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Research article

Extracellular vesicles-derived ferritin from lipid-induced hepatocytes regulates activation of hepatic stellate cells

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ABSTRACT

Introduction: and objectives: Extracellular vesicles (EVs) have emerged as key players in intercellular communication within the context of non-alcoholic fatty liver disease (NAFLD). This study aims to explore the intricate crosstalk between hepatocytes and hepatic stellate cells (HSCs) mediated by EVs in NAFLD.

Materials and methods: EVs ferritin was detected in hepatocytes stimulated with free fatty acids (FFA) as well as in NAFLD mice. Deferoxamine (DFO) was employed to reduce ferritin levels, while GW4869 was utilized to inhibit EVs. The impact of EVs ferritin on the HSCs activation was evaluated both *in vitro* and *in vivo*. Additionally, serum EVs ferritin levels were compared between NAFLD patients and controls.

Results: FFA treatment induces the formation and secretion of EVs and facilitates the release of ferritin from hepatocytes via EVs. Subsequently, EVs ferritin is hijacked by HSCs, prompting accelerated HSCs activation. Silencing ferritin with DFO and inhibiting EVs formation and

Abbreviations: EVs, extracellular vesicles; NAFLD, Non-alcoholic fatty liver disease; HSCs, Hepatic stellate cells; FFA, Free fatty acid; DFO, Deferoxamine; ROS, Reactive oxygen species; NASH, Non-alcoholic steatohepatitis; T2DM, Type 2 diabetes mellitus; FTH, Ferritin heavy subunit; FTL, Ferritin light subunit; HFD, High fat diet; CD, Chow diet; MCD, Methionine and choline deficient diet; MCS, Methionine-choline-supplement; NTA, Nanoparticle tracking analysis; BSA, Bovine serum albumin; Hep-EVs, EVs derived hepatocytes; Heps, Hepatocytes; α-SMA, α-smooth muscle actin; MTS, Masson's trichrome staining; Col1a1, Collagen type I α 1.

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secretion with GW4869 can reverse the effects of FFA treatment and disrupt the communication between hepatocytes and HSCs. Accumulation of ferritin leads to excessive reactive oxygen species (ROS) production, promoting HSCs fibrogenesis. Conversely, depleting EVs ferritin cargo restores liver function, concurrently mitigating NAFLD-associated fibrosis. Notably, NAFLD patients exhibit significantly elevated levels of serum EVs ferritin.
Conclusions: This study unveils a previously underestimated role of ferritin in HSCs upon its release from hepatocytes, emphasizing DFO as a promising compound to impede NAFLD advancement.

1. Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) has been rapidly escalating, emerging as a significant global public health concern [1,2]. It encompasses a spectrum of disorders, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma [3,4]. Compelling evidence indicates that NAFLD correlates with an approximately twofold higher risk of developing type 2 diabetes mellitus (T2DM), irrespective of obesity and other prevalent metabolic risk factors. Besides, patients with advanced liver fibrosis are at increased risk of developing T2DM. Furthermore, the resolution or amelioration of NAFLD has been linked to a decreased risk of T2DM, implying that liver-targeted interventions may mitigate the risk of T2DM. Given the myriad complications associated with diabetes, early intervention in NAFLD progression could forestall the onset of diabetes in patients [5,6].

Ferritin, a soluble iron-binding protein, act as a storage molecule for iron [7]. It comprises of two structurally distinct subunits: the heavy subunit (FTH) and the light subunit (FTL). FTH, possessing additional ferroxidase catalytic activity compared to FTL, aids in iron incorporation into ferritin. In normal human physiology, the majority of serum ferritin consists of FTL with low iron content. However, in pathological conditions, FTH levels is significantly increased [8]. FTH plays a regulatory role in immune function, liver cell apoptosis, and cell differentiation [9]. Serum ferritin serves as a potential biochemical marker widely used to assess NAFLD severity and is commonly included in standard examination for chronic liver diseases [10,11]. Clinical data indicate that higher ferritin levels correlate with more severe fibrosis in NAFLD patients [12,13] and increased mortality in those with decompensated liver disease [14]. Additionally, ferritin can exacerbate inflammatory reactions, contributing to HSCs activation by upregulating PKC ζ and MAPK signaling. It also induces NF-κB activation, thereby promoting the expression of pro-inflammatory mediators associated with fibrosis [9]. Meanwhile activated HSCs express specific receptors with high affinity for ferritin, and the binding of ferritin to HSCs depends on FTH [15]. Based on these findings, we are specifically detecting the heavy chain in EVs. Nevertheless, further exploration is needed to elucidate the source and function of highly expressed ferritin in NAFLD.

Extracellular vesicles (EVs) represent a class of extracellular nanovesicles (50–200 nm) released following the fusion of multivesicular bodies [16,17]. These EVs harbor diverse cellular molecules, including proteins and miRNAs [18–22]. And function as pivotal mediators of cell-cell communication by encapsulating and delivering bioactive components to influence biology and function. Consequently, EVEs assume a critical role in the pathogenesis of various diseases, including liver injury, and hold significance as biomarkers or therapeutic targets [22]. Within the realm of NAFLD, secreted EVs participate in intercellular signaling, potentially impacting disease progression [23,24].

Here, we present compelling evidence showcasing a notable increase in the circulation of EVs in both NAFLD patients and cell models, implying their involvement in NAFLD progression. Moreover, EVs derived from FFA-treated hepatocytes exhibit elevated levels of ferritin compared to controls. Mechanistically, our findings unveil that EVs derived from damaged hepatocytes contained ferritin and horizontally transferred it to HSCs and sensitized HSCs to fibrotic responses. This process maybe contingent upon reactive oxygen species (ROS). Lastly, we introduced DFO and GW4869 (EVs inhibition) to hepatocytes to impede the interplay between lipid-laden hepatocytes and HSCs. This novel discovery augments our understanding of how EVs-mediated ferritin affect the fibrosis progression in NAFLD.

2. Materials and methods

2.1. Patients

Patients aged 30–60 years, who underwent serum ferritin evaluation and conventional ultrasound assessment for NAFLD, were recruited at Tongji Hospital (Shanghai, China) from 2015 to 2023. The Healthy control individuals were selected from those undergoing routine physical examinations at Tongji Hospital. All patients had fulfilled the inclusion and exclusion criteria based on ultrasonography and eligibility criteria [25].

The exclusion criteria were positive serological markers for viral hepatitis B or C, alcohol intake, autoimmune hepatitis and human immunodeficiency virus, or other potential causes of liver disease.

2.2. Animal study

6–8 weeks male C57BL/6J mice were procured from Shanghai SLAC. To constructing NAFLD animal models, mice were randomly

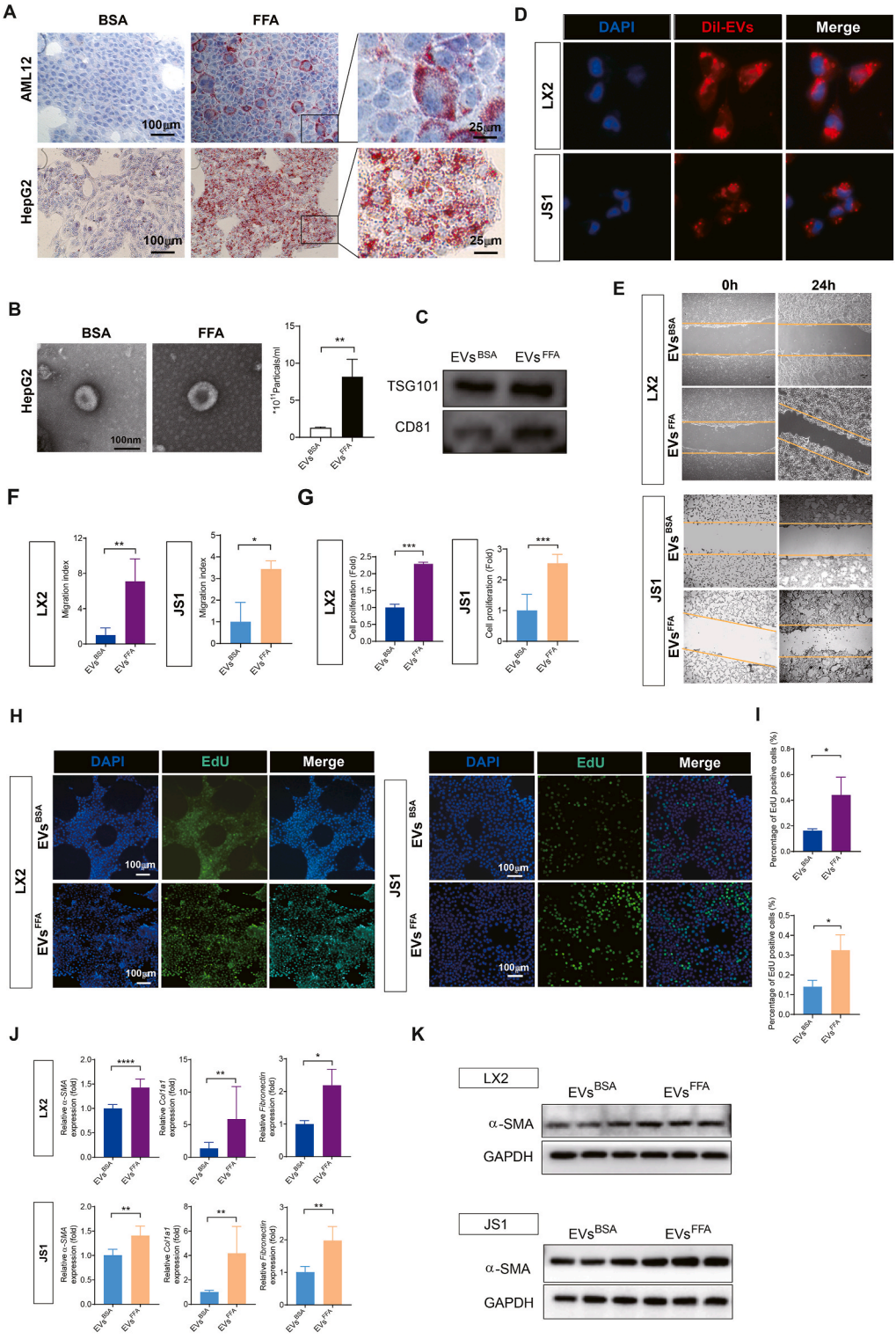

Fig. 1. FFA-induced hepatocytes EVs cause HSCs activation. (A) Hepatocytes were treated with 1 mM FFA for 24 h, lipid accumulation in HepG2 and AML12 was evaluated by Oil Red O staining (the scale bar indicates 100 μm). (B) EVs were isolated by differential ultracentrifugation, transmission electron was used for photomicrographs of hepatocyte EVs. N = 3 (left panel). NTA analysis demonstrates concentration of HepG2 EVs. N = 3 (right panel). (C) Expression of HepG2 EVs markers was measured by Western blot. (D) HSCs were incubated with Dil labeled-EVs for 6 h. LX2 and JS1 cells were visualized for EVs fluorescence (red) and DAPI (blue) by immunofluorescence microscopy. (E–F) Representative microphotographs of LX2 and JS1 I and corresponding quantification (F) graph of wound-healing assay. N = 3 independent experiments. (G) CCK8 assay of LX2

and JS1. N = 3 independent experiments. (H–I) Proliferate cells stained with EdU (H) and the percentage of positive cells (I) to total cells in LX2 and JS1. N = 3 independent experiments. (J) The mRNA expression of fibrogenic molecules by qPCR analysis in LX2 and JS1 subjected to Hep-EVs. N = 3 independent experiments. (K) The protein expression of α-SMA in LX2 and JS1 subjected to Hep-EVs. *P < 0.05 *, p < 0.01 **, p < 0.001 ****. FFA, free fatty acid; EVs, extracellular vesicles; NTA, nanoparticle tracking analysis; HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin.

allocated into four groups, each comprising five mice housed in individual cage. Two cages were assigned either a high fat diet (HFD) (TP23500; Trophic, China) or normal chow diet (CD group) for 16 weeks. The remaining two cages were provided with either a methionine and choline deficient diet (MCD) (TP3005G; Trophic, China) or methionine-choline-supplement (MCS) (TP3005GS; Trophic, China) diet for 8 weeks. Mice were housed in a specific pathogen-free facility at a 12-h light/dark cycle.

For DFO (Sigma-Aldrich, Germany) treatment, 6–8 weeks male C57BL/6J mice were randomly divided into four groups, with five mice housed together in each cage. Two cages of mice were fed with a HFD for 8 weeks, while the other two cages were fed with a MCD for 4 weeks. Within the HFD groups, one cage received DFO (20 mg/mouse) via intraperitoneal injection twice a week for 8 weeks, while the corresponding control cage was treated with PBS. The same treatment duration and method was applied to the MCD groups for 4 weeks (Fig. S). Livers samples from experimental mice were collected within 24 h after the final application for subsequent analysis.

2.3. EVs isolation

Following the specified treatments for 24 h, HepG2 (MD02, Cell Bank, Chinese Academy of Sciences, Shanghai, China) and AML12 cells (GNM42, Cell Bank, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Servicebio, Wuhan, China) without fetal bovine serum (FBS, Gibco, Waltham, USA) for 48 h. EVs were isolated from cell culture supernatants using a method of differential ultracentrifugation and characterized by nanoparticle tracking analysis (NTA) following established protocols [26].

For isolating EVs from patients serum, reagents were added to the serum samples and incubated at 4 °C for 30 min. The resulting pellets were recovered by centrifugation for 10 min at 10,000 g according to the manufacturer's established protocol (Umibio, Shanghai, China). The precipitated EVs were resuspended in PBS and are prepared for downstream analysis.

2.4. EVs uptake experiment

DiI (1 μM, Beyotime, Shanghai, China) was employed to label EVs, and DiI-labeled EVs were then incubated at 37 °C for 1 h. Excess dye was eliminated, and the precipitated EVs were subjected to three washes in PBS through ultracentrifugation at 100,000 g (Beckman Coulter). The labeled EVs were subsequently exposed to HSCs for 6 h following a modified version of the established protocol [27]. The HSCs were stained with DAPI to visualize the nucleus. The uptake of labeled EVs was visualized using confocal microscopy.

2.5. Statistical analyses

All statistical analyses are detailed in the figure legends. Values are presented as mean ± SD in this study. A significance level of $p < 0.05$ was considered statistically significant. Statistical analyses were conducted using GraphPad Prism 9.0 software. Differences between the two groups were compared, unpaired two–tailed Student's t–test for data with significantly different SDs were used. $P < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *.

For detailed methods and materials, please refer to supplemental materials.

3. Results

3.1. FFA-treated hepatocytes produced more EVs than vehicle-treated cells

Hepatocyte cell lines were cultured and supplemented with FFA to establish an *in vitro* high-fat model [28]. After 24 h, lipid droplets accumulated in the FFA-treated group compared to those treated with bovine serum albumin (BSA) (Fig. 1A). Subsequently, we isolated the EVs from FFA- and BSA-treated hepatocytes. Transmission electron micrographs revealed that EVs exhibited a size range of 50–200 nm and a mushroom shape morphology, as previously described (Fig. 1B). To assess whether FFA supplementation affects EV production, the number of EVs was analyzed. Interestingly, FFA-treated hepatocytes released a greater number of EVs compared to vehicle-treated cells, as indicated by nanoparticle tracking analysis NTA. Besides, we obtained the protein amount from the same volume of effluent EVs to compare the protein expressions of TSG101 and CD81, yielding the similar result (Fig. 1C). These findings suggest that FFA treatment induces a lipotoxic environment in hepatocytes, leading to increased EVs production.

3.2. FFA-induced hepatocytes EVs cause HSCs activation

We have confirmed that treating hepatocytes with FFA leads to an increased release of EVs into the supernatant, as documented in the literature [29] (Fig. 1C). To explore the impact of EVs derived from hepatocytes (Hep-EVs) on HSCs, we first evaluated whether EVs

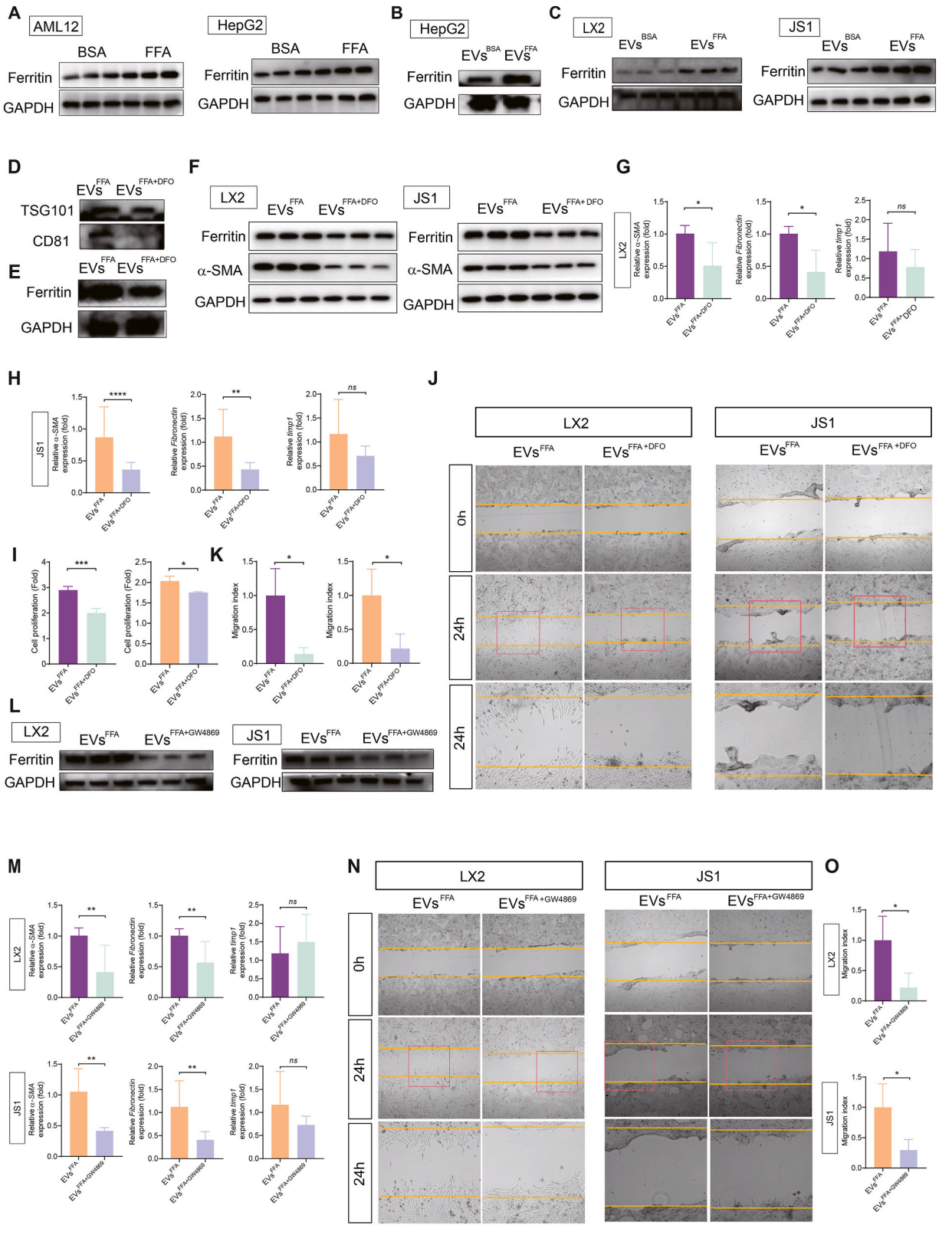

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Fig. 2. Fat-laden Hep-EVs contribute to the profibrogenic effects of HSCs by transferring ferritin. (A) Hepatocytes were treated with FFA for 24 h, and ferritin expression was analyzed by Western blot. (B) Expression of ferritin in EVs derived from HepG2 cells was measured by Western blot. (C) HSCs were exposed FFA- or BSA-treated Hep-EVs, and ferritin expression was analyzed by Western blot. (D) Expression of EVs markers from HepG2 cells was analyzed by Western blot. I Expression of ferritin in EVs derived from HepG2 cells with the listed treatment by Western blot. (F). Hepatocytes were incubated with FFA with or without DFO for 24 h, and EVs were isolated for the following experiments. HSCs were exposed to the listed Hep-EVs, and expression of ferritin and α-SMA was measured by Western blot. (G) The mRNA expression of fibrogenic molecules by qPCR analysis in LX2. (H) The mRNA expression of fibrogenic molecules by qPCR analysis in JS1. (I) CCK8 assay of LX2 and JS1. (J–K) Representative microphotographs of LX2 and JS1 (J) and corresponding quantification graph (K) of wound-healing assay. (L) Hepatocytes were incubated with FFA with or without GW4869 for 24 h, and EVs were isolated for the following experiments. HSCs were exposed to the listed Hep-EVs, and expression of ferritin was measured by Western blot. (M) The mRNA expression of fibrogenic molecules by qPCR analysis in LX2 and JS1. (N–O) Representative microphotographs of LX2 and JS1 (N) and corresponding quantification graph (O) of wound-healing assay. $P < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. Hep-EVs, extracellular vesicles derived hepatocytes; HSCs, hepatic stellate cells; FFA, free fatty acid; BSA, bovine serum albumin; DFO, deferoxamine.

derived from HepG2 or AML12 cells treated with FFA for 24 h could be taken up by HSCs. To investigate this, HSCs were exposed to DiI-labeled EVs (a representative lipophilic tracer). Our observations indicate that Hep-EVs were indeed internalized by HSCs following 24 h of incubation (Fig. 1D).

To delve deeper into the impact of Hep-EVs on HSCs activation, we conducted a scratch assay to evaluate migration, and utilized CCK8 and EdU staining to gauge proliferation of HSCs. The presence of EVs originating from FFA-treated hepatocytes notably augments HSCs migration (Fig. 1E and F), as evidenced by a significant increase in migrating cell numbers following exposure to EVs derived from lipotoxic hepatocytes compared to those from BSA-treated counterparts. Consistent with the scratch assay results, CCK8 (Fig. 1G) and EdU staining (Fig. 1H and I) revealed that EVs from FFA-treated hepatocytes potentially stimulate HSCs proliferation. Moreover, we explored the impact of Hep-EVs on modulating HSCs phenotype. Hep-EVs were incubated with HSCs cell lines for up to 24 h, revealing that mRNA expression of profibrogenic markers in HSCs, including α-smooth muscle actin (α-SMA), collagen-1a1, and fibronectin, was upregulated in EVs derived from lipid-accumulated hepatocytes compared to controls (Fig. 1J). These findings conclusively illustrate that extracellular vesicles (EVs) originating from hepatocytes treated with FFA bolster the expression of fibrosis markers in HSCs and trigger their activation [30] (Fig. 1K).

3.3. Fat-laden hepatocytes EVs contribute to the activation of HSCs by transferring ferritin

Serum ferritin acts as a proinflammatory cytokine during the activation of HSCs [9] and is linked to advanced liver fibrosis in hepatitis [31]. Circulating ferritin may be released from damaged hepatocytes, exerting paracrine effects on other cells [32]. Additionally, studies have demonstrated an increase in ferritin levels during the activation of primary HSCs *in vitro* [33]. Based on these reported literatures and our results, we hypothesized that hepatocyte-derived EVs, induced by lipotoxicity, prompt a phenotypical shift from quiescent to activated HSCs by transporting and delivering ferritin. To validate our hypothesis, we initially compared ferritin expression between lipotoxic hepatocytes and controls. Our results revealed elevated protein expression of ferritin in HepG2 and AML12 cells treated with FFA (Fig. 2A). Furthermore, EVs derived from these cells carried higher levels of ferritin (Fig. 2B). Subsequently, we exposed LX2 cells and JS1 cells to HepG2-EVs or AML12-EVs, respectively, and assessed the protein expression level of ferritin in HSCs. Our findings demonstrated a significant increase in ferritin levels in HSCs exposed to FFA-treated Hep-EVs (Fig. 2C).

DFO, a potent Fe (III) chelator produced by Streptomyces pilosus, binds with high affinity to ferritin, leading to its downregulation [34]. To validate ferritin as a cargo transferred by hepatocyte EVs, we employed DFO and observed a decrease in EVs production (Fig. 2D) accompanied by reduced ferritin content within EVs (Fig. 2E). Subsequently, we cocultured HSCs with hepatocyte-derived EVs treated with FFA alone or with FFA and DFO for 24 h. Remarkably, Remarkably, DFO treatment reversed the FFA-induced increase in EV production, but the addition of DFO to EVs resulted in elevated ferritin expression in HSCs, as previously mentioned. Conversely, DFO-treated hepatocyte EVs counteracted this effect (Fig. 2F). Concurrently, along with decreased ferritin expression in HSCs, we observed reduced fibrogenesis in LX2 and JS1 cells (Fig. 2G and H). Moreover, proliferation (Fig. 2I) and migration (Fig. 2J and K) of HSCs were diminished with EVs derived from hepatocytes treated with both FFA and DFO.

To verify whether EVs mediate ferritin transfer, we utilized GW4869, a potent inhibitor of neutral sphingomyelinase commonly used to inhibit EVs formation and secretion [35]. Notably, administration of GW4869 significantly mitigated the elevated ferritin effect in HSCs (Fig. 2L). Furthermore, similar to DFO, GW4869 inhibited fibrogenesis (Fig. 2M) and HSCs migration (Fig. 2N and O) of HSCs. These findings collectively demonstrated that ferritin transferred from lipotoxic hepatocytes via EVs triggers HSCs activation.

3.4. Ferritin transferred induced HSCs fibrogenic activation is ROS dependent

Ferritin levels have long been as indicative of hyperinflammation, yet their precise pathogenic roles in triggering cytokine storms remain elusive [36]. Previous studies have demonstrated ferritin's ability to promote inflammatory responses in a ROS-dependent manner [37]. Given that ROS serves as a driving force for the phenotypic transformation of HSCs from quiescent into pro-fibrogenic myofibroblasts [38] and activated HSCs isolated from NASH mice exhibit higher ROS levels than those from wild-type mice [33], we hypothesized that ferritin activates HSCs dependent on ROS in lipotoxic environment. As anticipated, ROS levels were elevated in LX2 (Fig. 3A and B) and JS1 (Fig. 3A–C) cells treated with EVs from fat-laden hepatocytes. Treatment with DFO, aimed at reducing ferritin levels in hepatocytes, correspondingly lowered ROS levels in LX2 (Fig. 3D and E) and JS1 (Fig. 3D–F) cells, suggesting

that ROS accumulation is crucial for ferritin-induced HSCs activation.

3.5. Ferritin is elevated in NASH patients and animal models

To investigate the role of ferritin in the development of NAFLD, we established two experimental NAFLD mouse models. Histological analysis revealed liver inflammation, steatosis, and injury in NAFLD mice, as evidenced by HE and Oil Red O staining (Fig. 4A). Liver Masson's trichrome staining (MTS) and Sirius Red staining, highlighting collagen in blue and pink hues (Fig. 4B and C), along with immunostaining for α-SMA (Fig. 4D), a marker of activated HSCs, exhibited significant enhancements in NAFLD mice. Furthermore, we compared ferritin expression between NAFLD mice and the control group. Western blot analysis revealed higher levels of ferritin in the livers of HFD (Fig. 4E) and MCD (Fig. 4F) mice compared to controls.

As previously demonstrated, ferritin may be transferred via increased EVs, prompting us to investigate the presence and levels of serum ferritin in NAFLD. Our results revealed a significant elevation in serum ferritin levels in NAFLD patients compared to controls [39] (Fig. 4G). Consistent with our *in vitro* findings suggesting a high-fat environment promotes EVs production and release, we observed significantly higher levels of EVs in the serum of NAFLD patients compared to healthy individuals. To further confirm ferritin transfer via EVs, we assessed ferritin expression in EVs. Remarkably, the expression of ferritin in serum EVs of NAFLD patients was significantly higher than that in healthy individuals (Fig. 4H). Taken together, these results suggest that released ferritin transferred by EVs induces fibrogenic activity.

3.6. DFO attenuates the progression of fibrogenesis in NAFLD

To further investigate whether reducing ferritin in HSCs could attenuate the establishment and progression of fibrosis in NAFLD, we administered intraperitoneal injections of DFO (20 mg/kg body weight, twice a week) to HFD-fed and MCD-fed mice [33]. Following 8 weeks of treatment in the HFD group and 4 weeks in the MCD group, DFO significantly reduced ferritin expression (Fig. 5A). Subsequently, we observed that DFO-treated mice exhibited alleviated liver injury, as evidenced by HE staining (Fig. 5B), and reduced fibrosis, characterized by lower expression of fibrogenesis-associated genes (Fig. 5C and D), as well as decreased intrahepatic collagen deposition visualized by MTS (Fig. 5E) and Sirius Red staining (Fig. 5F). Furthermore, immunostaining for α-SMA (Fig. 5G), a marker of activated HSCs, indicated that DFO attenuated HSCs activation.

4. Discussion

The intricate functional dynamics of the liver are tightly orchestrated by interactions among its various cellular populations, both parenchymal and non-parenchymal [40]. Paracrine interactions facilitated by EVs released by different liver cell types serve as crucial tools for intercellular communication. The distinct biomolecular cargo of EVs is known to be cell-specific and can influence the activation status of their parent cells [41]. However, the biological significance of EVs and their cargo in intercellular communication involved in HSCs activation during the development of NAFLD remains incompletely understood. In our study, we present a novel finding that the number of EVs is elevated in the supernatant of FFA-treated hepatocytes and in the serum of NAFLD patients. Additionally, we observed increased levels of serum ferritin in NAFLD patients compared to healthy individuals, indicating potential liver damage associated with metabolism. HSCs treated with EVs isolated from FFA-treated hepatocytes exhibited elevated levels of ferritin and enhanced fibrogenic activity, a process dependent on ROS production.

NAFLD has emerged as the most prevalent chronic liver disease. While often benign, some patients may develop hepatocellular injury and inflammation, termed steatohepatitis. Similar to other chronic liver conditions, this process can initiate abnormal wound healing responses leading to liver fibrosis [42]. Recent studies have shed light on the molecular and cellular mechanisms underlying

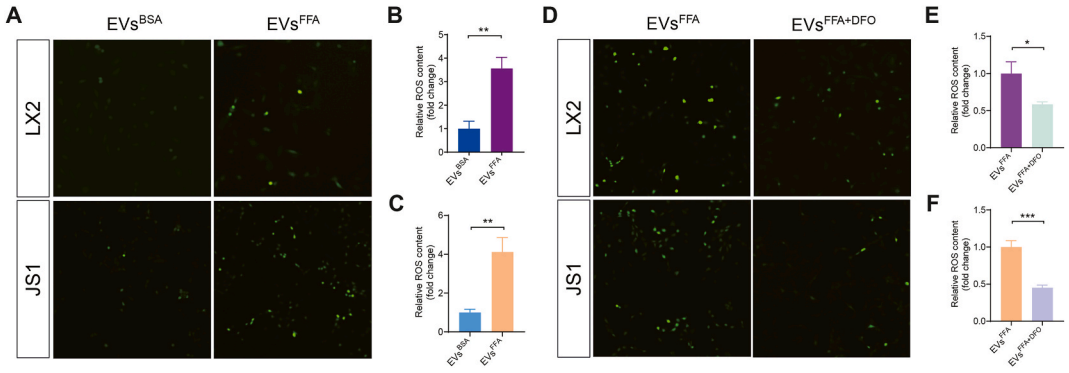

Fig. 3. Ferritin transferred induced HSCs fibrogenic activation is ROS dependent. (A) Effect of Hep-EVs treatment on ROS content in HSCs. (B–C) The quantification of ROS. (D) Hepatocytes were incubated with FFA with or without DFO for 24 h, and EVs were isolated for the following experiments. HSCs were exposed to the listed Hep-EVs, and ROS content was analyzed. (E–F) The quantification of ROS. $P < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. HSCs, hepatic stellate cells; ROS, reactive oxygen species; Hep-EVs, extracellular vesicles derived hepatocytes; DFO, deferoxamine.

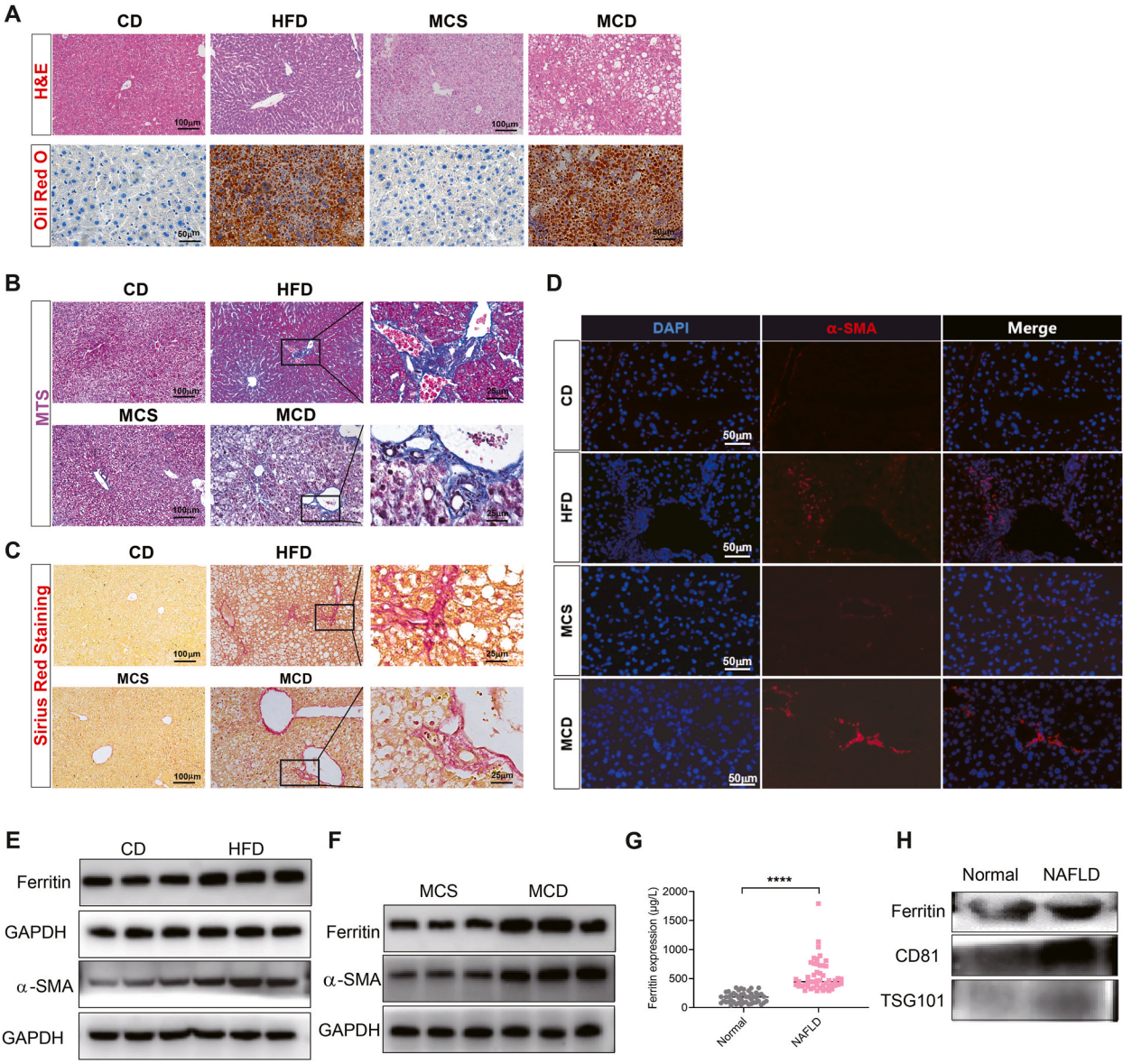

Fig. 4. Ferritin is elevated in NASH patients and animal models. (A–D) We constructed two NAFLD animal models induced by HFD and MCD. The control groups were induced by CD and MCS, respectively. Representative images of H&E, Oil Red O (A), MTS (B), Sirius red staining (C) and immunofluorescence for α-SMA staining (D) of liver tissue from NAFLD mice and controls (scale bars are shown). (E–F) The protein expression of ferritin and α-SMA in the listed groups. (G) The serum was collected from patients with NAFLD and healthy people, who had been evaluated for serum ferritin. (H) EVs were isolated from the serum and EVs population was analyzed by WB for CD81 and TSG101. The expression of ferritin in EVs was analyzed by Western blot. $P < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***. NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; HFD, high fat diet; MCD, methionine and choline deficient diet; CD, chow diet; MCS, methionine-choline-supplement; MTS, Masson's trichrome staining; α-SMA, α-smooth muscle actin; EVs, extracellular vesicles.

liver fibrosis progression in NAFLD [43,44], with particular emphasis on the interaction between various cell types and HSCs, the principal cells responsible for collagen deposition in the liver. Indeed, HSCs enhance their fibrogenic activity by engulfing cellular debris derived from apoptotic cells, which could be a mechanism by which hepatocyte apoptosis promotes fibrosis [45]. In delving deeper into the interaction between hepatocytes and HSCs, EVs have captured researchers' attention as promising mediators. However, the precise relationship between hepatocyte injury, HSCs phenotype modulation, and liver fibrosis in NAFLD remains incompletely understood.

EVs, small vesicles carrying cell-specific cargos, play a pivotal role in understanding chronic liver diseases [17,33]. They can be selectively absorbed by neighboring or distant cells away from their release, thereby reprogramming the characteristics of receptor cells with their bioactive compounds. This holds great significance in deciphering cell-cell interactions and the mechanisms underlying

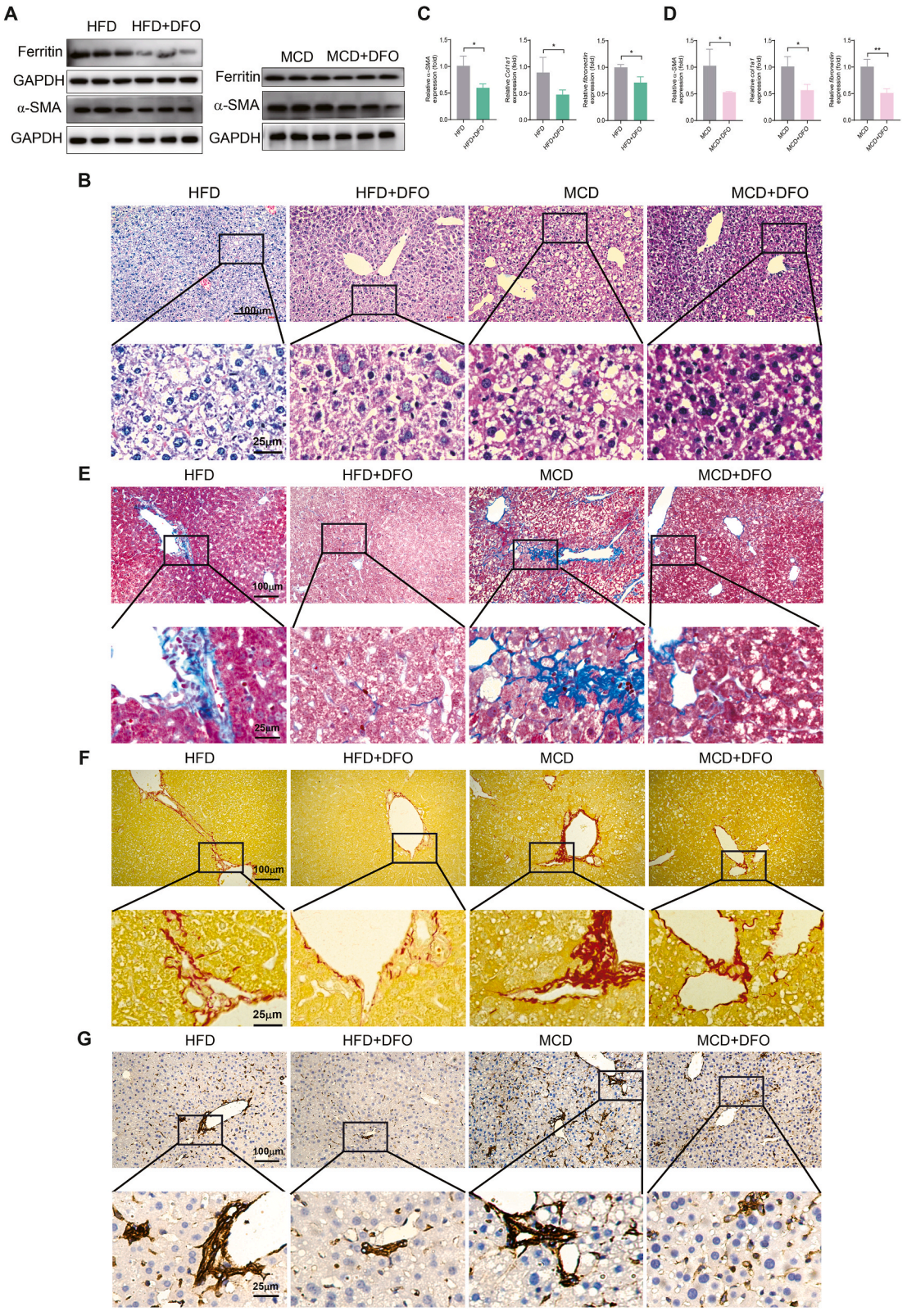

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Fig. 5. DFO attenuates the progression of fibrogenesis in NAFLD. (A) Hepatic expression of ferritin and α-SMA by Western blot analysis. (B) Representative images of H&E staining of liver tissue. (C–D) Hepatic mRNA expression of *α-SMA, Col1a1 and fibronectin* genes by RT-PCR in HFD (C) and MCD (D) groups. I Representative images of MTS of liver tissue. (F) Representative images of Sirius red staining of liver tissue. (G) Immunohistochemistry staining for α-SMA of liver tissue. *$p < 0.05$; **< 0.01; ***$p < 0.001$. DFO, deferoxamine; NAFLD, non-alcoholic fatty liver disease; α-SMA, α-smooth muscle actin; Col1a1, collagen type I α 1; HFD, high fat diet; MCD, methionine and choline deficient diet; MTS, Masson's trichrome staining.

chronic liver diseases. In recent years, there has been growing attention towards EVs as potential biomarkers and therapeutic targets, offering promising avenues for the prevention and treatment of chronic liver diseases [40,46]. However, the role of EVs in NAFLD remains poorly understood. In our study, we observed increased production of EVs from FFA-treated hepatocytes, with alterations in their contents likely induced by FFA-induced lipid accumulation. Thus, quantifying EVs numbers may serve as an important measure for assessing NAFLD progression. Released EVs can interact with target cells to deliver their cargo and transfer molecular information, facilitating cell-to-cell communication [46]. Building upon these findings, our *in vitro* model demonstrated that FFA treatment modifies EVs cargo expression patterns, leading to the transmission of signals that ultimately promote HSCs activation, thereby contributing to the progression from simple steatosis to NASH.

In the initial phases of our research, we observed a significant elevation of ferritin levels in both the serum of NAFLD patients and in EVs obtained from these patients. This led us to hypothesize that ferritin likely plays a crucial role in the progression of NAFLD. Previous studies have indicated that ferritin has the capability to activate signaling pathways in rat HSCs, thereby triggering the upregulation of pro-inflammatory mediators associated with HSCs activation and subsequent liver fibrosis [9]. However, the precise functions and mechanisms underlying the circulation of ferritin remain ambiguous. In our investigation, we discovered that heightened ferritin levels within HSCs can prompt the expression of fibrogenic genes and foster the proliferation of HSCs, ultimately culminating in their activation. During instances of liver injury, the release of ferritin predominantly originates from compromised hepatocytes or Kupffer cells, significantly influencing local ferritin concentrations and potentially exerting direct paracrine effects on neighboring cells [15,32]. Our findings also indicate that damaged hepatocytes release ferritin into the bloodstream, where it can be transported via EVs and subsequently intercepted by HSCs. This process appears to be reliant on ROS and can be impeded by EVs inhibitors and DFO. In summary, our study unveils a novel pathway that connects hepatic lipotoxicity in parenchymal cells to the activation of major fibrogenic cells within the liver, shedding light on potential key mechanisms underlying liver fibrosis in NAFLD. These findings bolster a model wherein an excess of toxic lipids prompts hepatocytes to release EVs, which are subsequently efficiently taken up by HSCs, thereby inducing their phenotypic transition into profibrogenic myofibroblasts. Crucially, this process involves the transport of ferritin cargo. Notably, the utilization of DFO effectively blocked this pathway. These revelations offer deeper insights into the pathogenesis of liver fibrosis in NAFLD and pinpoint potential molecular targets for anti-fibrotic therapeutic strategies.

Ethical statement

The use of human specimens was reviewed and approved by the Shanghai Tongji Hospital Ethics Committee (Approval No. 2023-008) in conformity with the ethical principles of the Helsinki Declaration and with Patients' consent and approval. All animal experiments were reviewed and approved by and in accordance with the guidelines of the Animal Experiment Committee of Tongji Hospital (Approval No. 2023-DW-0801-1231).

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

CRediT authorship contribution statement

Mengxue Sun: Methodology, Investigation. **Min Tang:** Writing – review & editing, Project administration, Investigation. **Yiting Qian:** Methodology, Formal analysis. **Guannan Zong:** Visualization, Validation, Methodology. **Gaowang Zhu:** Visualization, Validation. **Yan Jiang:** Writing – review & editing, Visualization. **Yingjie Mu:** Writing – review & editing, Validation. **Minjun Zhou:** Validation. **Qin Ding:** Writing – review & editing. **Hao Wang:** Writing – original draft, Software, Conceptualization. **Fengshang Zhu:** Funding acquisition, Conceptualization. **Changqing Yang:** Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2024.e33741.

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