

Targeting EIF5A improves liver fibrosis by inhibiting mitochondrial function of hepatic stellate cells

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Research Article

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Abstract

Background: Liver fibrosis (LF) is the pathological basis of a majority of chronic liver diseases, characterized by excessive activation of hepatic stellate cells (HSCs), inflammatory disorders and progressive scarring of the hepatic interstitial. Recently, single-cell RNA sequencing (scRNA-seq) technology highlighted a much greater diversity between quiescent HSCs (qHSCs) and activated HSCs (aHSCs) in the progression of LF. Our study aims to investigate the role of *Eif5a* in liver fibrosis.

Methods: Bioinformatic analysis showed that the expression of *Eif5a* was associated with aHSCs. Immunofluorescence staining and qRT-PCR assays were performed to detect the expression and localization of *Eif5a* in both in vitro and in vivo hepatic fibrosis models. CCK-8, EdU, wound healing and flow cytometry assays verified the effect of si-*EIF5A* on aHSCs. H&E staining, Masson staining and immunohistochemistry assays detected the function of AAV-sh-*Eif5a* on mouse liver fibrosis. Further, transmission electron microscopy, mitochondrial membrane potential, Mito-Tracker Green, OCR and ATP experiments confirmed the relationship between *EIF5A* and mitochondrial biogenesis.

Results: Functionally, the silencing of *Eif5a* inhibited proliferation, migration, differentiation, and extracellular matrix deposition in aHSCs from both human and mouse origin. In vivo experiments, we found that silencing *Eif5a* slightly alleviated CCL₄-induced liver fibrosis. Mechanistically, mitigating *EIF5A* triggered the mitochondrial morphology and function disorders to alleviate aHSCs activation.

Conclusions: In summary, our findings reveal that inhibition of *EIF5A* can alleviate LF through inhibiting mitochondrial function.

Introduction

Liver fibrosis (LF) is a common pathological feature of chronic liver diseases[1]. LF in early-stage could be reversed, but can not be reversed once it develops into cirrhosis and hepatocellular carcinoma[2]. Currently, the mortality rate secondary to cirrhosis continues to increase[3; 4]. However, there are still no effective therapeutic strategies [5; 6; 7]. Hepatic stellate cells (HSCs) are the dominant contributor for LF progression. HSCs could transdifferentiate into myofibroblasts in response to chemokines, cytokines and growth factors, leading to excessive extracellular matrix (ECM) accumulation and LF[8; 9]. Increasing evidence suggests that mitochondrial dysfunction is involved in the HSCs activation and contributed to LF progression[10; 11; 12]. Single-cell RNA sequencing (scRNA-seq) is a new technology, which achieving unprecedented accuracy in deciphering cell heterogeneity [13; 14]. Recently, several studies have revealed the function of hepatic mesenchymal cells by scRNA-seq, and found that portal vein-associated HSCs are the main contributors to collagen deposition in mice LF process [15]. Another study revealed the developmental trajectories of activated HSCs (aHSCs), and suggested that the different drivers of HSCs can affect the fibrosis process [16]. The scRNA-seq technique can be used to clarify the pathogenesis mechanism and find new therapeutic targets for LF.

The eukaryotic initiation factor (*EIF5A*) is the highly conserved protein in eukaryotes [17; 18]. The human *EIF5A* gene has been reported in a variety of tumors as an oncogenic gene [19; 20; 21]. Growing evidences also have shown that *EIF5A* plays a critical role in regulating mitochondrial metabolism [22; 23]. Cougnon M et al. demonstrated that inhibition of *EIF5A* retarded the function of mitochondria and decreased glucose reabsorption and increased glycolysis, and thus reducing oxygen consumption in mouse kidney [24]. Some studies demonstrated that *EIF5A* regulated oxygen consumption and reduced kidney fibrosis after transplant [25]. In addition, one study reported that hypusinated *EIF5A* is required for the translation of collagen protein [26]. These evidences suggests that *EIF5A* participated in organ fibrosis, but the relationship between *EIF5A* and LF is unclear.

In this study, we identified *EIF5A* as a critical pathogenic gene in LF by bioinformatic analysis and then explored its function and mechanism. We hope to elucidate the a *EIF5A*-involved pathogenic mechanism of LF and provide a favorable treatment strategy of LF.

Materials and methods

Bioinformation analysis

We downloaded the dataset numbered GSE171904 from gene expression omnibus database (GEO) database. The raw data was collected and visualized by uniform manifold approximation and projection (UMAP) technique [27]. Based upon the annotations of cell-type-specific label genes from previous study to distinguishing the aHSCs and qHSCs [28]. Unsupervised clustering visualized the similarity of different status from carbon tetrachloride-induced toxic injury (CCL₄) and bile duct ligation (BDL) models by the “vegan” of R package. Spearman’s correlation coefficients evaluated the co-expression *Eif5a* and fibrotic-genes. Then the “FindMarker” in Seurat with log FC.threshold = 0.5 was used to obtain differentially up-regulated genes in qHSCs and aHSCs respectively. Moreover, cell proliferation index evaluated the proliferation condition of qHSCs and aHSCs from two fibrotic models, the annotated genes of G1/S and G2/M phase from previous study [29].

We further divided into *Eif5a*-high and *Eif5a*-low groups according to the median value of *Eif5a*, and calculating the percentage of aHSCs and qHSCs between *Eif5a*-high and *Eif5a*-low groups. A majority of cell mitochondria and ATP-related terms of HSCs were loaded from gene ontology (GO) biological process (BP) by R package “msigdb”. Then, single sample gene set enrichment analysis (ssGSEA) algorithm of R package gene set variation analysis (GSVA) was utilized to calculate the correspondences between HSCs and mitochondrial and ATP-related pathway scores from CCL₄ and BDL models [30]. The above bioinformatic analysis provided reliable support for next study.

Mouse models of liver fibrosis

To match with the samples of bioinformation, CCL₄ and BDL were constructed. All experimental mice (C57BL/6 mice; 8 weeks old) were breed under room temperature and fed the same diet. CCL₄ model was

accomplished by intraperitoneal injection with CCL₄ (5 mL/kg) or same dose of saline. CCL₄: corn olive is 1:9, twice a week. BDL model mice were fur removed and anesthetized by intraperitoneal injection of avertin, and then performed a ligating the common bile duct surgical procedure. All mice were humanely sacrificed, and liver tissues and serum were prepared in 4% paraformaldehyde or -80°C refrigerator for subsequent study. All animal experimental protocols were approved by the Ethics Committee.

RNA interference in vitro and in vivo

To silence *Eif5a* in vivo, *Eif5a* inhibition was performed by tail-vein injection AAV8-sh-*Eif5a* (1.0×10^{12} plaque-forming units at a volume of 100 μ L) or AAV-sh-scramble (100 μ L) respectively. For interference *Eif5a* expression in vitro, small interfering RNAs (si-RNAs) were made by Hanbio biotechnology Corp. Si-*Eif5a* or si-negative control (si-NC) was transiently transfected into JS-1 or LX-2 cell using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfected cells were harvested for further experiences.

Cell culture and treatment

The mouse hepatic stellate cells (JS-1) and the human hepatic stellate cells (LX-2) were placed in DMEM culture medium containing 5% and 10% fetal bovine serum (FBS) and 1% penicillin streptomycin respectively. Transforming growth factor (TGF- β 1) (SigmaAldrich, USA) was applicated at a concentration of 20 ng/ml and cultured in the above complete medium for 24 h.

Real-time quantitative PCR (qRT-PCR)

Total RNA of tissues and cell samples was extracted by TRIzol reagent. NanoDrop 8000 was used to detected the concentration and purity of the RNA. RNA samples were reverse transcribed to cDNA according to the PrimeScript RT reagent kit. A reaction system (20 μ L) for each sample was prepared with cDNA, RNase free water, SYBR Green, and primers. A 7500 FAST real-time PCR System was used to detect RNA expression level. The data was analyzed by $2^{-\Delta\Delta CT}$ method. The primer sequences were listed in **Supplementary Table 1**.

Western blot

Total protein of tissues and cell samples was extracted by RIPA lysis buffer. BCA protein concentration reagent kit (Beyotime, Jiangsu, China) used to detected the concentration of the extracted proteins. 10% and 12% SDS–polyacrylamide gel was prepared to electrophoresis, and proteins were transferred to the PVDF membranes. 5% skim milk sealed membranes for 1 h, the membranes were tailored and incubated in different primary antibodies overnight, with Collagen I (Wanleibio, Shenyang, China), alpha smooth muscle actin (α -SMA) (Affinity, Shanghai, China), *Eif5a* (Proteintech, Wuhan, China), GAPDH (Abways, Shanghai, China), Membranes were washed with PBS-T and after incubated the second antibody. The proteins were detected and digitally analyzed by chemiluminescent signals.

Histopathological analysis and immunohistochemical staining

Liver tissues were fixed in 4% paraformaldehyde, dehydrated using different concentrations of ethanol and cut into 5 μ m paraffin sections for next experiments. Then hematoxylin and eosin (H&E) and Masson staining were performed to detect the assessment of inflammation and collagen deposition. Tissues sections were immersed in microwaved sodium citrate antigen repair solution to repair the antigen. Then the sections were washed with PBS and blocked with goat serum for 30 min, then incubated with primary antibodies overnight, PBS-T washed the sections, followed by continued incubation of second antibody. Finally, a light microscopy was used to take images.

EdU cell proliferation assay

The treated cells were inoculated into each well of 12-well plate. 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation kit (RiboBio, Guangzhou, China) was used to detect the cell proliferation. Cells were incubated with mixed A reagent, 4% paraformaldehyde, 0.5% TritonX-100 and Apollo Dye reagent were added in 12 wells according to the manufacturer's instructions. The nucleus was stained with DAPI. Fluorescence microscope was used to recorded the photographs.

Cell Counting Kit-8 (CCK-8) viability assay

Cells were cultured in 6-well plates and treated with TGF- β 1 (4 μ L) and transfection reagents. CCK-8 (200 μ L) (Meilunbio, Dalian, China) reaction solution was added the 6-well plates and incubated in a 37°C cell incubator for 30 min, after that the supernatant was transferred to a 96-well plate at a dose of 100 μ L per well. Microplate reader (BioTek, Richmond, USA) at 450 nm measured the OD values.

Immunofluorescence staining

The JS-1 and LX-2 cells were placed in the petri dishes. Cells were treated with different reagent according to different group. Then 4% paraformaldehyde was added to fixed cells at room temperature 30 min, 0.5% TritonX-100 and BSA were added to the each well for penetrated and blocked cells at room temperature. In turn, primary antibody α -SMA (Affinity, Shanghai, China) and secondary antibody were incubated, the nucleus was stained with DAPI for 15 min. Confocal microscopy (OLYMPUS, Japan) was used to recorded the fluorescence images.

Wound healing assay

Wound healing assay was implemented to verify the cell migration ability. The JS-1/LX-2 cells were placed in the 6-well plates. 200 μ L pipette tip was used to scratched wounds with confluency 90% for cells. Plates were washed with PBS until cell debris disappeared, then the medium with transfected reagents and TGF- β 1 was re-added. The microscope was used to recorded images at 0 h and 24 h for JS-1 cells, and recorded images at 0 h and 48 h for LX-2 cells.

Tissue hydroxyproline assay and serum aminotransferase assay

To detect the degree of liver fibrosis, hydroxyproline content assay was performed in vivo. 30 mg wet heavy liver tissue was provided and measured following the manufacturer's instructions of

hydroxyproline assay kit (Jiangcheng Bioengineering Nanjing China). Microplate reader (BioTek, Richmond, USA) at 550 nm evaluated the hydroxyproline content in tissue. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with the corresponded assay kit (Jiangcheng Bioengineering Nanjing China).

Cellular ATP assay

In order to identify the cellular ATP content, ATP (adenosine 5'-triphosphate) assay was measured. LX-2 cells were inoculated and transfected with siRNA and siNC respectively and treated with TGF- β 1. After 24 h, the cells were fully lysed with cell lysis solution RIPA (40 μ L) and the cell suspension was centrifuged, and then aspirated and the prepared ATP reaction working solution (80 μ L) (Beyotime, Jiangsu, China) was added. The ATP solution and cell supernatant were mixed and quickly added to the 96-well plate. The RLU values were measured using a Tecan Infinite M200 PRO luminometer.

Transmission electron microscopy

LX-2 cells with transfected siRNA and siNC were centrifuged, and the solution supernatant was discarded. The cell precipitates were solubilized by adding 2.5% glutaraldehyde, and then respectively transferred to 1.5 ml EP tubes and centrifuged again to fix the cells. Then the samples were dehydrated using different concentrations of ethanol, permeabilized with pure acetone and embedded. Samples were made into 70 nm sections and then 2% uranyl acetate-lead citrate double stained sections. Finally, images were captured under transmission electron microscopy (JEOL, Tokyo, Japan).

Flow cytometry assay

Flow cytometry method was prepared under the standard protocol. LX-2 cells transfected with siRNA and siNC for 24 hours were digested using EDTA, centrifuged and cell precipitates were collected. Then pre-cooled PBS was used to wash the cell precipitates. Cells were fixed using 70% ethanol for 24 h and pre-chilled PBS was washed again. Samples were stained with Propidium mix solution from Cell cycle analysis kit (Beyotime, Jiangsu, China) and then incubated in the dark. Finally, different phases of the cell cycle were analyzed by flow cytometry instruments.

Mitochondrial membrane potential and Mito-Tracker Green staining assay

The method of mitochondrial membrane potential was described by Dong et al's study[31]. Cultured LX-2 cells at 50% confluency were loaded with transfection reagents and TGF- β 1, and then loaded with TMRM (50 nmol/L, Invitrogen) and Hoechst 33342 (10 mg/mL, C0031, Solarbio, Beijing, China). The Mito-Tracker Green fluorescent label assay was used to detected mitochondrial morphology. Treated LX-2 cells was loaded with Mito-Tracker Green (50 nmol/L, Invitrogen). These images were recorded by Confocal microscopy (OLYMPUS, Japan) with a 20 $\times$ objective lens. The roundness was calculated by the according to the study[32].

Statistical analysis

All the statistical data were statistical at least three independent experiments and presented as the mean $\pm$ SEM. T-test was used for comparison between two groups, ANOVA test was considered for multiple group comparisons. P value < 0.05 was considered significant.

Results

The landscape of scRNA-seq in the HSCs of mouse hepatic fibrosis

As shown in the Fig. 1A-B, we attempted to visualize the distribution of HSCs for Oil, CCL₄ and BDL groups by UMAP method, and then annotated with the cell clusters according to previous study, the cells were divided into aHSCs and qHSCs[28]. In the graph, each point representing a cell. According the distribution of HSCs, we observed that aHSCs were more enriched in the CCL₄ and BDL groups, and the qHSCs were mainly enriched in the Oil group. Then, unsupervised clustering assay was visualized to compared the similarity of aHSCs and qHSCs in CCL₄ and BDL groups, as shown in Fig. 1C, where the vertical coordinates represent distances, indicating that aHSCs and qHSCs was divided significantly. The proliferation index of aHSCs was significantly increased in the CCL₄ and BDL groups. Similarly, the cell proliferation activity was higher in the CCL₄ group compared with the BDL group (Fig. 1D-E). Next, we also demonstrated the enrichment of aHSCs was higher in CCL₄ group than BDL model by evaluating the cell cycle (Fig. 1F-G).

Eif5a was contributed to aHSCs in mouse hepatic fibrosis

Based upon the results, we further identified significantly upregulated-genes between qHSCs and aHSCs from CCL₄ and BDL models (**Supplementary Table 2**). We referenced the previous description, the *Eif5a* was reported that prevented the kidney fibrosis[33; 34]. Moreover, several studies verified the gene play an oncogene in hepatocarcinoma through promoting epithelial mesenchymal transition, which illustrated that the gene could regulate the mesenchymal-associated proteins[35; 36; 37]. As shown in the Fig. 1H-I, the violin plot presented the expression of *Eif5a*, *Col1a1* and *Col1a2* and we found that the expression of *Eif5a* was higher in CCL₄-driven aHSCs than BDL-treated aHSCs. Moreover, the spearman's correlation coefficients showed that the co-expression relationship, which demonstrated the *Eif5a* was positively correlated with fibrosis-related genes. (Fig. 1J). Next, based on the median value of *Eif5a*, the samples were divided into *Eif5a*-high and *Eif5a*-low groups respectively, we found that a higher proportion of aHSCs in the *Eif5a*-high group (Fig. 1K-L).

Eif5a is positively enriched in mitochondrial-associated pathways

To further identified whether the *Eif5a* modulate the mitochondrial-associated genes, we picked out mitochondrial and ATP corresponded terms by R package msigdb (**Supplementary Table 3**), observing the *Eif5a*-high group was mainly enriched in the active mitochondrial module (Fig. 2A-B). Accordingly, we randomly selected several mitochondrial-related pathways in the CCL₄ and BDL models, mitochondrial-gene-expression, mitochondrial-transport, mitochondrial-membrane-organization, respectively. As

expected, we found that, comparing with the *Eif5a*-low group, *Eif5a*-high group followed by a higher score by the ssGSEA algorithm of the R package GSVA (Fig. 2C-D). These bioinformatic analysis results provided us abundant evidences, which inspirates us to hypothesize that *Eif5a* might play a regulatory role in liver fibrosis. We need further to demonstrated this find.

Eif5a is enhanced expression in liver fibrosis

To further explore the role of *Eif5a* in vivo and in vitro, CCL₄ and BDL liver fibrosis models were established. As shown in the representative images (Fig. 3A). The liver tissue surface in CCL₄ mice with scattered white granules and edges of liver was rounded, however, tissue morphology appearance in saline mice was smooth and with a reddish-brown color. BDL tissue showed obviously swollen gallbladder with a bottle green color compared to the sham mice. For the next step, we counted the body weight, liver weight and liver/body weight ratio (LV/BV) in different groups. The body weight of CCL₄ mice and BDL mice were decreased than individual normal groups (Fig. 3B). The liver weight of CCL₄ mice also decreased compared with that of normal mouse control mice, and the liver weight of BDL mice increased than sham group, it was considered that the ligated bile duct prevented bile flowing into the duodenum and thus stagnated in the liver (Fig. 3C). In contrast to the saline group, CCL₄ injection induced decreases of the LW/BW, BDL treatment group showed the upregulation of LV/BW (Fig. 3D). The liver of CCL₄ treatment and BDL intervene resulted remarkable histopathologic changes. As shown in the Fig. 3E, compared with the saline and sham mice, CCL₄ and BDL mice showed broadly clustered Kupffer cells infiltration by H&E staining. Moreover, the masson staining demonstrated the clustered collagen deposition in the liver paraffin sections of CCL₄ and BDL groups. Next, immunohistochemistry (IHC) detected the expression of *Eif5a* and fibrosis-related indicators, which including α -SMA and Collagen I. As shown by the sections, we found that the expression of α -SMA, Collogen I and *Eif5a* were all higher in CCL₄ and BDL group than saline and sham group (Fig. 3F). Moreover, the above results indicating that the fibrotic level in CCL₄ mice was higher than BDL mice according the fibrotic manifestations.

Thus, we performed the vitro study to further demonstrated the *Eif5a* expression. The JS-1 cells were treated with TGF- β 1 (20 ng/mL) and 20% serum for 24 h, qRT-PCR assay analyzed the expressions of fibrotic-related mRNAs, the data showed that the *Col1a1*, *Acta2*, and *Eif5a* level were significantly upregulated by induced TGF- β 1 and 20% serum (Fig. 3G). As shown the representative images, immunofluorescence co-localization demonstrated the subcellular localization of α -SMA and *Eif5a* and the proteins expression. The representative photographs speculated the α -SMA and *Eif5a* proteins levels were upregulated in TGF- β 1 and serum group compared with normal JS-1 cells, simultaneously, α -SMA was mainly expression in the cellular cytoplasm, and the *Eif5a* was localized in the both nucleus and cytoplasm in JS-1 cells (Fig. 3H). The above experiments speculated that the *Eif5a* expression was positively associated with fibrogenesis and could play a regulator role in liver fibrosis process. Next, we try to explore the function and mechanism of *Eif5a* in liver fibrosis.

Inhibition of *Eif5a* ameliorates fibrogenesis in TGF- β 1-treated JS-1 cells

We suppressed the expression of Eif5a by siRNA technology, and to clarified the role of Eif5a on fibrogenesis. The three small interfering RNAs were constructed. qRT-PCR assay showed that transfected siRNAs all suppressed Eif5a level and the siRNA-1 contributed to the best silencing efficiency in JS-1 cells (Fig. 4A). Then the siRNA-1 was applied to completed the subsequent experiments. CCK-8 assay was constructed to clarified cell vitality. The OD value showed that silencing of Eif5a restrained the cell viability in TGF- β 1 induced JS-1 cells (Fig. 4B). Next, we further applied an EdU method to identified the cell proliferation conditions in treated JS-1 cells by Eif5a interfering. As expected, treated TGF- β 1 could improve the cell proliferation, and transfected silencing Eif5a tended to restore cell proliferation by TGF- β 1 inducing (Fig. 4C-D). We then clarified the cells migration condition by wound healing experiments. The experiments clarified that the cell migration ability was upregulated by inducing TGF- β 1, however, si-Eif5a inhibited the activated cells migration ability (Fig. 4E-F). At the same time, qRT-PCR method analyzed the influence of Eif5a for profibrogenic-genes expression. As expected, treated TGF- β 1 could enhance the expression of fibrotic-marker mRNAs, which including Col1a1, Col3a1, Acta2, in addition, inhibition of Eif5a restored the upregulation of fibrotic-marker genes (Fig. 4G-J). Moreover, the representative images showed the changes of fluorescence intensity. TGF- β 1 group enhanced the protein expression of α -SMA, the silencing Eif5a slightly mitigated the enhanced expression of α -SMA, thus retard the differentiation into myofibroblasts (Fig. 4K).

Anti-fibrotic functions of EIF5A in TGF- β 1 induced LX-2 cells

Next, we continued to examine the role of the *EIF5A* on fibrogenesis in LX-2 cells. The expression of *EIF5A* was down-regulated by siRNA technology, and the qRT-PCR assay confirmed the inhibition efficiency (Fig. 5A). Treated in the same way as JS-1 cells, CCK-8 method was carried out to clarify the LX-2 cells viability, the TGF- β 1 treatment increased the cells viability, si-*EIF5A* mitigated the upregulation of cell viability by induced TGF- β 1 (Fig. 5B). In order to confirmed the effects of *EIF5A* by TGF- β 1-driven fibrogenesis, we found that TGF- β 1 trigger cells proliferation, whereas, inhibitory *EIF5A* attenuated the proliferation ability of activated LX-2 cells (Fig. 5C-D). Furthermore, wound healing results clarified that TGF- β 1 could increase cells migration ability, and silencing *EIF5A* reversed the upregulation of administration by TGF- β 1 in LX-2 cells. (Fig. 5E-F). Cell cycle assay further confirmed that si-*EIF5A* tend to prolong G1 phase and shortened S phase in treated LX-2 cells (Fig. 5G-H). These phenotypes results following our hypotheses, however, we still need to further clarify the role of *EIF5A* for fibrotic-genes.

The qRT-PCR assay results demonstrated that after knockdown of *EIF5A*, the fibrotic-related mRNAs were decreased (Fig. 6A-D). Consistent with the above results, the proteins expression of EIF5A, Collagen I, and α -SMA were overexpression in TGF- β 1 group, on the contrary, the transfected si-*EIF5A* into treated LX-2 cells suppressed the upregulation of these proteins by western blot assay (Fig. 6E-F). As shown in the representative confocal microscopic images, which showed that the si-*EIF5A* can reduce the α -SMA expression,

which illustrate the *EIF5A* silencing blunted the transition of hepatic stellate cells into myofibroblasts (Fig. 6G). Then we further explored the function of *EIF5A* in vivo experiments.

Silencing *Eif5a* mitigated liver fibrosis progress in CCL₄-treated mice

The liver fibrosis by driven-CCL₄ has been widely used in the animal experiments, so that we choose the method to accomplish the animal experiments[38]. To characterized the effect of *Eif5a* in vivo experiments, we injected an adeno-associated virus containing an *Eif5a* interference (AAV-sh-*Eif5a*) or AAV-sh-scramble (AAV-sh-scr) in CCL₄ mice. As illustrated in the Fig. 7A, the flowchart showing the process of treated mice. mice were randomly assigned to experimental groups, administered AAV-sh-*Eif5a* and AAV-sh-scr were injected by tail vein. After 30 days, 10% CCL₄ or the same dose of saline were treated by intraperitoneal injection and maintained 8 weeks, mice were euthanized and gutted liver tissue. As expected, the representative photographs of liver in CCL₄ group exhibited bumpy nodules, scattered white granular and rounded edges, sh-*Eif5a* group slightly alleviated the liver morphology appearance by treated CCL₄(Fig. 7B). Furthermore, we observed that fibrosis disorders could decrease the body weight, liver weight and LW/BW ratio, however, the depletion of *Eif5a* might attenuate the mouse poor healthy condition driven by CCL₄, which the body weight, liver weight were up-regulated, LW/BW ratio not showed the significantly statistics, compared with the AAV-sh-scr group (Fig. 7C-E).

In the following experiences, a series of pathological experiments corroborated the above results. H&E staining assay illustrated that slow down *Eif5a* had lower inflammatory cells infiltration than negative group, meanwhile, collagen deposition level in AAV-sh-*Eif5a* group also significantly down-regulated than negative control by Masson assay (Fig. 7F). Immunohistochemical staining demonstrated that the expression of *Eif5a*, α -SMA and Collagen I was up-regulated, and that such upregulation was alleviated in the silencing *Eif5a* mice (Fig. 7G). Liver-related serological tests could also assess the degree of liver fibrosis, the serum aminotransferase assay showed that AST and ALT were higher in CCL₄ mice than normal control, however, after being injected with AAV-sh-*Eif5a*, AST and ALT were downregulated compared with AAV-sh-scr group (Fig. 7H-I). Meanwhile, the hydroxyproline content also evaluated the degree of liver fibrosis. We found that application of CCL₄ increased the liver hydroxyproline content, and *Eif5a* silencing alleviated the hydroxyproline content by CCL₄ administration (Fig. 7J). Consistently, western blot experiments detected the expression of whole liver tissues, and confirmed that silenced expression of *Eif5a* reduced the expression of Collagen I, and α -SMA proteins (Fig. 7K-L). Based upon these results, we clarified that inhibitory *Eif5a* could retard liver fibrosis progress by CCL₄-induced mice. Taken together, the current study suggested that the *Eif5a* might be considered potential strategies for against liver fibrosis. Next, we will continue decipher the underlying mechanism between *Eif5a* and activated HSCs.

Silencing EIF5A alleviates fibrogenesis through inducing mitochondrial dysfunction

In previous study, *EIF5A* was reported to target gene in the renal fibrosis through involving into mitochondrial biogenesis[34; 39]. These studies inspires us that *EIF5A* could regulate the HSCs through interacting mitochondrial function. In the following experiences, we utilized the transmission electron microscopy to observed the change of mitochondrial morphology. As shown in the representative

photographs, Knockdown *EIF5A* into LX-2 cells lead to mitochondrial length shortened (Fig. 8A). Consistent with the results, the mitochondrial fluorescence images suggested that *EIF5A* inhibiting induced mitochondrial shattered by Mito-tracker Green FM prober, which increased the roundness of mitochondrial morphological correspondingly (Fig. 8B-C). Further, mitochondrial membrane potential results showed that *EIF5A* deficiency reduced the cellular mitochondrial membrane potential by TMRM staining in TGF- β 1-treated LX-2 cells (Fig. 8D-E). Mitochondrial respiratory function was analyzed by mitochondrial oxygen consumption rate (OCR) assay, and the statistical analysis showed that down-regulation of *EIF5A* could decrease cellular oxygen consumption rate for treated LX-2 cells (Fig. 8F-G). Therefore, the above results enlighten us to performed an energy assay to verify the changes in cellular energy metabolism. As shown in the Fig. 8H, TGF- β 1 + si-*EIF5A* group suppressed the cellular ATP generation than TGF- β 1 + si-NC group. Based upon these data, we considered that the *EIF5A* deficiency could induce mitochondrial disfunction and affected mitochondrial dynamics. The conclusion provides a decipherment of mechanism in activated HSCs.

Discussion

LF encompasses a spectrum of chronic liver injury, until now, there is still no clear clinical-drugs for against LF worldwide. HSCs as recognized as one major contributor, most targeting-therapies focus on the HSCs are being progressed. Xiuping et al. demonstrated that Metformin decreased the number of activated-HSCs through inducing cells cytochrome C release from mitochondrial into the cytoplasm, and targeted HSCs apoptosis[40]. Two similar experiences were manifested that administration of PPAR γ in HSCs and reduced the expression of Collagens, α -SMA and tissue inhibitor of metalloproteinase 1 [41; 42]. Recent single-cell RNA sequencing studies supported us to identified unique HSCs heterogeneity, and elucidated genetic tools to interference hepatic injury.

In our present study, we characterized the dataset, and the results showed that aHSCs were mainly clustered in both CCL₄ and BDL models through UMAP method. Then we screened the *Eif5a* gene, which is the unique protein identified that contains a post-translational activation through hypusination and regulated two key enzymes deoxyhypusine synthase (DHPS) and dexyhypusine hydroxylase (DOHH)[43]. The *Eif5a* was positively correlated with the degree of renal fibrosis through regulated the mitochondrial function, and participated in the development of various cells through manipulating the expression of fibronectin proteins[33; 35; 39]. Based on these evidences, which enlightens us to further explain the relationship between *Eif5a* and LF. Co-expression analyzed the *Eif5a* was positively related about fibrotic-genes. The enrichment analysis showed that the mitochondrial and ATP metabolism terms were activated in the *Eif5a*-high group. The above information indicates that *Eif5a* could hold potential to against LF.

To match with that the bioinformatic dataset, we have similarly established CCL₄ and BDL mouse LF models, JS-1 cells were driven by TGF- β 1 and serum, and showed that higher expression of *Eif5a* in LF. EdU and wound healing results suggested that silenced *Eif5a* ameliorated the proliferation and migration for activated JS-1 cells. More importantly, we verified that intervening *Eif5a* also alleviated the expression

of the *Col1a1* and *Acta2* by qRT-PCR assay. Among these, not only in activated JS-1 cells, but also attenuated fibrogenesis by TGF- β 1-driven LX-2 cells. Besides, we observed that intervening *Eif5a* also alleviated mouse LF progression. Then, based on the previous profiles, *Eif5a* was strongly associated with the mitochondrial biological function. Dong Ma et al. showed that *Eif5a* preserves mitochondrial integrity and involved in the mitochondrial fission in vascular smooth muscle cells[44]. Depletion of hypusinated *Eif5a* declined the mitochondrial respiration and accelerated the brain aging[45]. *Eif5a* protein isoform A depletion might slow-regulated mitochondrial biogenesis-related genes and modulated oxidative metabolism[22]. These studies testified to our findings, and suggested that *EIF5A* plays a vital role in liver injury, which is associated with mitochondrial regulation.

At the same time, it has been implicated that downregulation of mitochondrial function delays liver fibrosis through deficiency oxidative phosphorylation and reduced ATP synthesis in HSCs[32]. In the current study, we proved that the silenced *EIF5A* might induce mitochondrial morphological dynamics, interfered with mitochondrial respiratory feature, so that reduced the energy supply in LX-2 cells. Our study revealed that knockdown *EIF5A* improved cellular mitochondrial silencing, thus inhibiting cell proliferation, differentiation, delay cell cycle and collagen accumulation, which play a crucial role for LF.

However, our study has certain limitations. The more in-depth regulatory networks did not manifested about *Eif5a* influence mitochondrial biogenesis-related genes, limiting the demonstration of the relationship between *Eif5a* and mitochondrial dynamics for LF. In addition, liver puncture is rarely performed in clinical liver fibrosis, so that limiting us to verify the expression and relation of *Eif5a* in human liver fibrosis.

Conclusion

Taken together, our result here, investigated that inhibiting *Eif5a* play a protective role in both CCL₄-treated mouse LF and TGF- β 1-driven human and mouse HSCs, which through inducing mitochondrial disfunction and decline the cellular energy, thus retarded uncontrolled HSCs proliferation, differentiation, delaying cell cycle and collagen deposition. Overall, our discovery prompts a possible pathogenic mechanism for LF, proposing that *Eif5a* might be a genetic avenue for the therapeutic application of LF.

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Figures

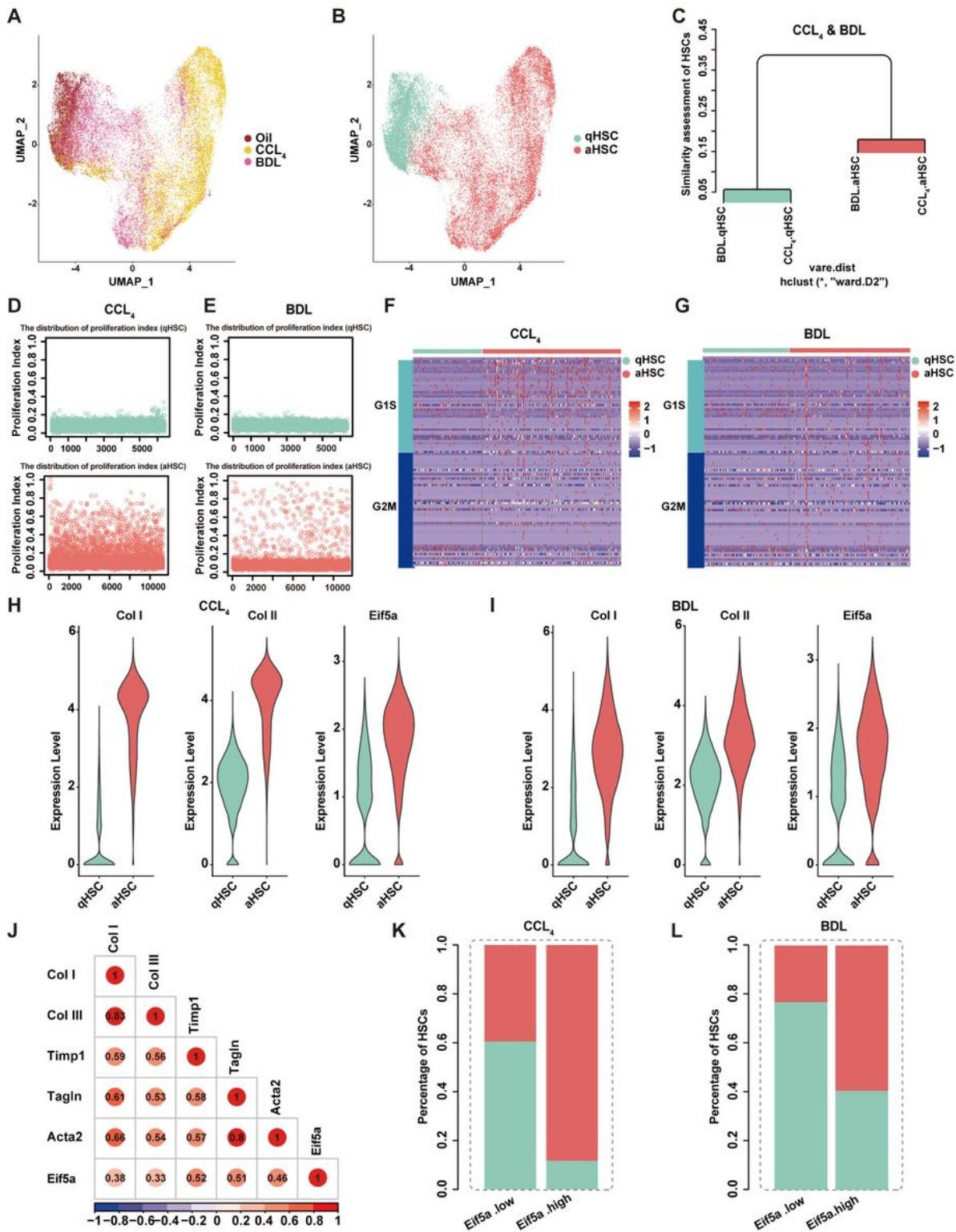


Figure 1

Generation of scRNA-seq dataset and identify in mouse hepatic stellate cells. (A) UMAP visualization profiled the cells clusters by scRNA-seq analysis. (B) The distribution of aHSCs and qHSCs between two groups by UMAP visualization assay. (C) Hierarchical clustering classified the similarity of differ status in the CCL₄ and BDL models. (D, E) The assessment of proliferation index for aHSCs and qHSCs between CCL₄ and BDL groups. (F, G) Heatmap showed that G1S and G2M phases of the cell cycle in aHSCs and

qHSCs from CCL₄ and BDL groups. (H, I) Violin diagram showed that the expression of *Col1a1*, *Col1a2* and *Eif5a* between aHSCs and qHSCs from CCL₄ and BDL groups. (J) Spearman's correlation analyzed the co-expression of the fibrotic genes and *Eif5a* gene. (K, L) Barplot evaluated the percentage of aHSCs and qHSCs in *Eif5a*-high and *Eif5a*-low group from CC1₄ and BDL groups.

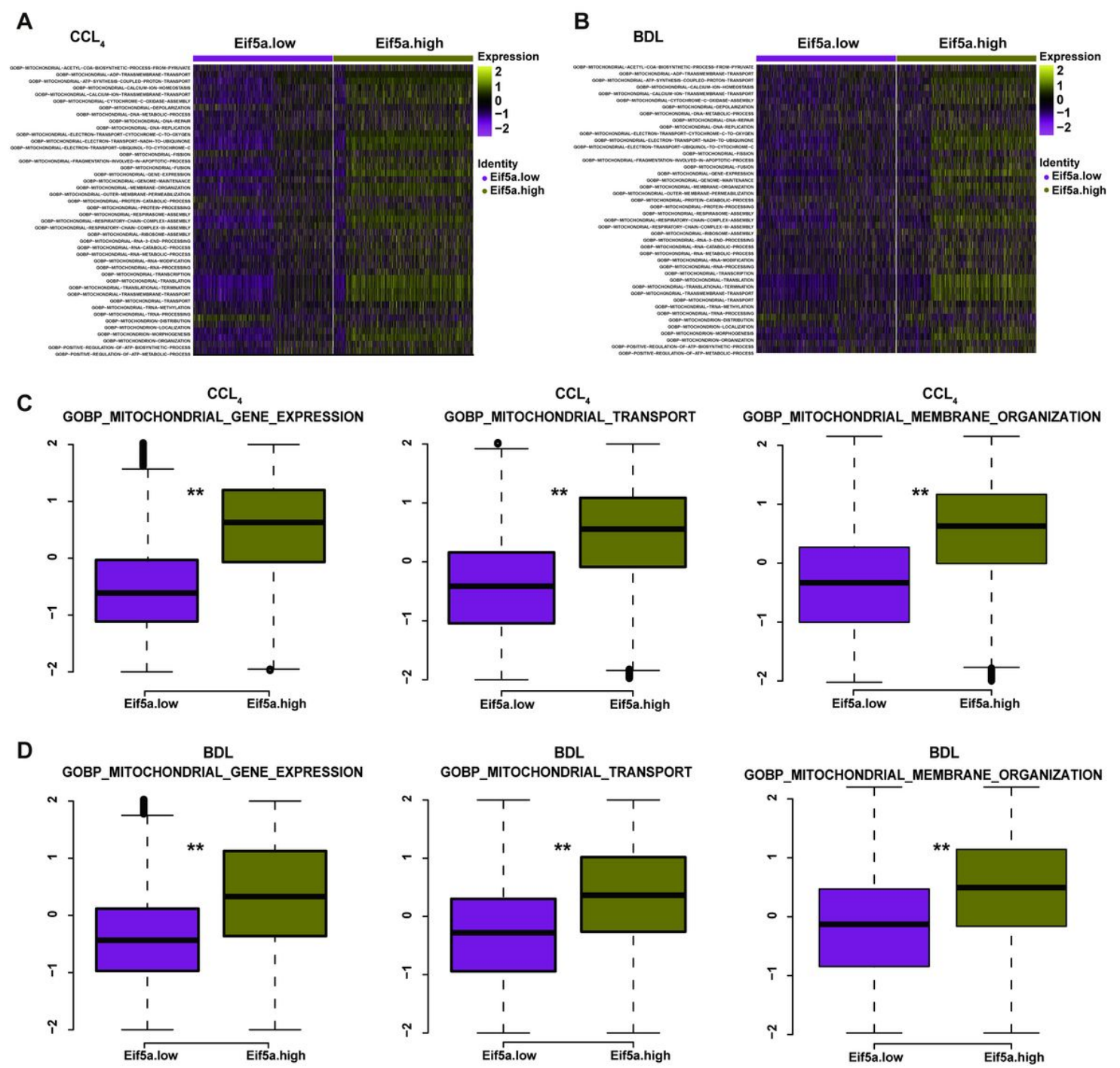


Figure 2

Functional enrichment confirmed that *Eif5a* affects the regulation of mitochondrial metabolism. (A, B) Heatmap comparing the activation of mitochondrial metabolism in *Eif5a*-high and *Eif5a*-low groups. (C,

D) Boxplot visualization of representative mitochondrial pathways in *Eif5a*-high and *Eif5a*-low expression groups (**P 0.01).

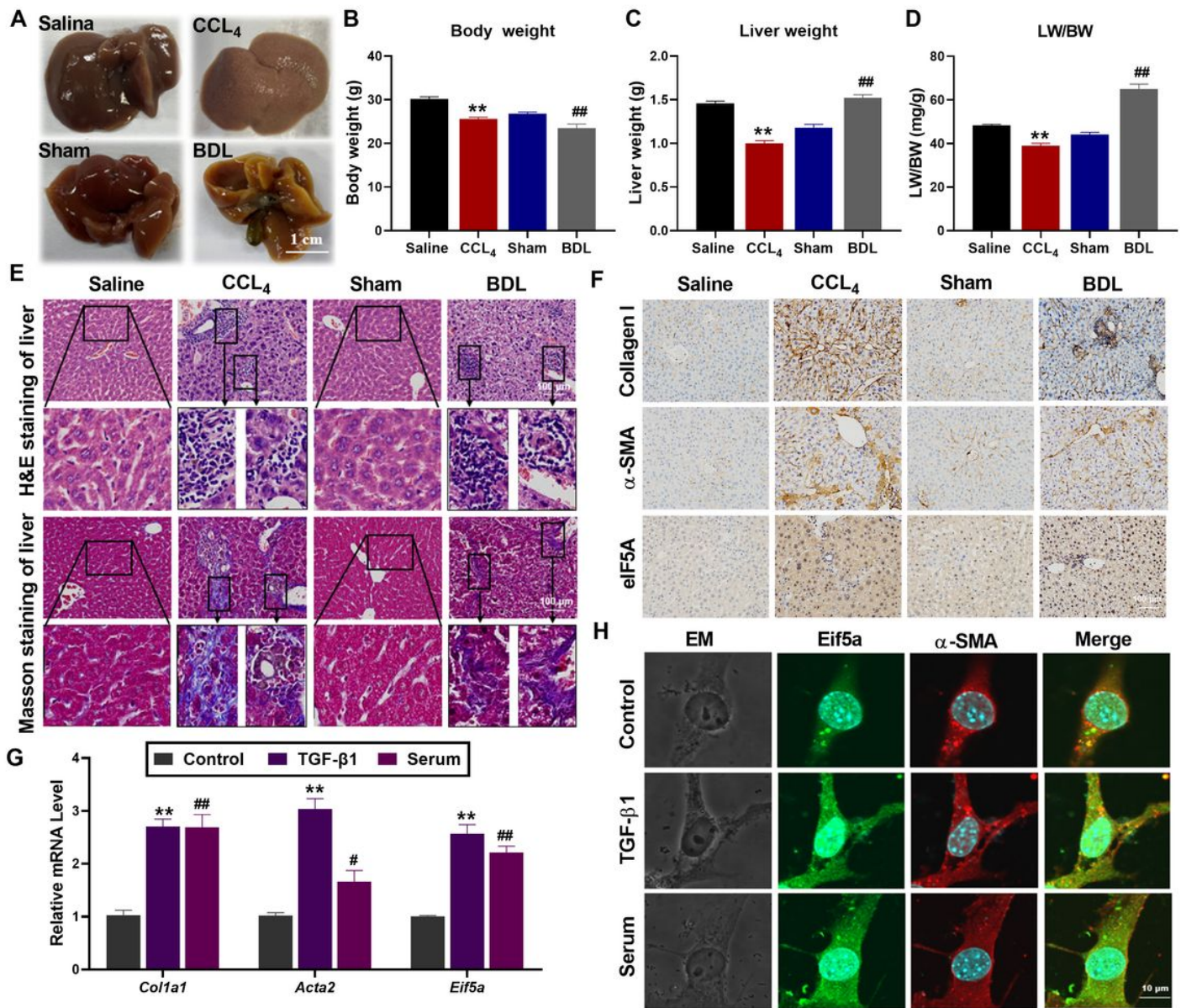


Figure 3

The expression of *Eif5a* is enhanced in mouse liver fibrosis and activated HSCs. (A) Representative images showed the liver morphology, by which CCL₄-induced and BDL-performed mouse liver fibrosis. (B, C, D) Statistical data showed the body weight, liver weight and LW/BW from different groups (n=5). (E, F) H&E and Masson staining detected the degree of inflammatory infiltration and collagen location, the framed area was magnificent. (G) qRT-PCR analysis the fibrotic-related mRNAs, *Col1a1*, *Acta2* and *Eif5a* expression after activated JS-1 cells (n=4). (H) Representative confocal fluorescence photographs demonstrated the subcellular co-localization of Eif5a (green), α-SMA (red) and DAPI (blue). *P<0.05, **P<0.01 vs Saline, #P<0.05, ##P<0.01 vs Sham.

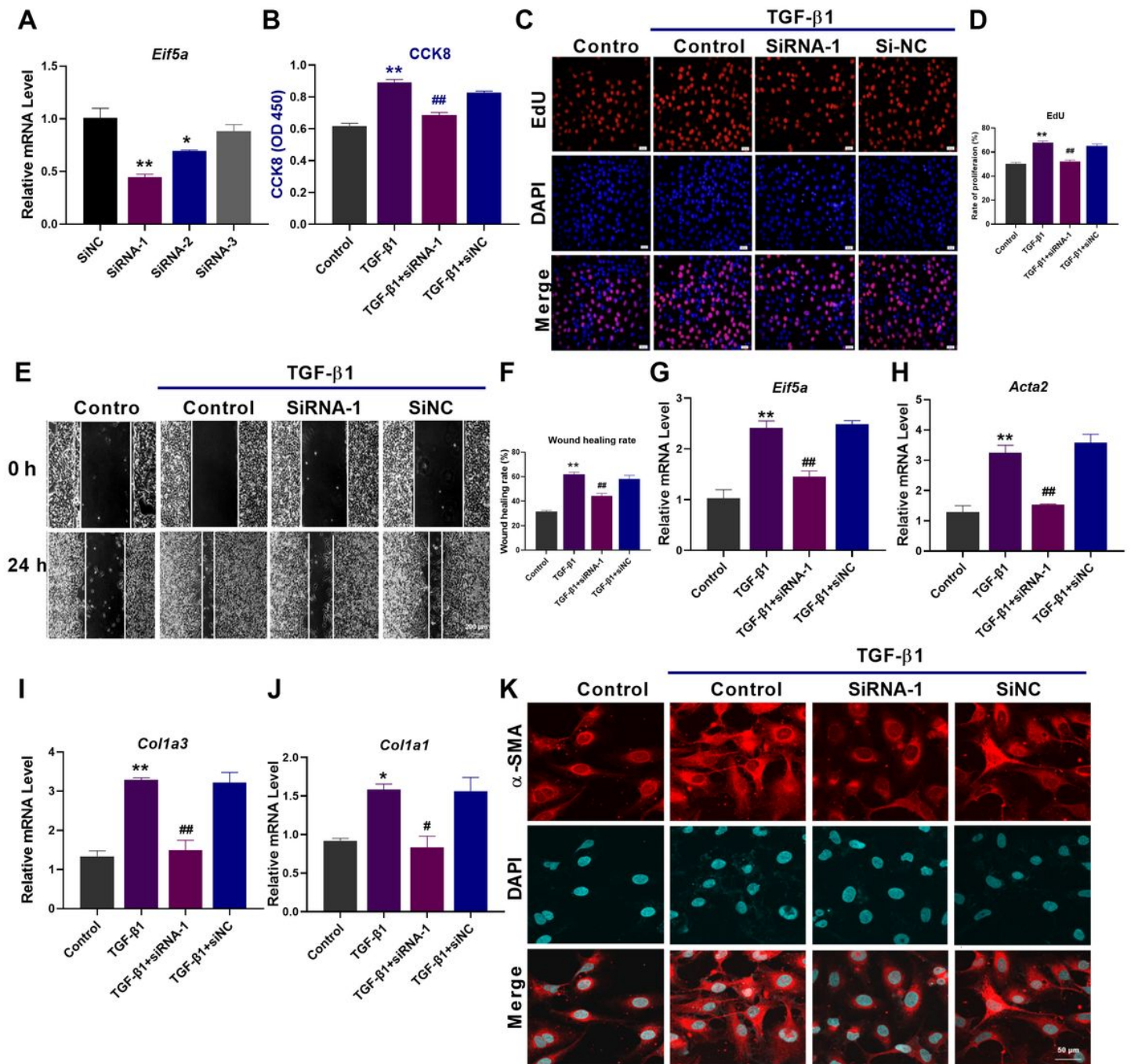


Figure 4

Silencing *Eif5a* attenuates TGF-β1-induced mouse hepatic stellate cells (JS-1) fibrosis. (A) qRT-PCR detected the silencing efficacy of siRNAs against *Eif5a* (n=3). (B) CCK-8 assayed the cell viability from different groups (n=10). (C-F) Silencing *Eif5a*-1 suppressed proliferation and migration phenotypes in TGF-β1-stimulated JS-1 cells by EdU assay and wound healing assays (n=7) (n=5). (G, J) The relative mRNAs expression of *Eif5a*, *Col1a1*, *Col3a1*, *Acta2* and by qRT-PCR results (n=3). (K) Silencing *Eif5a*-1 deficiency mitigated the fibroblast-myofibroblast transition driven by TGF-β1, as assessed by the

representative confocal immunofluorescence images. * $P < 0.05$, ** $P < 0.01$ vs control, # $P < 0.05$, ## $P < 0.01$ vs TGF- $\beta 1$ + siNC.

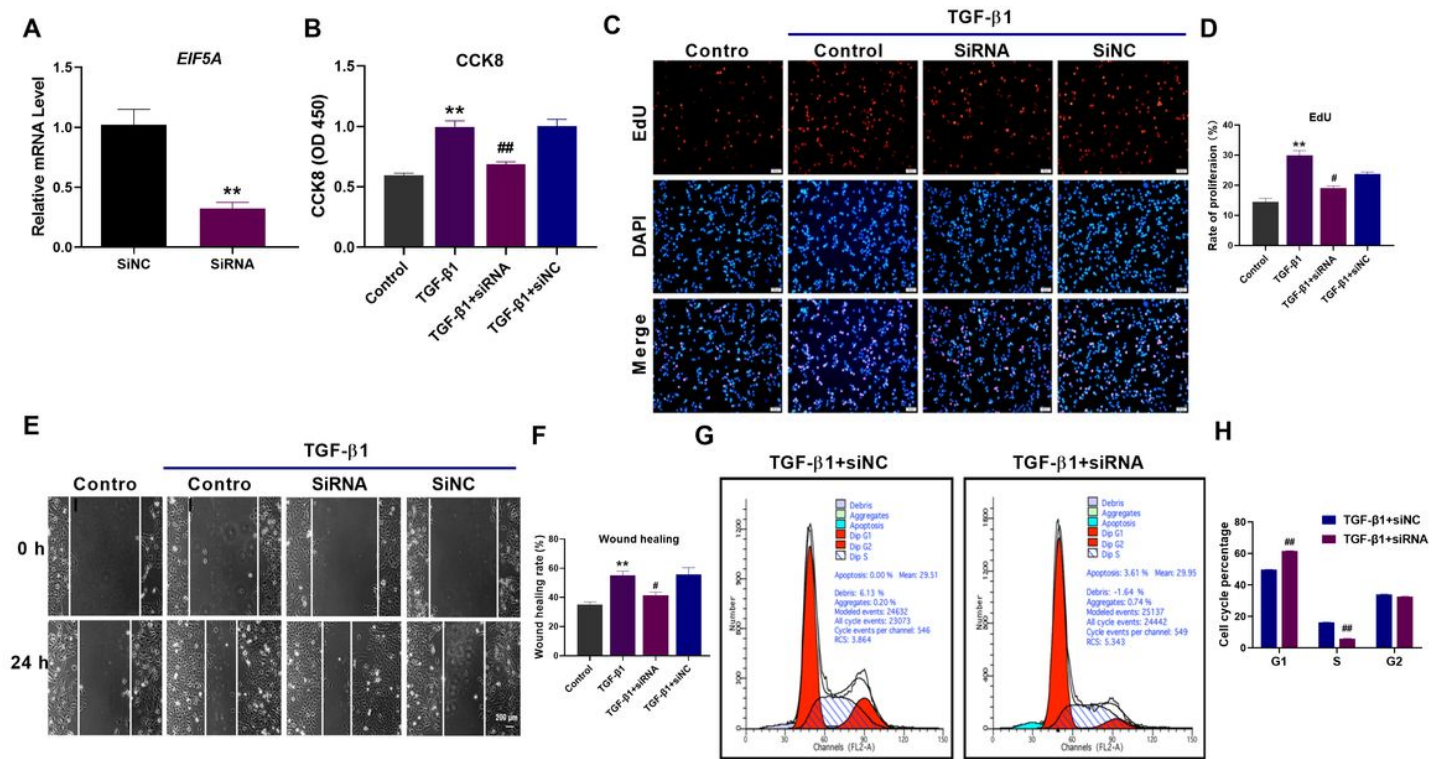


Figure 5

Down-expression of *EIF5A* mitigated TGF- $\beta 1$ -induced human hepatic stellate cells (LX-2) fibrosis. (A) qRT-PCR assay analyzed the silencing efficacy of si*EIF5A* against siNC in treated LX-2 cells (n=4). (B) Cell viability results from different group were showed by CCK-8 assay (n=8). (C, D) Cell proliferation rate was showed that silencing *EIF5A* inhibited activated cells proliferation by EdU assay (n=8). (E, F) Migration ability was analyzed that silencing *EIF5A* retarded the activated cells migration by wound healing assay (n=5). (G, H) Flow assays examined the G1, S and G2 phases of the cell cycle by TGF- $\beta 1$ -driven LX-2 cells (n=3). * $P < 0.05$, ** $P < 0.01$ vs control, # $P < 0.05$, ## $P < 0.01$ vs TGF- $\beta 1$ +siNC.

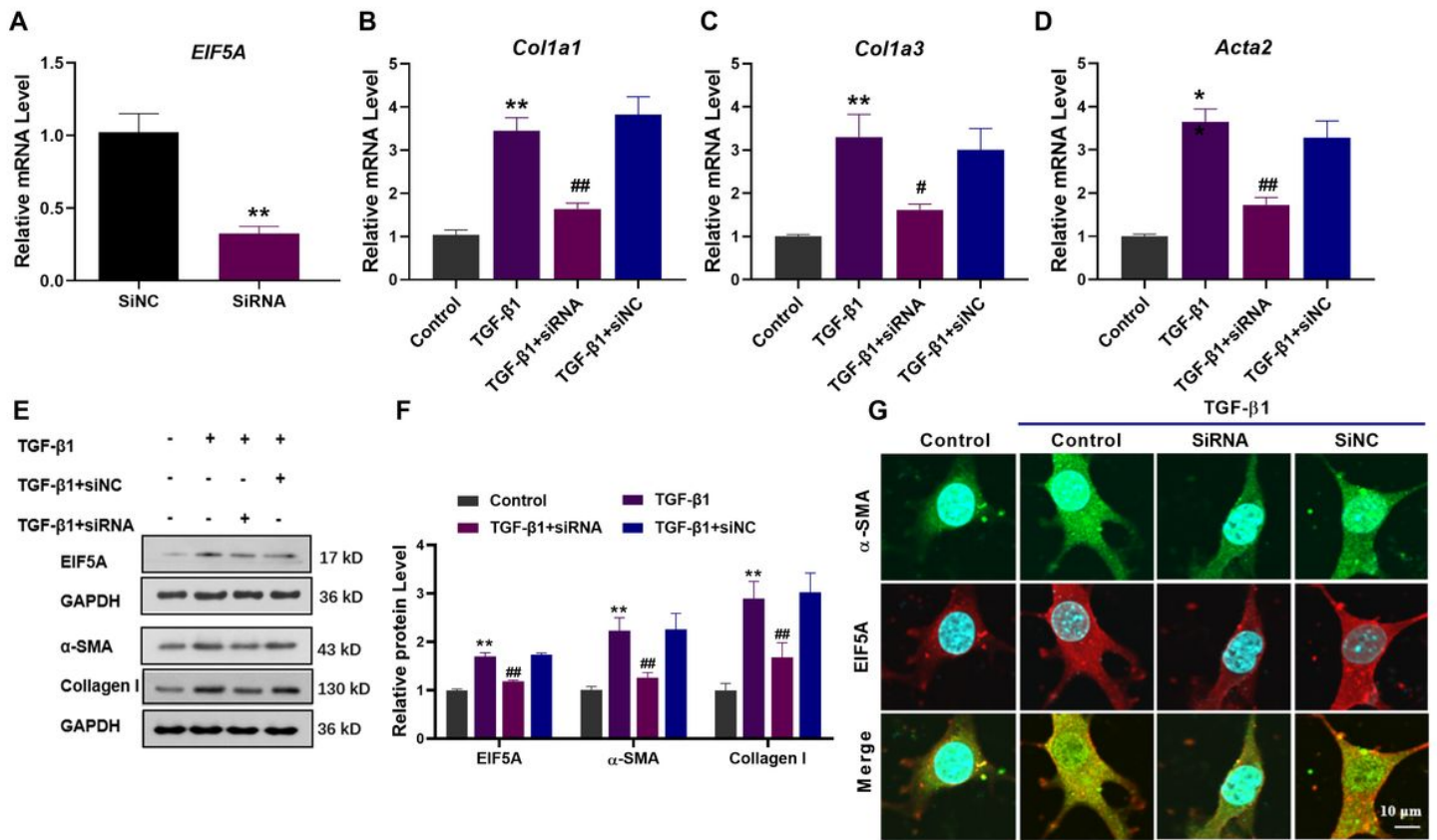


Figure 6

Downregulated *EIF5A* alleviated TGF-β1-induced fibrogenesis in LX-2 cells. (A-D) The relative mRNAs level of *Col1a1*, *Col3a1*, *Acta2* and *EIF5A* were showed by qRT-PCR results (n=6). (E, F) Western blot showed that the expression of related fibrogenesis proteins, including Collagen I, α-SMA and EIF5A (n=4). (G) The representative immunofluorescence photographs visualized co-expression of EIF5A and α-SMA in treated LX-2 cells. *P<0.05, **P<0.01 vs control, #P<0.05, ##P<0.01 vs TGF-β1+siNC.

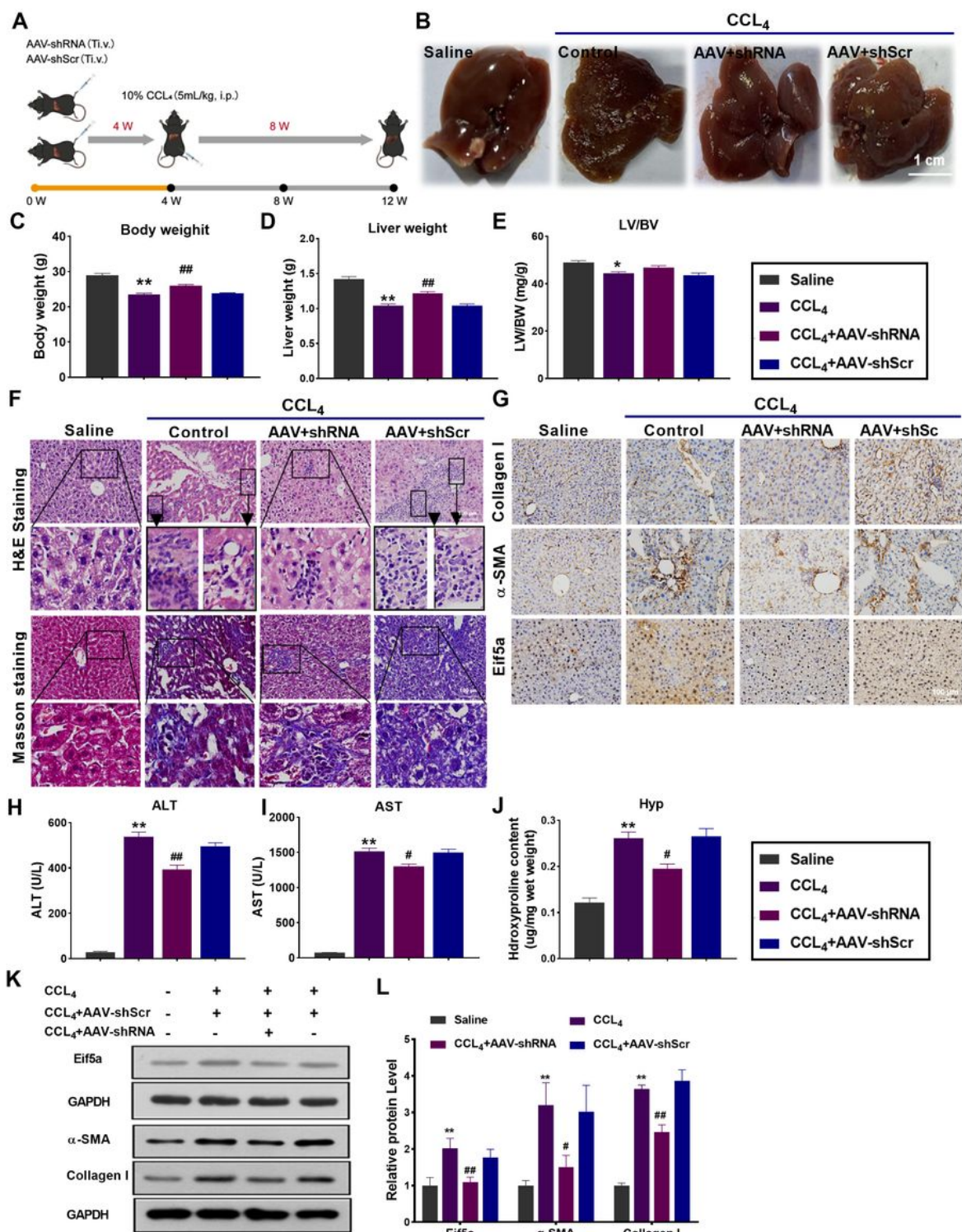


Figure 7

Decreased expression of *Eif5a* retards CCL₄-induced liver fibrosis in mice. (A) Schematic diagram of the experimental design. Mouse were infected with AAV-sh-*Eif5a* and AAV-sh-Scr 30 days ahead of CCL₄-induced treatment. (B) The representative images of mouse livers from were showed from different groups. (C-E) Graphical graphs showed the body weight, liver weight, liver weight/body weight (LV/BV) from different groups (n=5). (F) H&E and Masson staining showed the level of inflammatory infiltrates

and collagen deposition from different groups. (G) Immunohistochemistry staining was used to visualize proteins expression of Collagen I, α -SMA and Eif5a. (H, I) Mouse serology degree of ALT and AST was assayed in different group (n=5). (J) Hydroxyproline content of liver fibrosis tissues were analyzed (n=5). (K, L) Western blots analyzed the inhibited Eif5a mitigated the upregulation of fibrosis-related protein by CCL₄-induced mouse (n=3). *P<0.05, **P<0.01 vs Saline, #P<0.05, ##P<0.01 vs CCL₄+AAV-shScr.

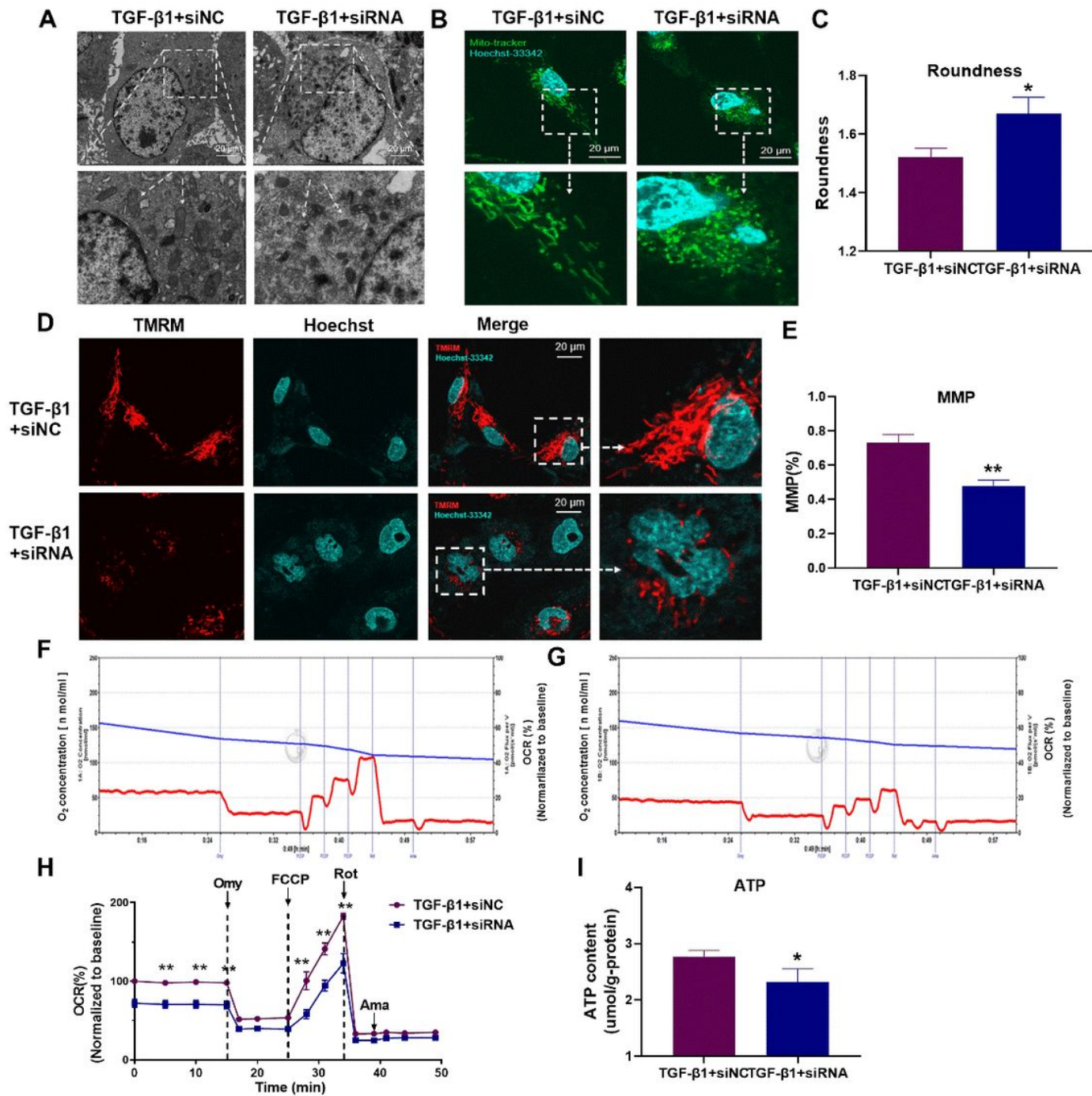


Figure 8

***EIF5A* deficiency inducing mitochondrial remodeling and dysfunction in TGF- β 1-induced fibrogenesis.** (A) Representative photographs elicited the down-regulated *EIF5A* triggered the mitochondrial morphological changes by transmission electron microscopy assay. (B, C) The mitochondrial fluorescence images also observed mitochondrial morphology changes in treated LX-2 cells with Mito-Tracker Green fluorescent labels (n=6). (D, E) Downregulation of *EIF5A* reduced mitochondrial membrane potential by TMRM staining in TGF- β 1 induced fibrogenesis (n=6). (F-H) The original representative images and statistical result of oxygen consumption of LX-2 cells treated with *EIF5A* interference in TGF- β 1 treated LX-2 cells (n=5). (I) Intracellular ATP levels after administration of interfering *EIF5A* by TGF- β 1 induced LX-2 cells (n=4). *P<0.05 vs TGF- β 1+siNC, **P<0.01 vs TGF- β 1+siNC.

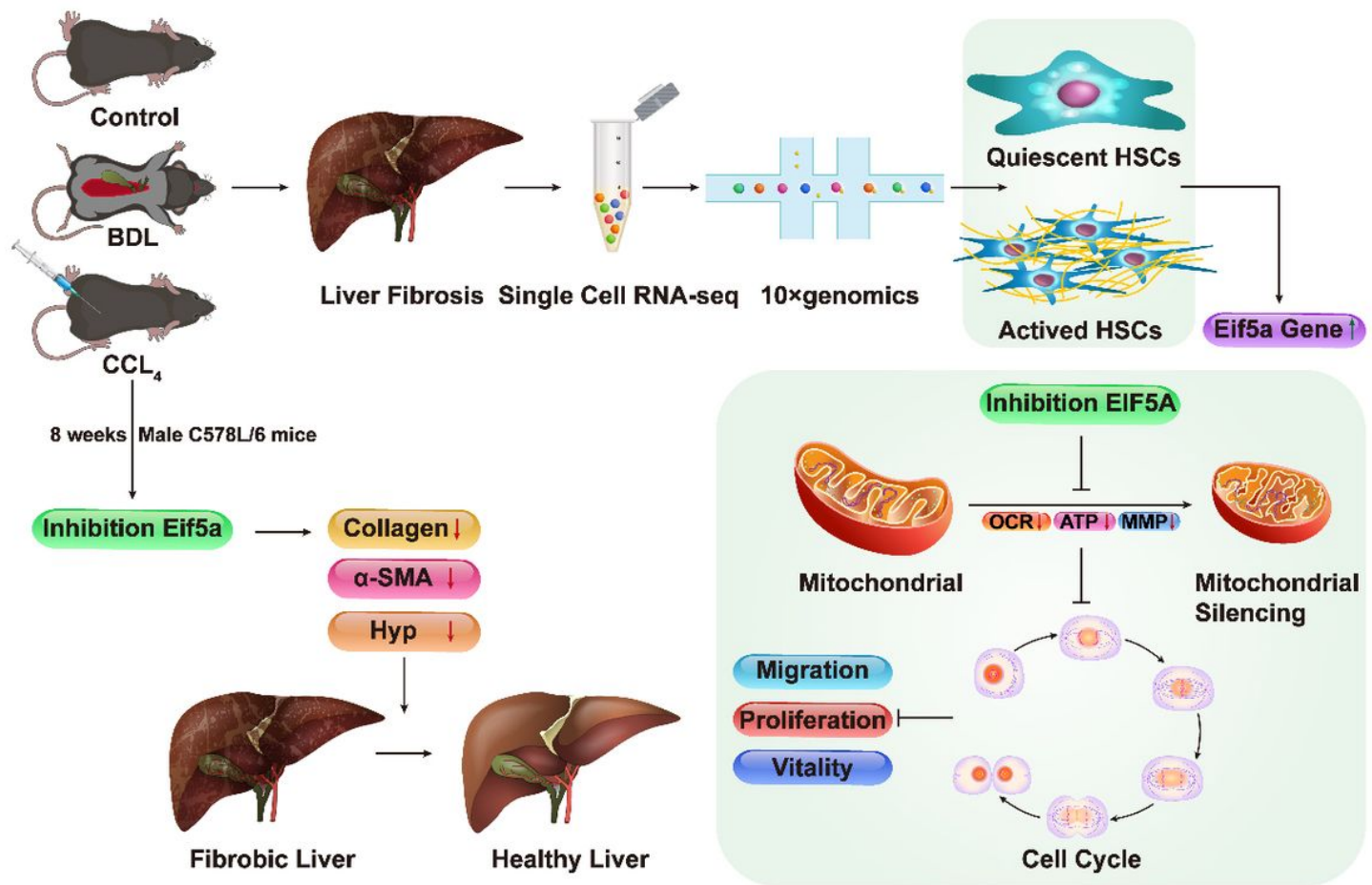


Figure 9

Schematic illustration of the *EIF5A* silencing alleviated liver fibrosis. Silencing *EIF5A* blunted the transition of qHSCs to aHSCs through regulating mitochondrial silencing, by which reducing Collagen, α-SMA and hydroxyproline deposition, as well as retarding the progression of liver fibrosis.

Supplementary Files

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