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ORIGINAL ARTICLE

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Mitochondrial fission in hepatocytes as a potential therapeutic target for nonalcoholic steatohepatitis

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Abstract

Aim: The mitochondria are highly plastic and dynamic organelles; mitochondrial dysfunction has been reported to play causative roles in diabetes, cardiovascular diseases, and nonalcoholic fatty liver disease (NAFLD). However, the relationship between mitochondrial fission and NAFLD pathogenesis remains unknown. We aimed to investigate whether alterations in mitochondrial fission could play a role in the progression of NAFLD.

Methods: Mice were fed a standard diet or choline-deficient, L-amino acid-defined (CDAA) diet with vehicle or mitochondrial division inhibitor-1.

Results: Substantial enhancement of mitochondrial fission in hepatocytes was triggered by 4 weeks of feeding and was associated with changes reflecting the early stage of human nonalcoholic steatohepatitis (NASH), steatotic change with liver inflammation, and hepatocyte ballooning. Excessive mitochondrial fission inhibition in hepatocytes and lipid metabolism dysregulation in adipose tissue attenuated liver inflammation and fibrogenesis but not steatosis and the systemic pathological changes in the early and chronic fibrotic NASH stages (4- and 12-week CDAA feeding). These beneficial changes due to the suppression of mitochondrial fission against the liver and systemic injuries were associated with decreased autophagic responses and endoplasmic reticulum stress in hepatocytes. Injuries to other liver cells, such as endothelial cells, Kupffer cells, and hepatic stellate cells, were also attenuated by the inhibition of mitochondrial fission in hepatocytes.

Conclusions: Taken together, these findings suggest that excessive mitochondrial fission in hepatocytes could play a causative role in NAFLD progression by liver inflammation and fibrogenesis through altered cell cross-talk. This study provides a potential therapeutic target for NAFLD.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; Atg5, autophagy-associated 5; α-SMA, α-smooth muscle actin; BEC, biliary epithelial cell; CDAA, choline-deficient, L-amino acid-defined diet; CK, cytokeratin; Col3a1, collagen 3a1; Col4a1, collagen 4a1; Cpt2, carnitine palmitoyltransferase 2; DMSO, dimethyl sulfoxide; Drp1, dynamin-related protein 1; ER, endoplasmic reticulum; FFA, free fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSC, hepatic stellate cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; mdivi1, mitochondrial division inhibitor 1; Mfn1/2, mitofusin1/2; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Nd1, NADH-ubiquinone oxidoreductase chain 1; Opa1, optic atrophy 1; PAS, periodic acid Schiff; Pgc1a, peroxisome proliferator-activated receptor gamma coactivator 1a; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; ROS, reactive oxygen species; TCA, tricarboxylic acid; TG, triglyceride.

KEYWORDS
autophagy, metabolic syndrome, mitochondrial dynamics, mitochondrial dysfunction, obesity

INTRODUCTION

Nonalcoholic fatty liver disease is the most common liver disease and is a well-known comorbidity of obesity. The prevalence of NAFLD continues to rise, in parallel with the increase in obesity worldwide.[1] Nonalcoholic fatty liver disease ranges from simple steatosis, which is a relatively benign form, to NASH, the progressive form. The mechanism underlying the progression of simple steatosis to NASH has not yet been fully characterized. It has been suggested that mitochondrial dysfunction is a causative factor for various human diseases, such as diabetes and cardiovascular diseases.[2] However, the association between mitochondrial dynamics, which include fission and fusion, and NAFLD progression remains largely unknown.

The quantity and quality of mitochondria are regulated by the balance of mitochondrial biogenesis, dynamics involving fission and fusion, and mitophagy/autophagy.[3] Excessive mitochondrial fission is associated with cellular dysfunction and disease progression in diabetes, cardiovascular diseases, and neurodegenerative diseases.[4–6] Recently, we and other research groups revealed that inhibiting excessive mitochondrial fission with mdivi1, a chemical inhibitor of mitochondrial fission, could prevent the progression of cardiovascular diseases by attenuating mitochondrial dysfunction, cellular senescence, and ER stress.[7–10] However, the beneficial effects of mdivi1 treatment are controversial because several reports have shown that mdivi1 treatment could cause cellular senescence, proton leak, and autophagic responses in specific conditions.[11,12] We hypothesized that the beneficial or detrimental effects of mdivi1 treatment could be determined by either the rebalance or imbalance of mitochondrial dynamics in the target cell during specific pathological conditions (stage of disease, injury level, and compensatory effects by fusion, biogenesis, and mitophagy). Thus, mdivi1 treatment could ameliorate NAFLD progression if excessive fission is induced in hepatocytes and other nonparenchymal cells in NAFLD pathogenesis.

Our aim was to clarify whether alterations in mitochondrial fission were associated with NAFLD progression using a CDAA diet-induced NAFLD/NASH model. We also investigated whether inhibiting mitochondrial fission by mdivi1 treatment could be beneficial in NAFLD/NASH pathogenesis by rebalancing mitochondrial dynamics.

METHODS

Detailed methodology is included in Document S1.

Animals

C57BL/6 mice (CLEA Japan) (male, 8 weeks old, 20–25 g) were used. Experimental protocols are shown in Figure S1A,B. All protocols were approved by the Animal Care and Use Committee of Kyoto University Graduate School of Medicine (Med Kyo 20558) and were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

Statistical analysis

Data represent the mean ± SEM. All data were analyzed using SPSS version 20.0 (SPSS Inc.). Statistical significance was determined using Student's *t*-test or ANOVA with Tukey's post-hoc test for multiple comparisons. For all analyses, statistical significance was set at $p < 0.05$.

RESULTS

Enhanced mitochondrial fission is associated with pathological changes in various liver cells and disease progression in the early stage of NAFLD.

To evaluate the effectiveness of mdivi1, we first undertook immunohistochemical analysis of Drp1, a key mediator for fission, in the livers of mice fed a standard diet containing vehicle or mdivi1 for 1 week and found that the Drp1 level in the liver was only slightly decreased in mdivi1-treated mice compared to vehicle-treated mice (Figure S2). To address the confounding effects of CDAA diet and the adverse effects of mdivi1, we next evaluated food and water intake, body and organ weights, and serum biochemical changes in mice treated with mdivi1 with or without CDAA feeding (Figure S1). Results showed that after CDAA diet feeding and mdivi1 treatment, there were no significant changes in food and water intake, body weight, or liver, spleen, and epididymal fat weight per body weight ratio (Figures S3,S4A,B). Similarly, no significant changes in serum biochemical parameters were found in the mdivi1-treated group when compared to those in the vehicle-treated group, except for a slight decrease in triglyceride level after mdivi1 treatment (Figure S4C). These results suggest that mdivi1 treatment did not induce liver or systemic injuries in this study setting.

Mitochondrial fission is generally enhanced in pathophysiological conditions, such as impaired bioenergetics and apoptosis induction, compared to that in physiological states.[13,14] The effects of mdivi1 tend to be increased with the severity of the injuries because the capacity of mitochondrial fission by Drp1 upregulation is dependent on the magnitude of the injuries.[15] Therefore, we hypothesized that the difference in Drp1 expression between the vehicle and mdivi1 treatment groups is augmented by CDAA diet feeding. Indeed, Drp1 expression level was enhanced with 1 week of CDAA diet but decreased in mdivi1-treated mice. Hence, the difference in Drp1

expression between the two treatment groups was augmented by CDAA feeding (Figure S5).

In general, pathological changes in chronic liver injuries are frequently associated with aberrant enhancement of mitochondrial fission.[16] In addition, small mitochondria have reduced oxygen production capacity compared to large mitochondria, even when the total mitochondrial masses are similar, probably due to the reduced energy production capacity of smaller mitochondria.[17] Therefore, it is thought that the blockade of mitochondrial fission by mdivi1 treatment could attenuate the pathological changes in NAFLD progression. To address this hypothesis, we next undertook macroscopic, biochemical, histological, and ultrastructural analyses in CDAA-fed mice treated with vehicle or mdivi1 for 1 week (Figures S6–9). Although no significant changes in macroscopic or biochemical parameters were observed between the two groups (Figure S6), histological analyses showed that the areas stained with PAS, a marker of glycogen storage capacity, and the reticulin-stained area, an immature collagen deposition area, were significantly reduced followed mdivi1 treatment (Figure S7). In line with these minor beneficial changes in histological parameters, ultrastructural analyses showed that the loss of porosity area in LSECs, an LSEC injury marker, as well as the enlarged bile canalicular diameters and loss of microvilli in hepatocytes, bile canalicular injury markers, were attenuated by mdivi1 treatment (Figure S8). Furthermore, collagen deposition in the space of Disse, infiltration of neutrophils and activated KCs, inflammatory responses, and robust collagen release by myofibroblasts mainly derived from HSCs were attenuated by mdivi1 treatment (Figure S9). These findings suggest that enhanced mitochondrial fission contributes to disease progression in the early stage of NAFLD by pathological cell cross-talk between various liver cells and could be partially attenuated by mitochondrial fission inhibitors.

Enhanced mitochondrial fission in hepatocytes and dysregulation of lipid metabolism contribute to pathological systemic changes in early-stage NASH

To understand the importance of mitochondrial roles in the different liver cells in the physiological state, we undertook electron microscopic analyses in healthy mice (Figure 1a). We found that the number of mitochondria in hepatocytes was higher than in other liver cells, suggesting the importance of mitochondria in hepatocytes.

To evaluate the effects of mdivi1 treatment on the liver in the early stage of NASH, we next carried out a macroscopic analysis of liver samples from mice fed a CDAA diet containing vehicle or mdivi1 for 4 weeks. The livers of vehicle-treated mice showed an enlarged size and pale color compared to those of healthy controls, suggesting hepatomegaly and steatosis (Figure S10A). In addition, the liver size was not enlarged in mdivi1-treated mice compared to the vehicle-treated group, suggesting amelioration of hepatomegaly. We next undertook immunohistochemical and western blot analyses of Drp1 and found that Drp1 protein expression was substantially reduced by mdivi1 treatment (Figure S10B–D). To clarify whether mdivi1 treatment could inhibit mitochondrial fission by blocking Drp1, we carried out an ultrastructural analysis of liver samples from mice fed a standard diet containing vehicle or mdivi1 for 4 weeks. Consistent with our expectations, detrimental changes in mitochondrial dynamics in hepatocytes, including excessive mitochondrial fission (as reflected by mitochondrial content, mitochondrial area reduction, increased number of mitochondria per field, and deformed mitochondria), were attenuated by mdivi1 treatment (Figure 1a,b). Liver sinusoidal endothelial cells, KCs, and HSCs are abundant nonparenchymal cells in the liver and have a major role in NAFLD progression.[18] Therefore, we also analyzed mitochondrial changes in these cells (Figures 1b,c,S11). However, contrary to our expectations, neither detrimental changes in mitochondrial dynamics by CDAA feeding nor beneficial effects of mdivi1 were identified in these cells, except for an increased number of deformed mitochondria in LSECs by CDAA feeding, indicating that the effects of mdivi1 treatment were specific to hepatocytes in this protocol.

To clarify whether mdivi1 treatment exerts beneficial effects in the early stage of NASH, we next undertook macroscopic and biochemical analyses in mice fed CDAA for 4 weeks with or without mdivi1 treatment. Although mouse body weights were not significantly different between the two groups, hepatomegaly, portal hypertension, and visceral fat accumulation (as reflected by the liver, spleen, and epididymal fat weight per body weight ratio) were significantly lower in the mdivi1-treated group than in the vehicle-treated group (Figure 2a). Furthermore, biochemical analysis revealed beneficial changes in mdivi1-treated mice, as shown by significant reductions in serum levels of alanine aminotransferase and alkaline phosphatase, total cholesterol, triglyceride, free fatty acid, total bilirubin, and glucose (Figure 2b). To clarify whether the improvement in lipid and glucose metabolisms was influenced by the effects of mdivi1 on adipose tissue and insulin resistance, we undertook macroscopic, histological, biochemical, and qRT-PCR analyses (Figure 2c–e). We found that mdivi1 induced a slight improvement in adipose tissue inflammation, mRNA expression levels of fatty acid oxidation, fatty acid synthesis and lipid storage, and lipid metabolism and mitochondrial biogenesis (Cpt2a, Fas, and Pgc1a, respectively). These findings suggest that enhanced mitochondrial fission in hepatocytes and dysregulation of lipid metabolism in adipose tissue contributes to pathological systemic changes in the early stage of NASH.

Enhanced mitochondrial fission in hepatocytes contributes to NASH development

To determine whether mdivi1 treatment could reverse liver microscopic changes in the early stage of NASH, we undertook histological analysis of liver samples from mice fed a standard diet containing vehicle or mdivi1 for 4 weeks. We found that the grades of steatosis, inflammation, hepatocyte ballooning, and the stage of fibrosis were significantly aggravated by 4 weeks of CDAA diet feeding (Figure 3). Although the steatosis scores were not changed by mdivi1 treatment, the other scores significantly improved following the treatment.

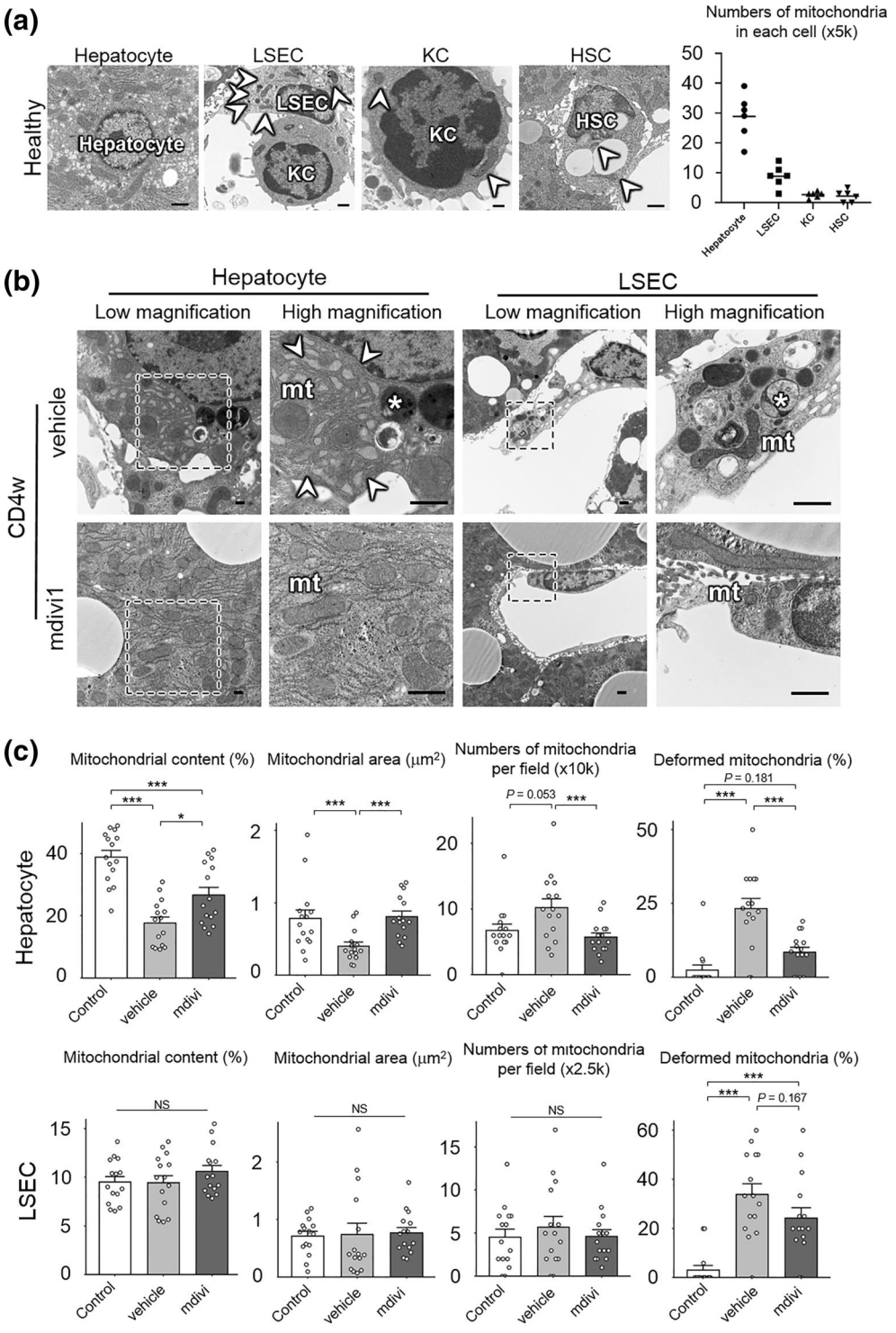

FIGURE 1 Mitochondrial division inhibitor 1 (mdivi1) attenuates mitochondrial dynamics of hepatocytes but not liver sinusoidal endothelial cells (LSEC) in nonalcoholic fatty liver disease progression. (a) Transmission electron microscopy analyses of liver samples from standard diet-fed healthy control mice. Arrowheads indicate mitochondria. Numbers of mitochondria in each cell were analyzed in six randomly chosen fields. HSC, hepatic stellate cell; KC, Kupffer cell. (b) Transmission electron microscopy analyses of liver samples from standard or choline-deficient, L-amino acid-defined diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Scale bar, 1 μm. Arrowheads indicate swollen endoplasmic reticula. Asterisks show autophagosomes. mt, mitochondria. (c) Mitochondrial content and area, numbers of mitochondria per field, and deformed mitochondria (e.g., megamitochondria, loss of cristae) in hepatocytes and LSECs were analyzed in 15 randomly chosen fields. Data represent mean ± SEM. Statistical analyses were carried out by one-way ANOVA with Tukey's post-hoc test. $^{*}p < 0.05$, $^{***}p < 0.001$. $n = 3$ for all groups. NS, not significant

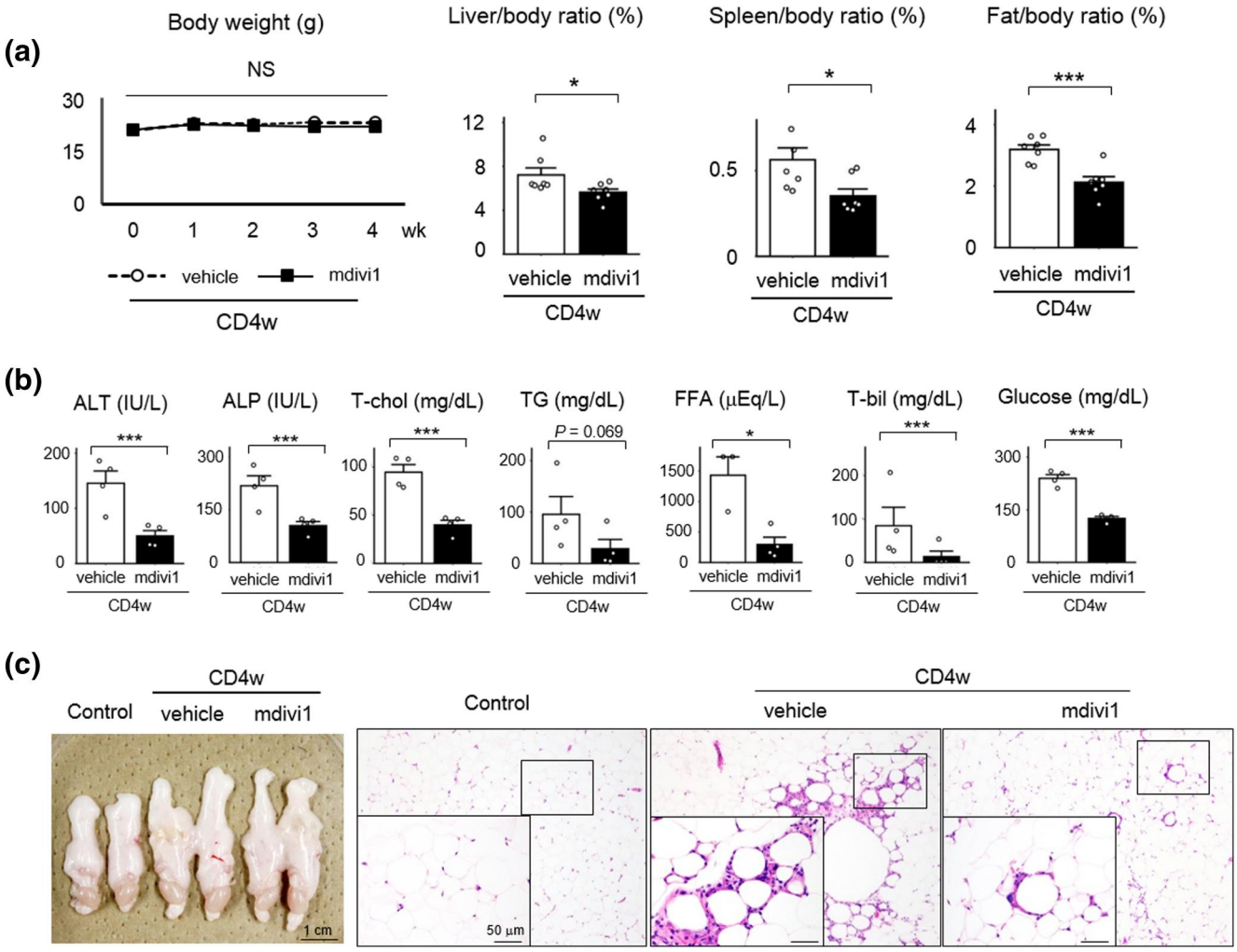

FIGURE 2 Mitochondrial division inhibitor 1 (mdivi1) attenuates systemic injuries and lipid metabolism in nonalcoholic fatty liver disease progression. (a) Body weight of choline-deficient, L-amino acid-defined (CDAA) diet-fed mice treated with vehicle or mdivi1 for 0–4 weeks. Liver/body, spleen/body, and fat/body ratios of CDAA diet-fed mice treated with vehicle or mdivi1 for 4 weeks. (b) Serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-chol), triglyceride (TG), free fatty acid (FFA), total bilirubin (T-bil), and glucose in CDAA diet-fed mice treated with vehicle or mdivi1. (c) Macroscopic and hematoxylin–eosin (H&E) staining images of epididymal fat in control and CDAA diet-fed mice with vehicle or mdivi1 treatment for 4 weeks. (d) Quantitative reverse transcription–polymerase chain reaction analysis of epididymal fat samples from standard or CDAA diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Markers of fatty acid oxidation (*Atgl*, *Hsl*, *Cpt1a*), fatty acid uptake (*CD36*), fatty acid synthesis and lipid storage (*Fas*), lipid metabolism and mitochondrial biogenesis (*Pgc1a*), mitochondrial bioenergetics (*Ucp2*), activated macrophage (*F4/80*), inflammation (*Tnfα*, *IL6*), mitochondrial fission (*Drp1*), and mitochondrial fusion (*Mfn1/2*) are shown. Gene expression levels are normalized to *β*-actin. (e) Serum levels of glucose and insulin and homeostasis model assessment-insulin resistance (HOMA-IR), an index of insulin resistance, of standard or CDAA diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Data represent mean ± SEM. Statistical analyses were carried out by using Student's *t*-test or one-way ANOVA with Tukey's post-hoc test where appropriate. $n \geq 3$ for all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant

Moreover, the significantly elevated levels of glycogen storage markers, as reflected by the PAS-positive stained area, and the fibrotic area, represented by the Azan-positive stained area, were attenuated by mdivi1 treatment. Immature collagen accumulation, as indicated by the reticulin-stained area, was predominantly aggravated by 4 weeks of CDAA feeding, compared to mature collagen deposition. Both mature and immature fibrotic changes were attenuated by mdivi1 treatment. These findings suggest that excessive mitochondrial fission mainly contributes to the early stage of NASH, rather than the simple steatosis stage.

Enhanced mitochondrial fission in hepatocytes leads to various cell injuries in the early stage of NASH

Under physiological conditions, various parenchymal and non-parenchymal cells mutually regulate their function and maintain homeostasis in the liver.[19] However, in pathophysiological states, impaired function of one cell type could lead to synergistic impairments of all types of liver cells through altered cell cross-talk, entering the vicious cycle, thus promoting disease progression.[20] Theoretically, these pathological changes depend on the magnitude

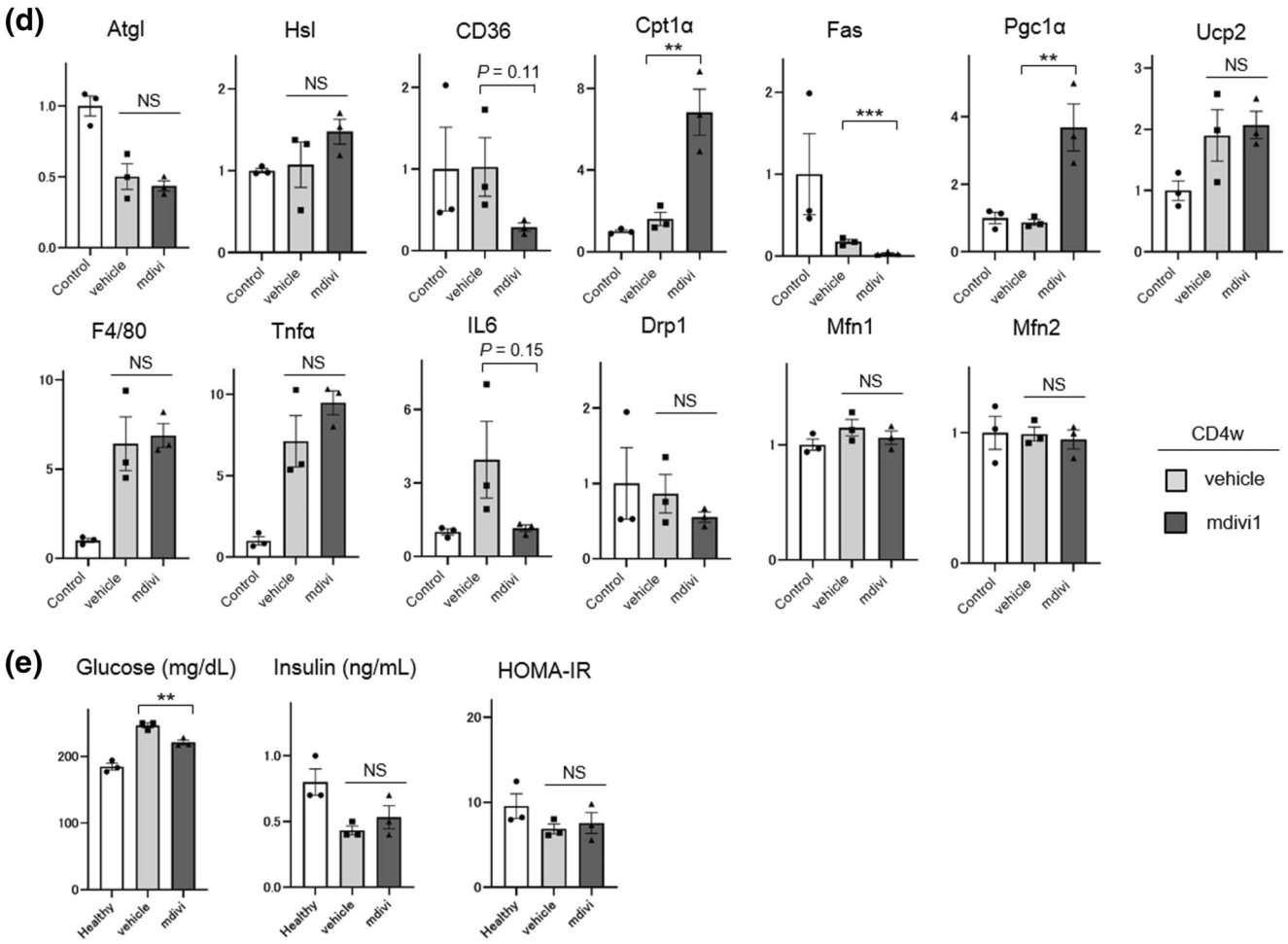

FIGURE 2 (Continued)

and duration of liver damage. Therefore, to determine whether the excessive mitochondrial fission in hepatocytes was involved in the impairment of other liver cells in the early stage of NASH, we undertook ultrastructural and immunohistochemical analyses of the livers of CDAA-fed mice treated with vehicle or mdivi1 for 4 weeks. As expected, the pathophysiological changes in various cells worsened in mice fed CDAA for 4 weeks compared to those in mice fed CDAA for 1 week, and mdivi1 treatment improved the morphological appearance in all types of liver cells, including LSECs, KCs, HSCs, and hepatocytes (bile canaliculi), as shown by scanning electron microscope and transmission electron microscope analyses (Figures 4a,S12). In line with the results of ultrastructural analyses, immunohistological analyses of activation markers in LSECs, KCs, BECs, and HSCs showed that the increased positively stained area of all markers was significantly decreased by mdivi1 treatment (Figure 4b). We confirmed that the positively stained area of all markers in the vehicle groups was similar to that in mice fed CDAA for 4 weeks without vehicle treatment (data not shown). These results suggest that excessive mitochondrial fission in hepatocytes leads to the dysregulation of various liver cells in the early stage of NASH.

Autophagy and ER stress are associated with enhanced mitochondrial fission in hepatocytes in early-stage NASH

To determine whether excessive mitochondrial fission could be involved in the organellar injuries in the early stage of NASH,[21,22] we carried out western blot and qRT-PCR analyses of liver samples from CDAA-fed mice treated with vehicle or mdivi1 for 4 weeks. Western blot analysis showed that the protein expression of α-SMA, an HSC activation marker, tended to increase in CDAA-fed mice with vehicle treatment and was slightly, but not significantly, decreased by mdivi1 treatment (Figure 5a,b). In line with the histological and ultrastructural analyses, qRT-PCR analysis revealed that the mRNA expression levels of interleukin 6 (an inflammation marker), Col4a1, Col3a1, and transforming growth factor β (fibrogenesis markers), and vascular endothelial growth factor receptor 2 (an LSEC injury marker) tended to decrease in the mdivi1-treated group (Figure 5c). Moreover, the expression levels of Pgc1a (a mitochondrial biogenesis marker), Mfn1/2 and Opa1 (fusion markers), Atg5 (an autophagy marker), and X box-binding protein 1 and C/EBP homologous protein (ER stress markers) were substantially decreased by mdivi1 treatment. mRNA

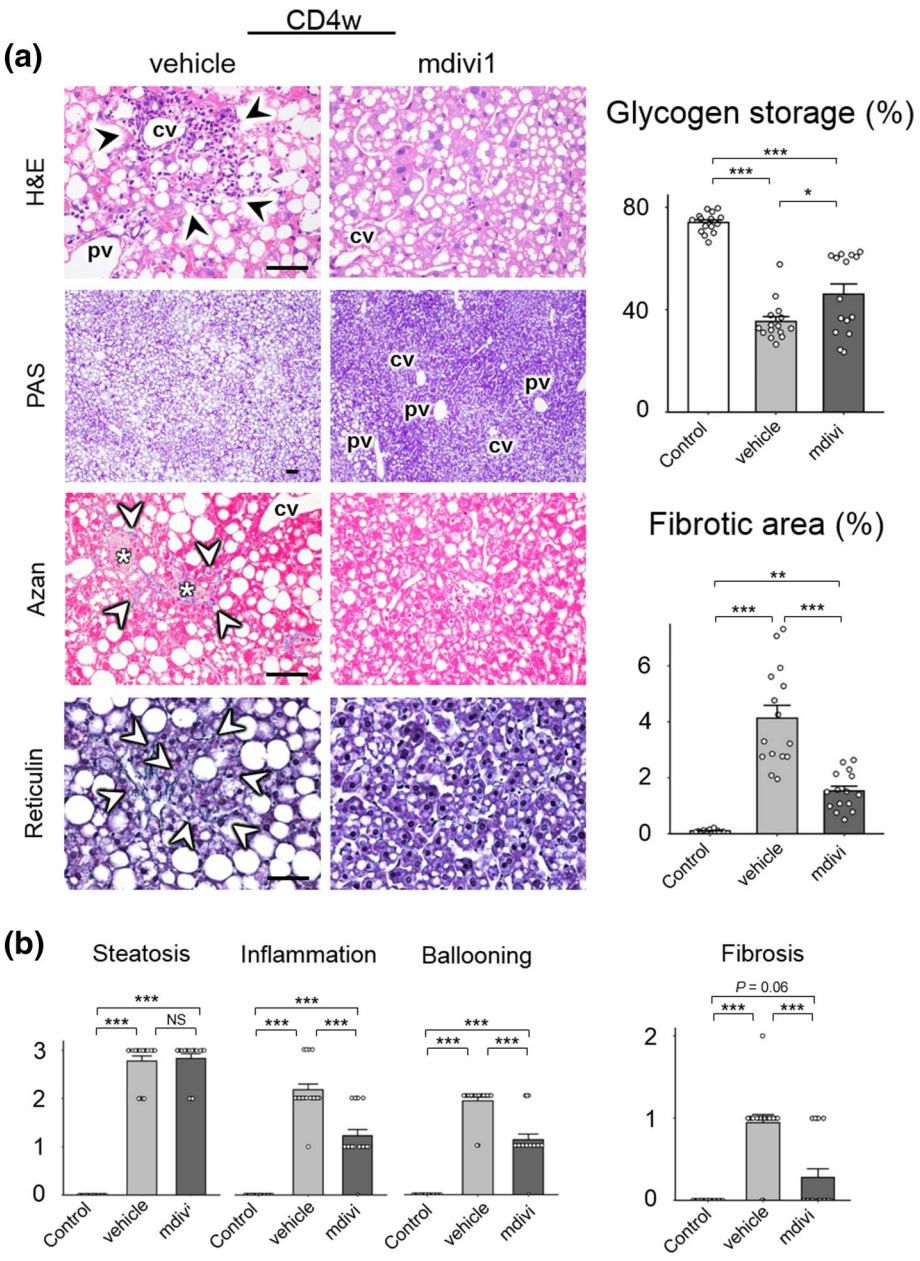

FIGURE 3 Mitochondrial division inhibitor 1 (mdivi1) attenuates liver histological changes in nonalcoholic fatty liver disease (NAFLD) progression. (a) Hematoxylin–eosin (H&E) staining, periodic acid Schiff (PAS) staining, Azan staining, and reticulin staining of liver samples from choline-deficient, L-amino acid-defined diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Black arrowheads indicate inflammatory infiltrations. White arrowheads indicate collagen fiber depositions. Asterisks show lipid-laden macrophages. Scale bar, 50 μm. Glycogen storage (PAS-positive) and fibrotic area (Azan-positive) were analyzed in 15 randomly chosen fields from each group. cv, central vein; pv, portal vein. (b) Grades of NAFLD activity scores (steatosis, inflammation, and ballooning) and fibrosis stage. Data represent mean ± SEM. Statistical analyses were carried out by using one-way ANOVA with Tukey's post-hoc test. $n \geq 3$ for all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. NS, not significant

expression levels of Cpt2 (a mitochondrial fatty acid oxidation marker) were substantially, but not significantly, increased by mdivi1 treatment. Contrary to our expectation, the mRNA expression of the ROS production markers cytochrome oxidase subunit 4 and NADH-ubiquinone oxidoreductase chain 1 increased following mdivi1 treatment. Theoretically, Drp1 mRNA expression should be increased by mdivi1 treatment, as the inhibition of Drp1 protein expression could induce negative feedback and thus lead to increased Drp1 mRNA expression; however, mdivi1 treatment resulted in a significant decrease in Drp1 mRNA expression. We confirmed that these unexpected changes were not dependent on the changes in housekeeping gene expression between β-actin and GAPDH, suggesting that these changes could be the unfavorable or compensatory effects of mdivi1 treatment, rather than accidental errors due to multiple comparisons (data not shown). These findings suggest that excessive mitochondrial fission in hepatocytes leads to further impairment of autophagy, mitophagy, and ER stress in the early stage of NASH.

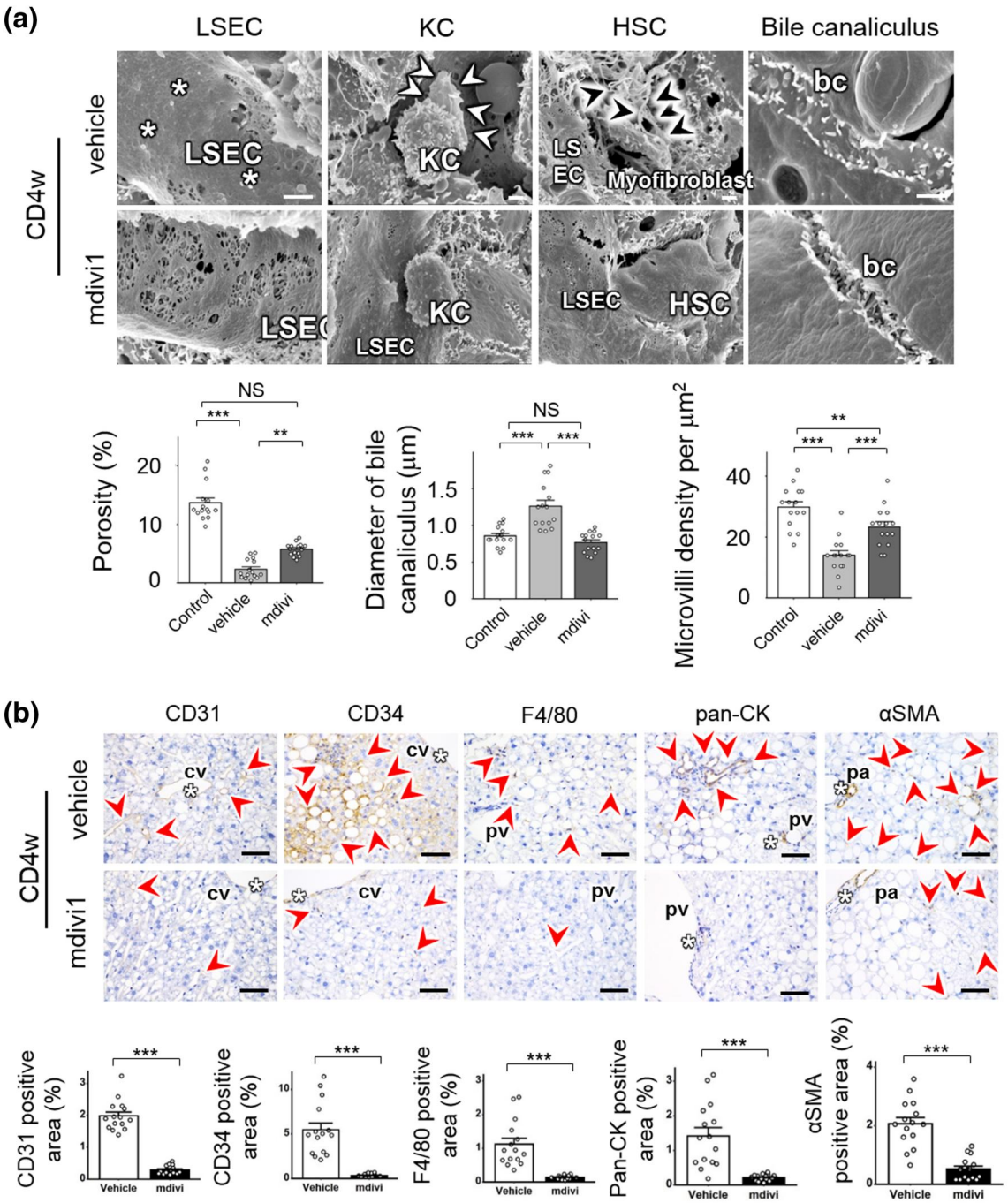

FIGURE 4 Mitochondrial division inhibitor 1 (mdivi1) attenuates histological and ultrastructural changes in various liver cells in nonalcoholic fatty liver disease progression. (a) Scanning electron microscopy analyses of liver samples from choline-deficient, L-amino acid-defined (CDAA) diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Scale bar, 1 μm. White arrowheads indicate elongated pseudopods of a Kupffer cell (KC). Black arrowheads indicate collagen fibers released by a myofibroblast. Asterisks show the loss of fenestrae. Porosity, diameter of bile canaliculus, and microvilli density per μm^2 were analyzed in 15 randomly chosen fields. bc, bile canaliculus. HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell. (b) Immunohistochemical analyses of liver samples from CDAA diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Liver sections were stained with the liver sinusoidal endothelial cell injury markers CD31 and CD34, activated macrophage marker F4/80, bile duct epithelial cell injury marker pan-cytokeratin (CK), and the activated HSC marker α-smooth muscle actin (α-SMA), with hematoxylin counterstaining. Arrowheads indicate positive cells in each immunohistochemical staining. Asterisks show intrapositive control cells, such as portal vein endothelial cells in CD31 and CD34 staining, normal bile duct epithelial cells in pan-CK staining, and normal smooth muscle cells of hepatic arteries in α-SMA staining. Scale bar, 50 μm. Positive area was analyzed in 15 randomly chosen fields. Data represent mean ± SEM. Statistical analyses were carried out by Student's *t*-test or one-way ANOVA with Tukey's post-hoc test where appropriate. $n \geq 3$ for all groups. $^{**}p < 0.01$, $^{***}p < 0.001$. cv, central vein; NS, not significant; pa, portal artery; pv, portal vein

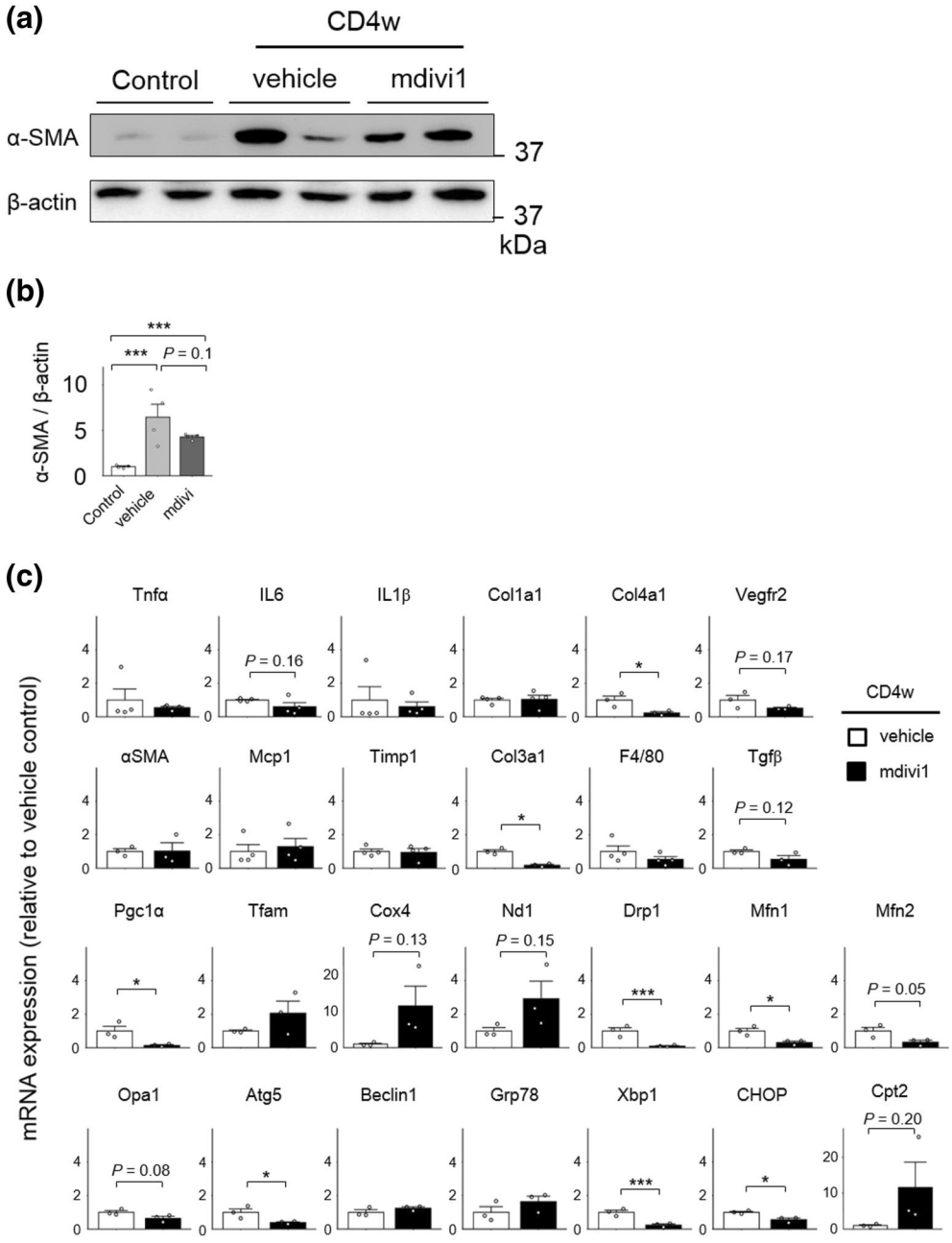

FIGURE 5 Mitochondrial division inhibitor 1 (mdivi1) rebalances mitochondrial dynamics and attenuates inflammatory and fibrogenic responses in nonalcoholic fatty liver disease progression. (a) Western blot analysis of the activated hepatic stellate cell marker α-smooth muscle actin (α-SMA) and *β*-actin (loading control) in liver samples from standard or choline-deficient, L-amino acid-defined (CDAA) diet-fed mice treated with vehicle or mdivi1 for 4 weeks. (b) Densitometric analyses of the intensities of each band normalized to *β*-actin intensity. Data represent mean ± SEM. $n = 4$ for all groups. NS, not significant. (c) Quantitative reverse transcription–polymerase chain reaction analysis of liver samples from CDAA diet-fed mice treated with vehicle or mdivi1 for 4 weeks. Gene expressions are normalized to *β*-actin. Data represent mean ± SEM. Statistical analyses were carried out by using Student's *t*-test or one-way ANOVA with Tukey's post-hoc test where appropriate. $n \geq 3$ for all groups. $^{*}p < 0.05$, $^{***}p < 0.001$

Enhanced mitochondrial fission in hepatocytes in late-stage NASH leads to further aggravation of liver fibrogenesis

To clarify whether mdivi1 could improve liver injuries even after the occurrence of progressive fibroinflammatory changes in the liver, we undertook macroscopic, serum biochemical, histological, ultrastructural, and qRT-PCR analyses in 12-week CDAA-fed mice treated with vehicle or mdivi1 for the last 4 weeks of the experiment (Figures 6,S13). The mdivi1 treatment resulted in a partial but meaningful improvement in macroscopic, biochemical, histological, ultrastructural, and gene expression changes due to CDAA-induced NAFLD development despite aggressive liver fibrogenesis.

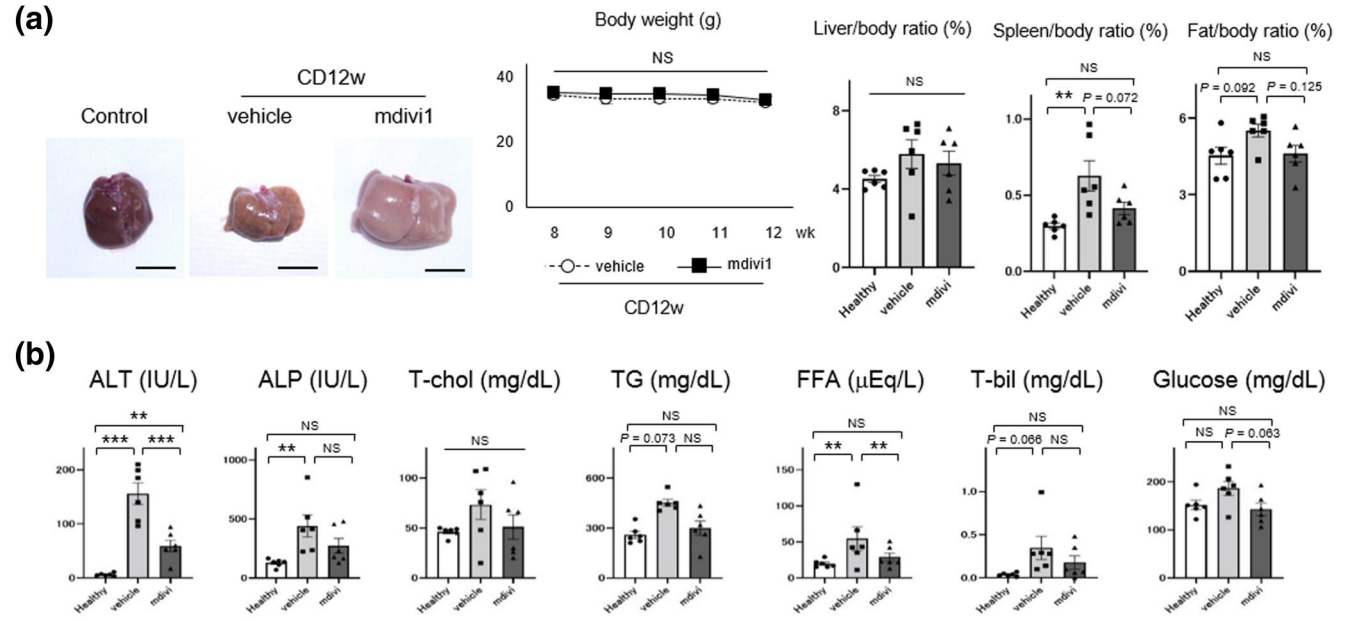

FIGURE 6 Mitochondrial division inhibitor 1 (mdivi1) attenuates liver and systemic injuries in nonalcoholic fatty liver disease (NAFLD) progression even after progressive liver fibrogenesis. (a) Macroscopic images, body weight, and liver/body, spleen/body, and fat/body ratios at 12 weeks in standard or choline-deficient, L-amino acid-defined (CDAA) diet-fed mice treated with vehicle or mdivi1 for the last 4 weeks. (b) Serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-chol), triglyceride (TG), free fatty acid (FFA), total bilirubin (T-bil), and glucose of CDAA diet-fed mice treated with vehicle or mdivi1. (c) Hematoxylin–eosin (H&E) staining, periodic acid Schiff (PAS) staining, Azan staining, and reticulin staining of liver at 12 weeks in CDAA diet-fed mice treated with vehicle or mdivi1 for the last 4 weeks. Black arrowheads indicate inflammatory infiltrations. White arrowheads indicate collagen fiber depositions. Asterisks show lipid-laden macrophages. Scale bar, 50 μm. Glycogen storage (PAS-positive) and fibrotic area (Azan-positive) were analyzed in 15 randomly chosen fields from each group. Grades of NAFLD activity scores (steatosis, inflammation, and ballooning) and fibrosis stage. Quantitative reverse transcription–polymerase chain reaction analysis of livers from 12-week standard or CDAA diet-fed mice treated with vehicle or mdivi1 for the last 4 weeks. Expression of markers of fibrogenesis (*Col1a1*, *Col1a2*, *α-SMA*, and *Tgfβ*) is shown. Gene expression levels are normalized to *Gapdh*. Data represent mean ± SEM. Statistical analyses were carried out using Student's *t*-test or one-way ANOVA with Tukey's post hoc test where appropriate. $n \geq 3$ for all groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. cv, central vein; NS, not significant; pv, portal vein

DISCUSSION

The current study revealed that excessive mitochondrial fission in hepatocytes was associated with NAFLD pathogenesis. The inhibition of excessive mitochondrial fission by mdivi1 treatment attenuated disease progression, not only in the early NASH stage but also after the progressive fibrosis stage. Taking these results together, we propose that excessive mitochondrial fission in hepatocytes contributes to deleterious changes in other organelles and abnormal activation of various types of liver cells, leading to liver inflammation, fibrogenesis, and systemic pathophysiological responses in the early and chronic stages of NASH (Figure 7). In addition to hepatocyte damage, dysregulation of lipid metabolism could be involved, at least in part, in liver and systemic pathologies. We also highlight the potential therapeutic implications of inhibiting abnormal mitochondrial fission enhancement in patients in the early and chronic stages of NASH.

Hepatocytes maintain liver homeostasis by regulating the balance between lipid influx, secretion, lipogenesis, and metabolism under physiological conditions.[23] However, in pathological states, such as overnutrition, sedentary lifestyle, and genetic predisposition, excess lipid accumulation occurs in hepatocytes. The current study suggested that excessive mitochondrial fission in hepatocytes could be slightly induced by dysfunctional steatotic hepatocytes. If hepatocytes suffered from persistent pathophysiological stresses, excessive fission was further aggravated, leading to other organellar injuries, such as ER stress and autophagy/mitophagy. However, in contrast to the impaired mitochondrial dynamics in hepatocytes, the balance between fission and fusion in other liver cells (LSECs, KCs, HSCs) is relatively maintained. Organellar injuries in hepatocytes induced damage in other liver cells, such as BECs, LSECs, and KCs. Hepatocyte death and KC activation induced liver inflammation through the recruitment and activation of lymphocytes and neutrophils. After the inflammatory responses, myofibroblasts dedifferentiated from HSCs and secreted disproportionate collagen fibers. Pathological changes in the liver induced by excessive mitochondrial fission in hepatocytes and dysregulation of lipid metabolism[24] in adipose tissue led to further liver damage and also systemic detrimental changes, such as visceral fat accumulation, splenomegaly, hyperlipidemia, and hyperglycemia. Previous studies have shown that these inflammatory and fibrogenic responses can eventually lead to cirrhosis and hepatocellular carcinoma.[25] Inhibition of mitochondrial

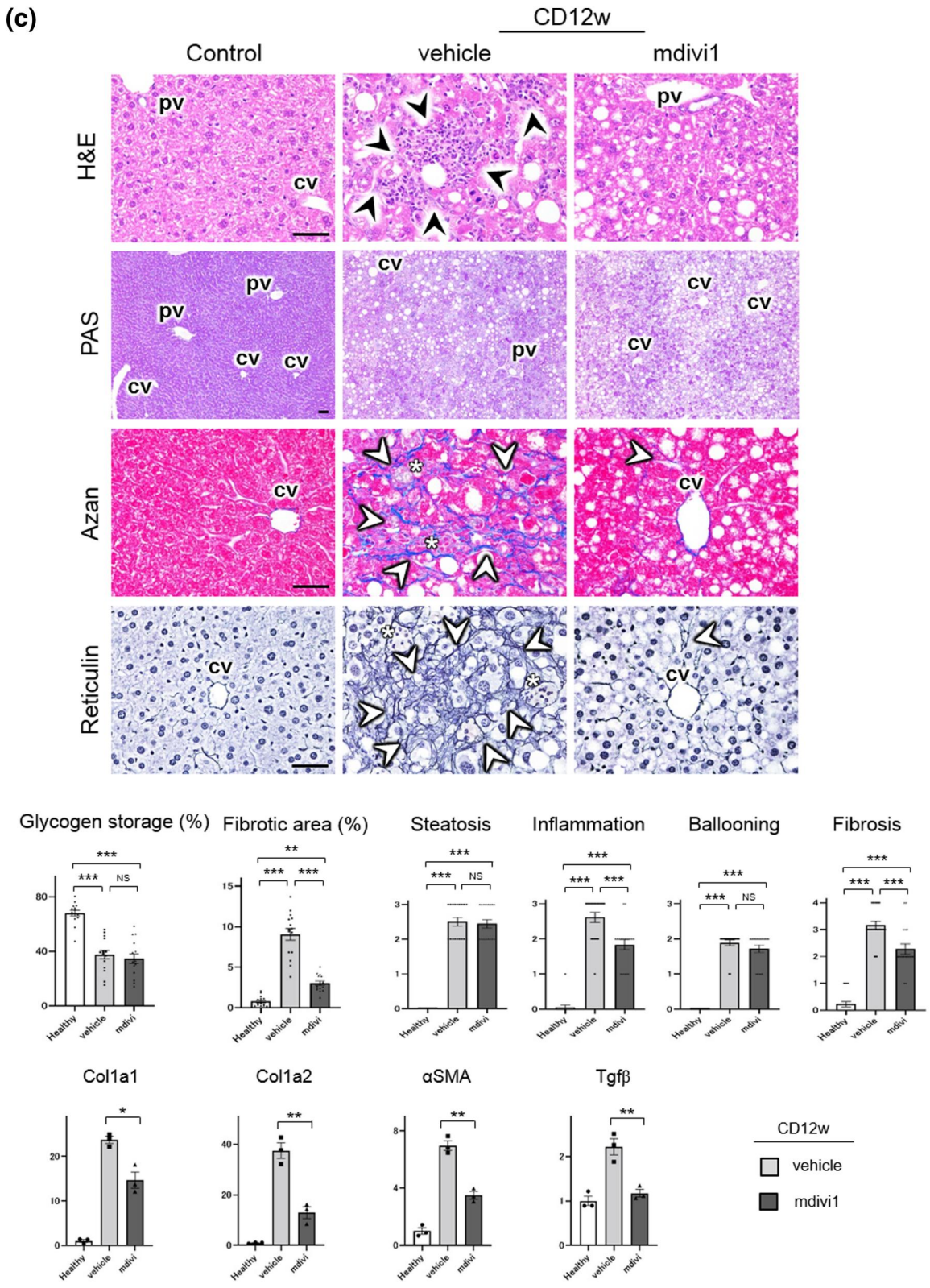

FIGURE 6 (Continued)

fission by mdivi1 treatment can slow down or prevent liver injuries by attenuation of inflammation, fibrosis, and systemic injuries, thereby improving patient outcomes.

The mitochondria are multifunctional organelles that play vital roles in energy production, ROS production, cell differentiation, autophagy, and apoptosis.[26] Therefore, it is not surprising that

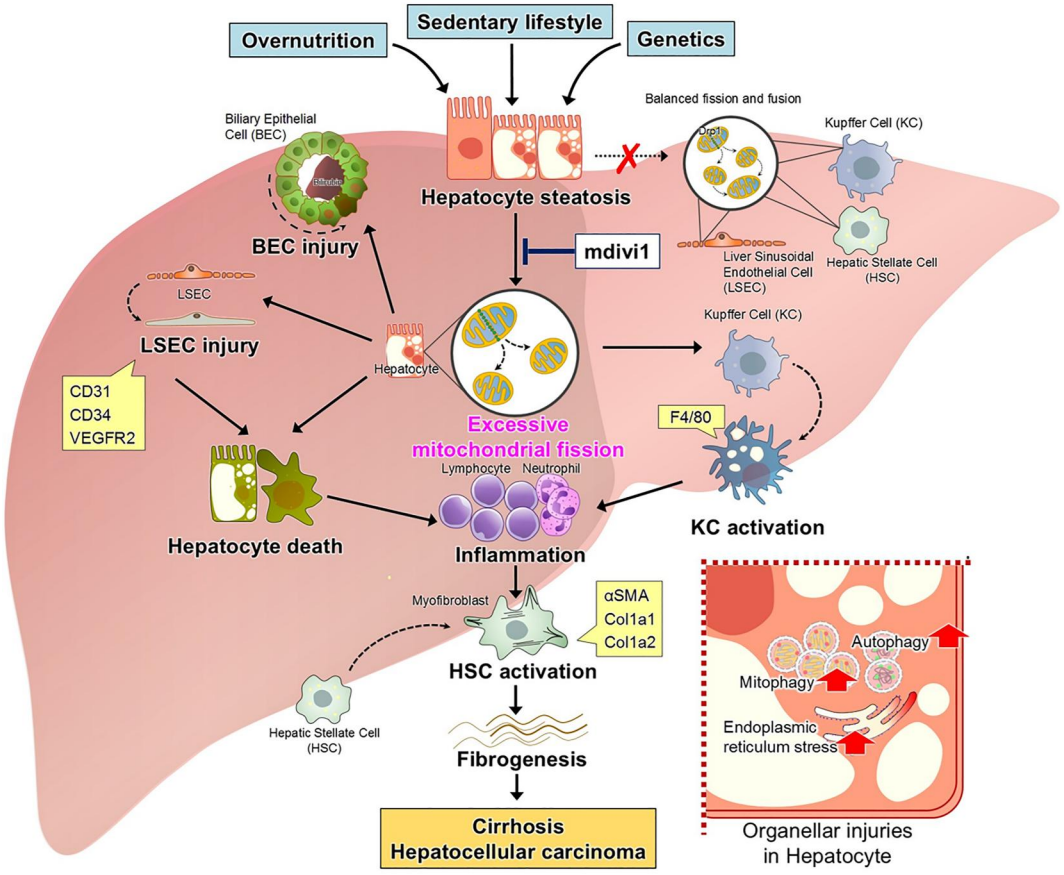

FIGURE 7 Proposed roles of mitochondrial fission in nonalcoholic fatty liver disease (NAFLD) progression. Overnutrition, sedentary lifestyle, and genetic predisposition could lead to hepatocyte steatosis. These abnormal conditions could lead to excessive mitochondrial fission in hepatocytes but not in other liver cells such as liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), or hepatic stellate cells (HSCs). Excessive mitochondrial fission in hepatocytes initiates autophagy and mitophagy, and endoplasmic reticulum stress in hepatocytes, then results in hepatocyte death, biliary epithelial cell (BEC) injury, LSEC injury, and KC activation. Hepatocyte death and KC activation enhance liver inflammation through recruitment of inflammatory cells, such as lymphocytes and neutrophils. Subsequently, HSCs are activated by these pathophysiological responses and thus promote liver fibrogenesis by releasing disproportionate collagen fibers and a large amount of the extracellular matrix, ultimately resulting in cirrhosis and hepatocellular carcinoma. Mitochondrial division inhibitor 1 could prevent the initiation of liver inflammation and the subsequent liver fibrogenesis and carcinogenesis in NAFLD by blocking excessive mitochondrial fission in hepatocytes and dysregulation of lipid metabolism in adipose tissue due to overnutrition, sedentary lifestyle, and genetic predisposition. α-SMA, α-smooth muscle actin; Col1a1/a2, collagen 1a1/a2; VEGFR2, vascular endothelial growth factor receptor 2

alterations in mitochondrial function can induce not only rare genetic mitochondrial diseases but also various common diseases, including NAFLD.[27,28] In 1998, Day et al. first proposed the "two-hit theory" and described that mitochondrial dysfunction was involved in NAFLD pathogenesis.[29] Currently, the "multiple parallel theory" has been well accepted for complex NAFLD pathogenesis; according to the theory, diverse parallel insults, including mitochondrial ROS production, could contribute to NAFLD progression.[30] However, the relationship between mitochondrial fission and NAFLD development remains elusive. To this end, we investigated the precise role of mitochondrial fission in NAFLD pathogenesis. Recently, using a genetic approach, Song et al. revealed that the imbalance between mitochondrial fission and fusion rather than defects, either fission or fusion, could be deleterious in cardiomyopathy pathogenesis.[31] Therefore, they suggested that balancing rather than correcting mitochondrial dynamics could be a promising therapy against cardiomyopathy progression. Similar mechanisms might be involved in the liver.[32,33] Thus, it is worthwhile to conduct research using these genetic engineering approaches. However, they present critical disadvantages for research focused on common diseases, such as NAFLD, because of their low feasibility for gene therapies due to high cost and the narrow range of therapeutic adaptation.[34] For these reasons, we chose mdivi1 as a candidate drug and investigated the role of mitochondrial fission in NAFLD pathogenesis. However, mdivi1 treatment in the simple steatosis phase of CDAA-induced NAFLD resulted in only partial attenuation of the disease, which could have sufficient beneficial effects in the early phase of NASH. Therefore, inhibition of excessive mitochondrial fission in hepatocytes could be a potential therapeutic option for patients in the early stages of NASH. Moreover, because mdivi1 could also improve liver injuries in the 12-week CDAA model, inhibition of excessive mitochondrial fission in hepatocytes could be useful even

after the occurrence of progressive fibro-inflammatory changes in the liver. However, it should be noted that qRT-PCR analysis in the 4-week CDAA model showed an unexpected increase in the mRNA expression of ROS production markers and decreased expression of Drp1. It is not known whether these changes were adverse effects of mdivi1 treatment or compensatory effects, such as enhancement of energy supply by rapid TCA cycle turnover with secondary release of ROS production[35] and rebalancing effects by actively decreasing gene expression of Drp1 against pathological decreased expression of fusion-associated genes such as *Mfn1/2* and *Opa1*.[36] Future studies using drug treatment that could influence TCA cycle turnover and mitochondrial fusion are needed to answer these questions.

Nonalcoholic fatty liver disease ranges from simple steatosis to NASH, a progressive stage with high mortality in liver and cardiovascular diseases. To date, various factors that contribute to the development of NASH from simple steatosis have been reported; these factors include mitochondrial dysfunction, oxidative stress, ER stress, apoptosis, and autophagy/mitophagy.[37] However, the mechanisms underlying the progression from simple steatosis to NASH have not been fully characterized. So far, we have focused on spatiotemporal changes in various diseases, especially in the early stages, because of the potentially high preventability of disease progression due to early diagnoses and interventions.[18,38,39] We hypothesized that mitochondrial fission is involved in the initiation of NASH. However, we found that neither excessive mitochondrial fission in hepatocytes nor that in LSECs largely contributed to NASH initiation from simple steatosis. Although it is unknown why pathological enhancement of mitochondrial fission did not largely contribute to NASH initiation, several compensatory changes in the mitochondria could rescue the excessive mitochondrial fission.[40] Further studies focusing on other mitochondrial changes, such as fusion, biogenesis, autophagy/mitophagy, and respiratory capacity, are needed to elucidate the roles of mitochondria in NASH initiation.

Another limitation of our study is that, although our study revealed that excessive mitochondrial fission aggravates NAFLD progression, we did not examine whether similar mechanisms could be involved in other NAFLD models. In addition, we did not undertake an in vitro study to investigate the detailed mechanisms underlying the effects of mdivi1 on hepatocytes in NAFLD. Future studies using different models or in vitro experiments are needed to validate our results.

In conclusion, our study provides novel insights into the causative relationship between alterations in mitochondrial fission and NAFLD/NASH pathogenesis. The mitochondria are indispensable for maintaining cellular functions, and the imbalance of mitochondrial dynamics in one type of cell could lead to other cellular injuries in the organ through altered cell cross-talk, inflammation, and fibrogenesis, eventually resulting in systemic pathological phenotypes. Therefore, rebalancing mitochondrial dynamics could be a promising therapeutic approach for NAFLD.

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CONFLICT OF INTEREST

None declared.

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