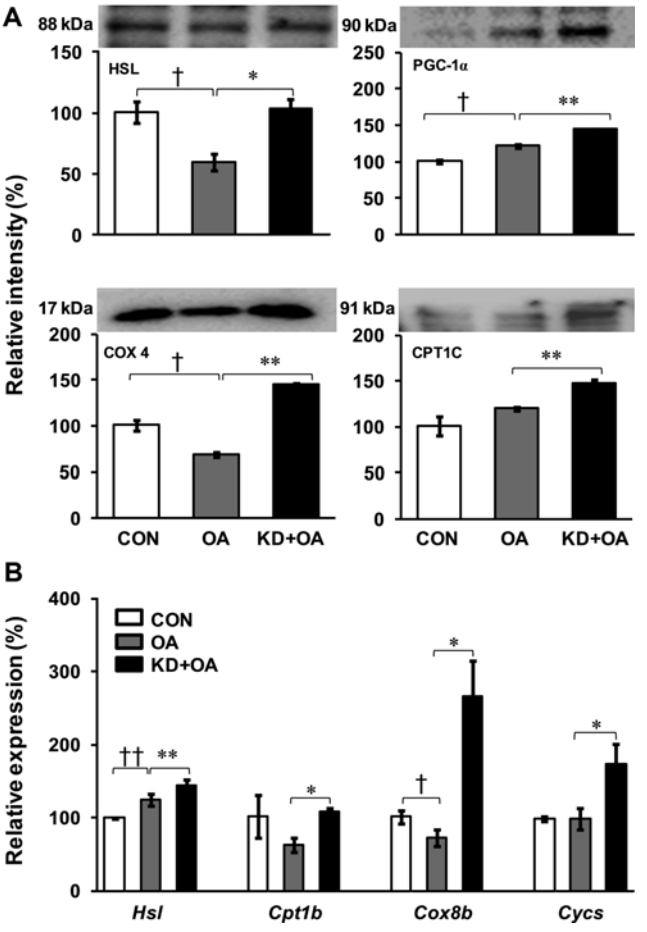

Fig. 5. Knockdown of *Dj1* regulates lipolytic and mitochondrial markers at the protein (A) and mRNA (B) levels. Relative expression values (%) of protein and mRNA were normalized to β-actin. Band densities were calculated using ImageMaster 2D software version 4.95, and relative intensity (%) values of protein were normalized to β-actin. Statistical significance between each group was calculated by Student's *t*-test. Significance between control and oleic acid (OA)-treated cells was set at either $^{\dagger}p < 0.05$ or $^{\dagger\dagger}p < 0.01$. Significance between OA-treated control and OA-treated knockdown cells was set at either $^{*}p < 0.05$ or $^{**}p < 0.01$.

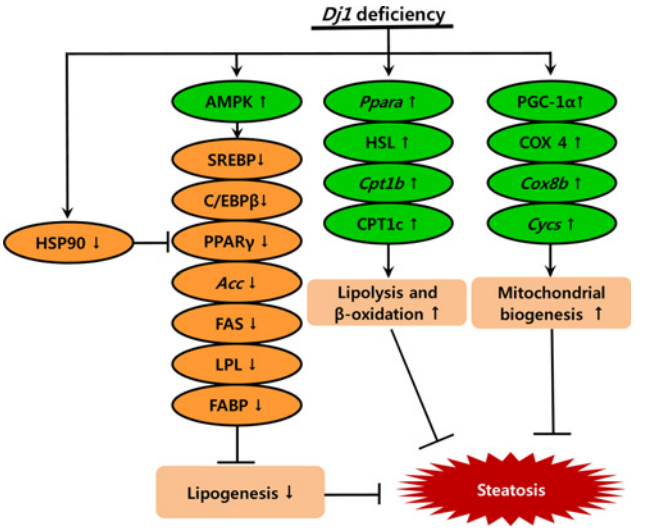

Fig. 6. Suggested molecular mechanism underlying DJ-1-dependent regulation of hepatosteatosis.

4. Discussion

In the present study, we evaluated for the first time the effect of DJ-1 and its potential mechanism underlying metabolic regulation in hepatosteatosis. We observed marked reduction of lipogenesis, enhanced β-oxidation, as well as improved mitochondrial biogenesis after silencing of *Dj1* in hepatocytes. Previous studies reported elevated levels of DJ-1 in adipose and liver tissues of diet-induced obese mice [22] and SD rats [19], which drove us to hypothesize that reduced DJ-1 expression may help to reduce lipid accumulation in hepatocytes. We observed that knockdown of *Dj1* markedly reduced accumulation of lipid droplets in hepatocytes. It has been documented that DJ-1 is associated with lipid rafts in astrocytes [25], and a recent report demonstrated marked reduction of lipid droplets in adipocytes after knockdown of *Dj1* [26]. However, to date, it remains to be determined whether DJ-1 regulates lipid homeostasis in the liver.

Accumulation of TGs in the liver is a common symptom of metabolic liver disease such as hepatic steatosis [27]. Similarly, dietary cholesterol and free fatty acids are important risk factors for progression to hepatic inflammation in diet-induced non-alcoholic steatohepatitis (NASH) [28]. In this study, we observed reduced accumulation of TGs, total cholesterol, and free fatty acids in OA-treated *Dj1* knockdown cells as compared to OA-treated control cells. In support of our findings, previous reports have demonstrated reduced levels of hepatic TGs, cholesterol, and free fatty acids in C57BL/6J mice for recovery from steatosis [2,28]. An imbalanced lipid profile may cause degenerative brain diseases [29], and deletion of Parkinson's disease-associated protein Parkin (Park2) can result in reduced hepatic TG and free fatty acid levels in mice [29]. Moreover, DJ-1 and Parkin are linked and maintain a parallel relationship in Parkinson's disease [30]. Taken together, DJ-1 also has the capacity to control accumulation of TGs, cholesterol, and free fatty acids in hepatocytes. Thus, our findings demonstrate the role of DJ-1 in OA-induced lipid homeostasis of hepatocytes.

We next determined the expression and phosphorylation levels of AMPK, which is an essential player in energy homeostasis with potential to control obesity [31]. Regarding the importance of AMPK as a metabolic master switch, it has been shown to regulate various intracellular signaling pathways, including cellular uptake of glucose and its transporters as well as β-oxidation and mitochondrial

biogenesis [32,33]. We observed down-regulation and phosphorylation of AMPK in DJ-1-deficient cells treated with OA, suggesting a potential role for DJ-1 in overcoming steatosis. Similar to our observations, it has been reported that phosphorylation of AMPK inhibits expression of SREBP, which helps to attenuate hepatic steatosis in mice and *in vitro* [34,35]. These results suggest that silencing of *Dj1* results in AMPK phosphorylation, which diminishes steatosis through regulation of SREBP.

We next speculated that knockdown of *Dj1* may alter hepatic lipid metabolism through down regulation of steatosis markers. Interestingly, we observed decreased expression of SREBP1, C/EBPβ, and PPARγ, indicating that DJ-1 may have restricted lipid accumulation by inhibiting expression of these marker proteins. SREBP1 is a member of the basic helix-loop-helix-leucine zipper family of transcription factors, which not only activates several lipogenic enzymes involved in hepatic fatty-acid synthesis [36] but also plays a crucial role in cholesterol synthesis and accumulation of TGs in the liver [37]. Further, SREBP1 regulates lipogenesis by using dietary carbohydrate sources [10]. In this study, we observed marked reduction of SREBP expression at both the mRNA and protein levels after silencing of *Dj1*. Consistent with our findings, earlier studies have reported enhanced expression of SREBP1 under steatotic conditions in human patients [38,39], and reduced expression of SREBP was observed *in vitro* and in SD rats after recovery from steatosis [40,41]. Hence, our results suggest that knockdown of *Dj1* inhibits the expression of SREBP that may further reduce the lipid content in OA-induced hepatocytes.

We next identified the effect of *Dj1* on the expression levels of C/EBPβ, PPARγ, and *Ppara* in relation to steatosis and fatty acid uptake. Recent evidences have implicated that reduced expression of C/EBPβ is a necessary event in abrogating steatosis. Earlier studies demonstrated altered expression of C/EBPβ *in vitro* and in the liver of transgenic mice during steatosis [42,43]. However, deletion of C/EBPβ prevented hepatic steatosis by controlling hepatic TG levels as well as altering PPARγ and PPARα expression levels in C57Bl/6J mice [44]. Our data suggest that reduced C/EBPβ expression after knockdown of *Dj1* is an important event to overcoming OA-induced steatosis.

Previous studies have indicated that PPARγ is an important player of fatty acid uptake and transport [45]. Interestingly, we detected reduced protein expression of PPARγ but increased mRNA expression of *Ppara*, which helps to reduce fatty acid uptake and ultimately prevent expression of lipogenic factors. In line with our observations, altered expression of PPARγ has been reported in the liver of morbidly obese women [46] and diet-induced male C57BL/6J mice [2]. On the other hand, it has been reported that suppression of PPARα causes hepatic steatosis [47]. It is obvious that deletion of PPARγ delays hepatic TG uptake and ultimately prevents steatosis in diet-induced transgenic obese mice [45]. An earlier report demonstrated that C/EBPβ regulates PPARγ and TGs to promote hepatic lipogenesis in mice [44]. Therefore, we suggest that the inverse relationship between PPARγ and *Ppara* after knockdown of *Dj1* may restore the protective ability of hepatocytes against OA-induced steatosis.

Next, we examined the effect of *Dj1* on the expression of heat shock protein 90 (HSP90) in OA-induced hepatocytes. HSP90 is an emerging therapeutic target in liver and metabolic diseases [48]. In this study, we observed that knockdown of *Dj1* subsequently reduced protein levels of HSP90, which is considered as an important modulator of steatosis. Recent reports have shown that inhibition of HSP90 blocks PPARγ signaling in primary hepatocytes [49] as well as reduces steatosis and macrophage activation in obese C57BL/6 mice [48]. Further, it has been previously documented that knockout of DJ-1 destabilizes HSP90 [50], thereby controlling PPARγ expression. Taken together, these results suggest a possible role for DJ-1 in alleviating steatosis in OA-induced hepatocytes.

Further, we investigated whether silencing of *Dj1* reduces expression levels of lipogenic markers in cultured hepatocytes. Previously, it was established that during the course of steatosis, stimulation of FAS, ACC, L-FABP, FABP4, and LPL expression reflects increased fatty acid synthesis [46,51,52]. In our study, silencing of *Dj1* led to reduced expression of lipogenic markers. In line with our findings, reduced levels of lipogenic markers were observed as compared to control steatosis *in vitro* [53] and SD rats [54]. In addition, an earlier study reported that DJ-1 deficiency led to reduction of adipogenesis and diet-induced inflammation in mice [26]. Collectively, these results imply that silencing of *Dj1* significantly contributes to reduced lipogenesis.

Since hepatic steatosis is caused by increased lipogenesis and decreased lipolysis, we hypothesize that silencing of *Dj1* is necessary to reduce hepatic fat deposition through hormone-sensitive lipase (HSL)-dependent lipolysis. HSL is a rate-limiting enzyme of intracellular TG metabolism as well as a major regulator of fatty acid mobilization in adipose tissue and other metabolic tissues [55]. Silencing of *Dj1* elevated expression of HSL at both the mRNA and protein levels, resulting in activation of lipolysis. Increased HSL levels may be responsible for transport of fatty acids to the liver, resulting in increased hepatic lipolysis and fatty acid oxidation. Thus, with respect to our observations, we conclude that deletion or silencing of *Dj1* may negatively

and positively regulate lipogenic and lipolytic markers, respectively. These results led us to hypothesize that such molecular events may result in reduction of fatty acid accumulation in OA-induced hepatocytes.

Another remarkable outcome of this study is that *Dj1* silencing increased expression of PGC-1α, a master regulator of mitochondrial biogenesis and energy metabolism. In general, elevated expression of PGC-1α is required for mitochondrial biogenesis and function [56,57]. Moreover, PGC-1α is involved in adaptation to fasting and gluconeogenesis, which is the *de novo* synthesis of glucose in the liver [58]. Parallel to our findings, other studies have reported that elevated levels of PGC-1α protect the liver from steatosis by increasing mitochondrial biogenesis [58,59]. It is well documented that DJ-1 deficiency leads to abnormal mitochondrial morphology and dynamics as well as reduced mitochondrial membrane potential in mouse brain and neuronal cell lines [18,60]. At the same time, these defects can be rescued by overexpression of Parkin (Park2) or PINK1 [61]. Therefore, we hypothesize that selective inhibition of DJ-1 may help to increase the mitochondrial number, thereby increasing mitochondrial biogenesis through an unknown mechanism in OA-induced hepatocytes. However, further detail studies will be required to prove this hypothesis.

Additionally, silencing of *Dj1* led to increased expression of some important mitochondrial marker genes and proteins (*Cpt1b,* CPT1C, COX 4, *Cox8b,* and *Cycs*). CPT1 is the key enzyme of the mitochondrial fatty acid β-oxidation pathway [62,63], and its dysregulation causes impaired mitochondrial biogenesis through COX 4 in leptine deficiency-mediated nonalcoholic steatohepatitis [64]. Collectively, enhanced expression of these mitochondrial markers reflects improved mitochondrial β-oxidation through silencing of *Dj1*, suggesting that DJ-1 has the capacity to control steatosis by minimizing lipogenesis and elevating β-oxidation.

5. Conclusion

Our data suggest that knockdown of *Dj1* can induce alleviation of steatosis by reducing lipogenesis and augmenting mitochondrial biogenesis. Hence, DJ-1 is a key player involved in metabolic homeostasis and pathophysiology of hepatosteatosis.

Author Contribution

HNC and JWY designed the experiments. HNC performed the experiments and analyzed the data. JWY wrote the paper and responsible for guiding the work.

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Nomenclature

ACBP/*Acbp* : Acyl-CoA-binding protein/-encoding gene
Acaca : Acetyl-CoA carboxylase-encoding gene
AMPK/*Ampk* : 5' adenosine monophosphate-activated protein kinase/-encoding gene
C/EBPβ/*Cebpb* : CCAAT/enhancer-binding protein beta/-encoding gene
Clone 9 : C9
Cpt1b : Carnitine *O*-palmitoyltransferase 1 isoform b-encoding gene
CPT : Carnitine O-palmitoyltransferase
CYCS/*Cycs* : Cytochrome complex/-encoding gene
COX 4 : Cytochrome oxidase subunit 4
Cox8b : Cytochrome oxidase subunit 8b-encoding gene
DJ-1/*Dj1* : Parkinson disease 7/-encoding gene
FABP : Fatty-acid-binding protein
FAS/*Fasn* : Fatty acid synthase/-encoding gene
HSL/*Hsl* : Hormone-sensitive lipase/-encoding gene
HSP90 : Heat shock protein 90
LPL/*Lpl* : Lipoprotein lipase/-encoding gene
PGC-1α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR/*Ppar* : Peroxisome proliferator-activated receptor/-encoding gene
SREBP1/*Srebpf1* : Sterol regulatory element-binding protein/-encoding gene

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