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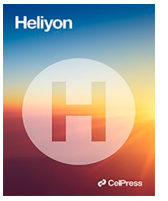

Research article

CD47, a novel YAP target gene, contributes to hepatic stellate cell activation and liver fibrosis induced by high-fat diet

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ABSTRACT

Activated hepatic stellate cells (HSCs) have been widely recognized as a primary source of pathological myofibroblasts, leading to the accumulation of extracellular matrix and liver fibrosis. CD47, a transmembrane glycoprotein expressed on the surface of various cell types, has been implicated in non-alcoholic fatty liver disease. However, the precise role of CD47 in HSC activation and the underlying regulatory mechanisms governing CD47 expression remain poorly understood. In this study, we employed single-cell RNA sequencing analysis to investigate CD47 expression in HSCs from mice subjected to a high-fat diet. CD47 silencing in HSCs markedly inhibited the expression of fibrotic genes and promoted apoptosis. Mechanistically, we found that Yes-associated protein (YAP) collaborates with TEAD4 to augment the transcriptional activation of CD47 by binding to its promoter region. Notably, disruption of the interaction between YAP and TEAD4 caused a substantial decrease in CD47 expression in HSCs and reduced the development of high-fat diet-induced liver fibrosis. Our findings highlight CD47 as a critical transcriptional target of YAP in promoting HSC activation in response to a high-fat diet. Targeting the YAP/TEAD4/CD47 signaling axis may hold promise as a therapeutic strategy for liver fibrosis.

1. Background

Nonalcoholic fatty liver disease (NAFLD) is a progressive disorder that is strongly associated with dysregulation of lipid metabolism. It is characterized by the accumulation of lipids, formation of lipid droplets, and hepatocellular injury, including hepatocellular steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma. The global incidence of NAFLD is currently estimated to be 25 %, leading to a significant increase in the economic burden associated with this disease [1]. Liver fibrosis represents a crucial stage in the advancement of NAFLD and is closely linked to the development of cirrhosis and potentially

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hepatocellular carcinoma. Despite ongoing clinical trials, there are presently no approved pharmacological interventions for NASH treatment, primarily due to an incomplete comprehension of the underlying mechanisms and progression of liver fibrosis [2–4].

Liver fibrosis is a dynamic and reversible process characterized by excessive accumulation of extracellular matrix (ECM) in the liver. Activated hepatic stellate cells (HSCs) is now well established as the primary source of ECM in liver tissue and the key driver of hepatic fibrosis [5]. HSCs reside in the disse space and remain in a quiescent state under normal conditions. However, in response to liver injury, HSCs undergo activation and begin to secrete ECM proteins. Initially, this activation and ECM secretion contribute to the healing process and restoration of the damaged liver tissue. However, prolonged and excessive activation of HSCs, characterized by increased proliferative activity, can lead to the excessive deposition of collagen and other ECM components, ultimately resulting in the development of liver fibrosis [6]. Therefore, the development of anti-fibrotic therapies that specifically target the activation of HSCs represents a promising treatment strategy. However, the process of HSC activation is intricate, involving multiple signaling pathways and mediators, such as transcriptional regulation and receptor-mediated signals. There is thus an urgent need for further research to elucidate the molecular mechanisms underlying HSC activation. Such studies will contribute to a better understanding of liver fibrosis pathogenesis and aid in the identification of potential therapeutic targets for anti-fibrotic interventions.

CD47 is a membrane receptor glycoprotein that plays diverse roles in cellular processes such as cell migration, cell signaling, apoptosis, and immunomodulation. Structurally, it consists of a heavily glycosylated N-terminal IgV domain and five transmembrane helices domains [7,8]. CD47 whole-body knockout mice performed lower blood sugar levels and increased insulin sensitivity in a high-fat diet-induced obesity model [9]. While mice with diet-induced NASH showed lower levels of inflammation and fibrosis after treatment with CD47 antibodies [10]. Previous studies have reported that the increased expression of CD47 in necrotizing hepatocytes prevents macrophages from phagocytosis in mice model, which aggravates inflammation and hepatic fibrosis [11]. In patients with NAFLD, the expression of CD47 protein in the erythrocyte membrane has been found to decreased, which is potentially due to the increased inflammation [12]. These studies confirmed that CD47 can be associated with hepatic fibrosis, although the direct role of CD47 in high-fat diet-induced HSCs activation remain unclear. In parallel, further investigation is required to elucidate the mechanisms involved in the regulation of CD47 expression during this process.

In this study, we employed single-cell RNA sequencing analysis and observed a significant increase in CD47 expression in HSCs induced by a high-fat diet. Functional experiments showed that CD47 knockdown effectively inhibited HSC proliferation and induced apoptosis by suppressing the AKT/mTOR signaling pathway. Furthermore, we identified CD47 as a downstream target gene regulated by the YAP/TEAD4 signaling pathway. Notably, pharmacological inhibition of YAP protected mice from liver fibrosis and led to a reduction in CD47 expression in HSCs. Our findings highlight the crucial role of CD47 in HSC activation and suggest that it can serve as a promising target for anti-fibrotic therapies. Inhibitors targeting CD47 or strategies aimed at selectively blocking the YAP/TEAD4/CD47 signaling axis in HSCs hold potential as effective anti-fibrotic therapeutic approaches.

2. Materials and methods

2.1. Animals

C57BL/6J male mice were purchased from Hua Xing Laboratory Animal Co. in Henan Province, China. The 4-week-old mice were randomly divided into a normal diet group (ND) and a high-fat diet (HFD, protein, 20 %; fat, 60 %; carbohydrates, 20 %; HFK Bioscience, Beijing, China) for a period of 20 weeks, (n = 5/group). All mice used in this study were treated humanely according to institutional animal care guidelines, and the study was approved by the Animal Ethics Committee of the Fifth Affiliated Hospital of Zhengzhou University (Ethics No:KY2022045). All experimental protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals developed by the Ministry of Science and Technology of China.

2.2. cDNA library construction and single-cell RNA-sequencing

Fresh livers from ND and HFD groups were dissociated into single cell suspensions. After dissociation, single-cell suspensions were evaluated for viability (>90 %) and were immediately loaded on the Chromium Single Cell Controller Instrument following the manufacturer's instructions (10 × Genomics, Pleasanton, CA, USA). Library and sequencing were performed on a NovaSeq 6000 platform. scRNA-seq libraries construction and downstream data analysis were performed by Shanghai Biotechnology Corporation. All raw sequences were submitted to Genome Sequence Archive (Accession Number: CRA013859).

2.3. Cell culture and transfection with siRNA

T6 and LX-2 cells were obtained from pre-frozen storage in the laboratory of the Fifth Affiliated Hospital of Zhengzhou University. T6 and LX-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with antibiotics (100 μg/ml penicillin and 100 U/ml streptomycin) and 10 % fetal bovine serum (Gibco, Thermo Fisher). CD47 small interfering (siRNA) (5′-GGAAUGACCUCUUUCACCATT-3′; siYAP (5′-GACAUCUUCUGGUCAGAGATT-3′); siTEAD4 (5′-GGAACAAACUGUGCCUGAA-3′); siYAP (5′-GACAUCUUCUGGUCAGAGATT-3′); orscramble nonsense siRNA (′5′-UUCUCCGAACGUGUCACGUTT-3′) were transiently transfected to cells with Lipofectamine 3000 according to the manufacturer's instructions. Cells were collected and analyzed at 48 h after siRNA transfection. Primary HSCs (rHSC-primary) were isolated from normal liver tissue or HFD liver tissue by collagenase digestion followed by centrifugation in an OptiPrep gradient, as previously described [13]. Cells were maintained at 37 °C in a humidified incubator with 5 % of CO_2.

2.4. Histology and immunohistochemistry

Liver tissues were fixed in 4 % paraformaldehyde and embedded in paraffin. Tissue sections (4 μM) were used for Sirius red and Masson staining to examine the extent of liver fibrosis. For immunofluorescence, liver sections were deparaffinized followed by antigen retrieval. Slides were incubated with primary antibodies, including Anti-CD47 (1:500, proteintech), anti-α-SMA (1:500, proteintech) at 4 °C overnight. The Cy3 and Alexa Fluor® 488-conjugated secondary antibody was incubated for 2 h at room temperature. The cell nucleus was stained by DAPI (4',6-diamidino-2-phenylindole). Images were captured under a microscope (Nikon, Tokyo).

2.5. Western blotting analysis

Cells were rinsed with PBS and were lysed in lysis buffer (50 mM Tris-HCl, pH7.5, 1 mM EDTA, 1 % NonidetP-40, 150 mM NaCl, 10 % glycerol) containing protease inhibitors for 30 min on ice. Cell lysis supernatant was collected after centrifuge and applied to 10 % SDS-PAGE gels. The primary antibodies CD47, α-SMA, YAP, and GAPDH (proteintech, USA) were probed with antibodies. Blots were visualized by chemiluminescence. Quantitative determination of band intensity involved using software ImageJ.

2.6. Quantitative real-time PCR (qPCR)

Total RNA was extracted from cell lines or primary HSCs by using TRIzol reagent (Invitrogen, USA) and reverse-transcribed into complementary DNA (TaKaRa, Japan). Quantitative real-time PCR involved the SYBR green-based assay with LightCycler 480 II (Roche, USA). The GAPDH was used as an internal reference. The sequences of primers for qPCR were in Supplementary Table 1.

2.7. Plasmid construction and dual luciferase reporter assay

The activated YAP-overexpression plasmid (pCDNA-YAP^{S127A}) was a gift from Professor Xiangzhan Zhu at Children's Hospital Affiliated of Zhengzhou University. The proximal promoter fragment of CD47 gene covering −2713 to −1 base pair was cloned into PGL4 basic luciferase reporter vector (Promega). For luciferase reporter assay, pcDNA-YAP ^{S127A}, pGL4-CD47 promoter luciferase reporter plasmids together with pRL Renilla luciferase reporter vector was transfected into T6 cells in 24-well plates. The Dual Luciferase Assay System (Promega; E1910) kit was employed to detect the luciferase activity according to the manufacturer's instructions. After a 36-h transfection period, the cells were harvested immediately upon the addition of passive lysis buffer. This was done by vigorously scraping the cells with a disposable plastic cell lifter. To ensure complete cell lysis, the cell lysate underwent 1 or 2 freeze-thaw cycles. Subsequently, a mixture of 20 μL of cell lysate and 100 μL of luciferase substrate Reagent A was prepared in a sample plate and promptly detected using a luminometer (referred to as "ValueLuciferase"). Following that, 100 μL of Renilla luciferase substrate Reagent B was added to the sample plate and briefly vortexed for mixing. The sample was then immediately detected using a luminometer (referred to as "ValueRenilla"). The relative luciferase activity was calculated as the ratio of ValueLuciferase to ValueRenilla.

2.8. Chromatin immunoprecipitation (ChIP) assay

T6 cells were subjected to cross-linking using 1 % formaldehyde at room temperature, and the cross-linking reaction was terminated after 10 min by treating the samples with glycine. Subsequently, the samples were sonicated using a Sonicator (Diagenode) to fragment the DNA into fragments ranging from 100 to 500 base pairs. The resulting DNA fragments were then incubated with protein A/G beads conjugated to TEAD4 antibody. Following the immunoprecipitation step, the DNA fragments were eluted and subjected to decrosslinking to reverse the cross-linking process. These eluted DNA fragments served as templates for reverse transcription polymerase chain reaction (RT-PCR) analysis. The primer sequences used for quantitative PCR (qPCR) can be found in Supplementary Table 2.

2.9. Statistical analysis

All values were presented in the figures as mean ± SD, with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns, no significance. The number of animals (n) used in the experiments can be found in the figures. Two groups of a single variable were compared using unpaired, two-tailed Student's *t*-test. Statistical significance analysis was performed with GraphPad Prism software.

3. Results

3.1. CD47 is highly expressed in HSCs induced by HFD treatment

To investigate the expression of CD47 in HSCs between the ND and HFD groups, we conducted single-cell transcriptome profiling of liver samples from both groups. Each group consisted of three biological replicates. The profiling was performed using a 10X Genomics system. After applying quality filtering, we obtained a total of 17,260 cells from the HFD group and 20,734 cells from the ND group. These cells were classified into 11 distinct cell populations (Fig. 1A). Further analysis involved comparing the proportions of these 11 cell populations between the HFD and ND groups. Interestingly, we observed a significant increase in the proportion of monocyte cells

specifically in the HFD group, while the proportions of other cell types remained comparable between the two groups (Fig. 1B). Although the number of HSCs did not differ significantly between the HFD and ND groups, transcriptome sequencing data analysis revealed a substantial upregulation of CD47 expression in HSCs from the HFD group (Fig. 1C and D). Additionally, HSCs isolated from ND mice exhibited lower levels of CD47 expression compared to those from HFD mice (Fig. 1E).

3.2. Knockdown of CD47 prevents HSCs activation

To assess the impact of CD47 on HSCs activation, the expression of fibrosis-related markers α-SMA and COL1A1 was detected by RT-PCR and western blot after CD47 knockdown. The results showed that the mRNA levels of α-SMA and COL1A1 were significantly reduced after CD47 knockdown compared with siNC group ($P < 0.001$) (Fig. 2A). In addition, the fluorescence intensity of α-SMA was significantly decreased in T6 cells after CD47 knockdown compared with that in control group (Fig. 2B). Similarly, the protein expression of α-SMA was also significantly decreased after CD47 knockdown (Fig. 2C).

3.3. CD47 promoted HSCs activation via regulating AKT/mTOR signaling

To further investigate the specific regulatory role of CD47 in HSCs activation mechanism, we conducted an analysis of the top 20 differentially regulated signaling pathways in HSCs derived from ND and HFD groups. Through KEGG pathway enrichment analysis, we identified significant differences in the apoptosis and mammalian target of rapamycin (mTOR) signaling pathways between the two groups (Fig. 3A). To explore the functional consequences of CD47 silencing on HSCs, we examined the proportion of apoptotic T6 cells after CD47 knockdown using flow cytometry (Fig. 3B). Notably, we observed an increased proportion of apoptotic T6 cells following CD47 silencing. This finding suggests that CD47 may play a role in regulating apoptotic processes in HSCs(Fig. 3B). Furthermore, the mTOR signaling pathway was found to be a central node in the regulatory network of cellular processes governing cell growth. Activation of the mTOR pathway leads to increased cell proliferation, accompanied by an increase in cell number and size. These observations indicate a potential involvement of CD47 in modulating the mTOR signaling pathway, thereby influencing the growth

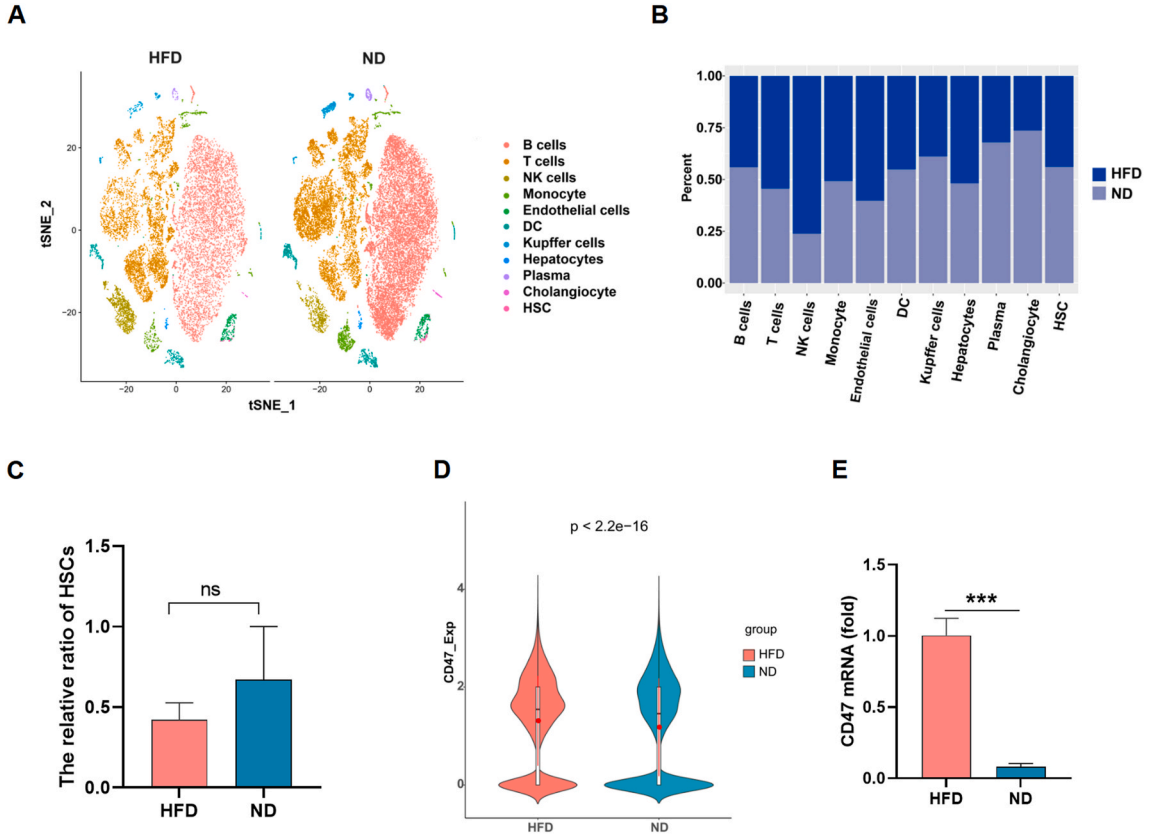

Fig. 1. Cell landscapes of mice livers based on single-cell transcriptome profiles. (A)t-SNE plots of single-cell clusters. The Seurat algorithm was used to visualize the clustering of all cells 20,734 derived from ND and 17,260 single cells derived from HFD mice.**(B)** Depicting the ratio of different cell subtype in ND vs. HFD. **(C)** Depicting the ratio of HSCs subtype in ND vs. HFD. **(D)** Expression of CD47 mRNA enriched in HSCs from ND and HFD based on RNA-Seq data. **(E)** Quantification of CD47 mRNAs in primary HSCs from ND and HFD. The ns refers to non-significant findings,***$P < 0.001$ (n = 3 mice/group).

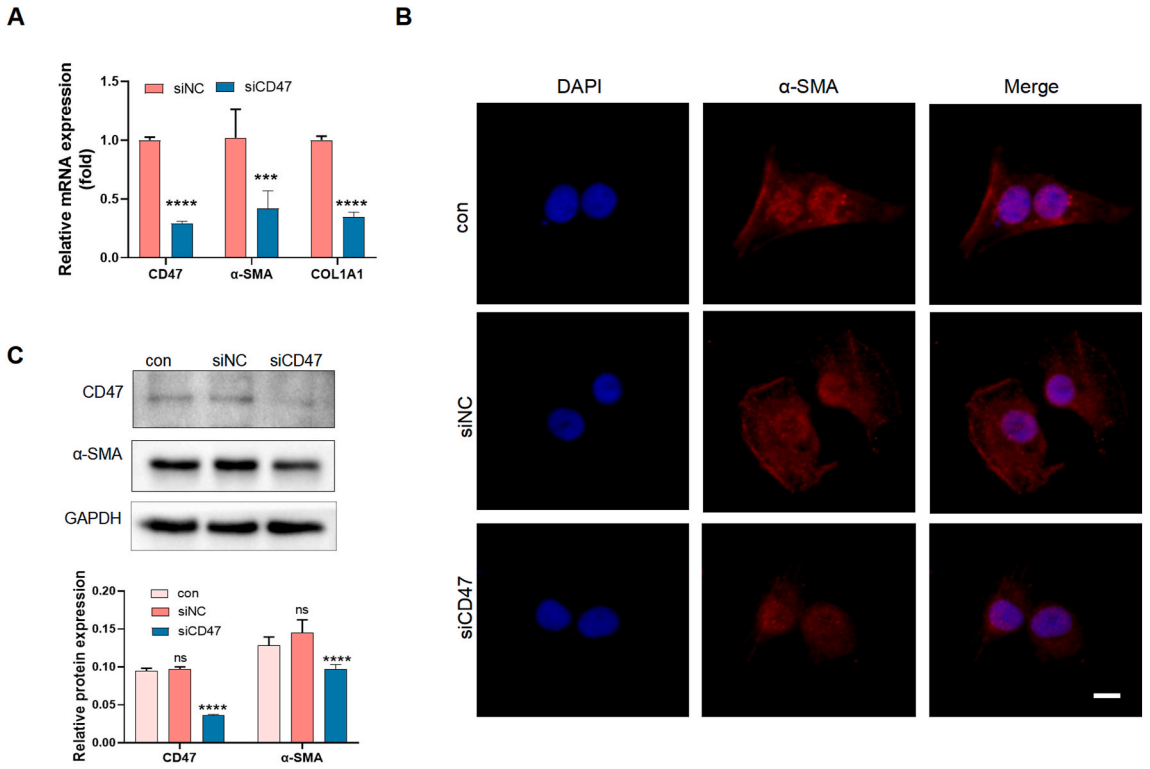

Fig. 2. Knockdown of CD47 significantly inhibited HSCs activation. (A) qPCR detected the mRNA levels of fibrosis-related genes α-SMA and COL1A1 in T6 cells after CD47 knockdown. **(B)** Representative immunofluorescence images of T6 cells immunoassayed with anti-α-SMA. Scale bar, 50 μm. **(C)** The protein expression of CD47 and α-SMA were analyzed by western blot (The original blot is provided in the Supplementary file). The experiments were independently repeated three times. The ns refers to non-significant findings; ****$P < 0.0001$, compared with siNC group.

and activation of HSCs [14]. Then the expression levels of AKT, mTOR, p-AKT, p-mTOR, and CD47 were detected after CD47 knockdown using western blot. The results showed that the expression of p-AKT and p-mTOR was significantly reduced after CD47 knockdown (Fig. 3C). Meanwhile, the effect of CD47 knockdown on cell viability was detected using CCK-8 (Fig. 3D and E); The cell viability was significantly reduced in CD47 knockdown group compared with siNC group ($P < 0.05$). These data indicated that CD47 knockdown inhibited HSCs activation, which can be related to the activity of AKT/mTOR signaling pathway.

3.4. CD47 is a direct downstream target of YAP in HSCs

The Hippo/YAP pathway is a signaling pathway that integrates diverse mechanical and biochemical signals to orchestrate cell state transitions. It has garnered significant recognition as a crucial regulatory pathway in the context of liver fibrosis [15]. Immunofluorescence and western blot analysis showed that the knockdown of YAP significantly inhibited the expression of CD47 and α-SMA (Fig. 4A and B). Furthermore, CCK-8 assay showed that the cell viability was significantly reduced in YAP knockdown group compared with it from siNC group (Fig. S1A). Notably, a potential binding site (BS) (−1984bp-1973bp) for TEAD4 that is a transcriptional co-activator of YAP, in the promoter region of CD47 which were analyzed from JASPAR database (Fig. 4C). The dual-luciferase reporter assay showed that YAP positively regulated the expression of CD47 ($P < 0.01$) (Fig. 4D). In parallel, ChIP assay showed that TEAD4 directly can bind to the promoter of CD47 (Fig. 4E). Consistent with results above, YAP knockdown or disrupting YAP/TEAD4 interaction by Verteporfin significantly inhibited the expression of CD47 and reduced cell viability in HSCs (Figs. S1B–E).

3.5. Pharmacological inhibition of YAP prevents HSCs activation and alleviates liver fibrosis

To explore the role of YAP/TEAD4 in CD47-related HSCs activation and liver fibrosis in vivo, HFD mice were treated by Verteporfin, a YAP/TEAD4 inhibitor. C57BL/6 mice were fed with HFD for 10 weeks, followed by treatment with Verteporfin for 10 weeks (100 mg/kg, i.p. at 3-day intervals, with continued HFD feeding). We found that the expression of α-SMA and CD47 was more detectable in the livers in HFD mice than that in ND mice. However, Verteporfin treatment significantly blocked the upregulation of α-SMA and CD47 (Fig. 5A). We also detected the expression of CD47 and α-SMA in primary HSCs isolated from ND and HFD mice. Verteporfin treatment decreased the activation-responsive mRNA expression of fibrotic genes, such as CD47, α-SMA, and CTGF (Fig. 5B). As compared with HFD mice, Verteporfin treatmentalleviate HFD-induced liver fibrosis, as shown by Sirius red and Masson staining (Fig. 5C and D).

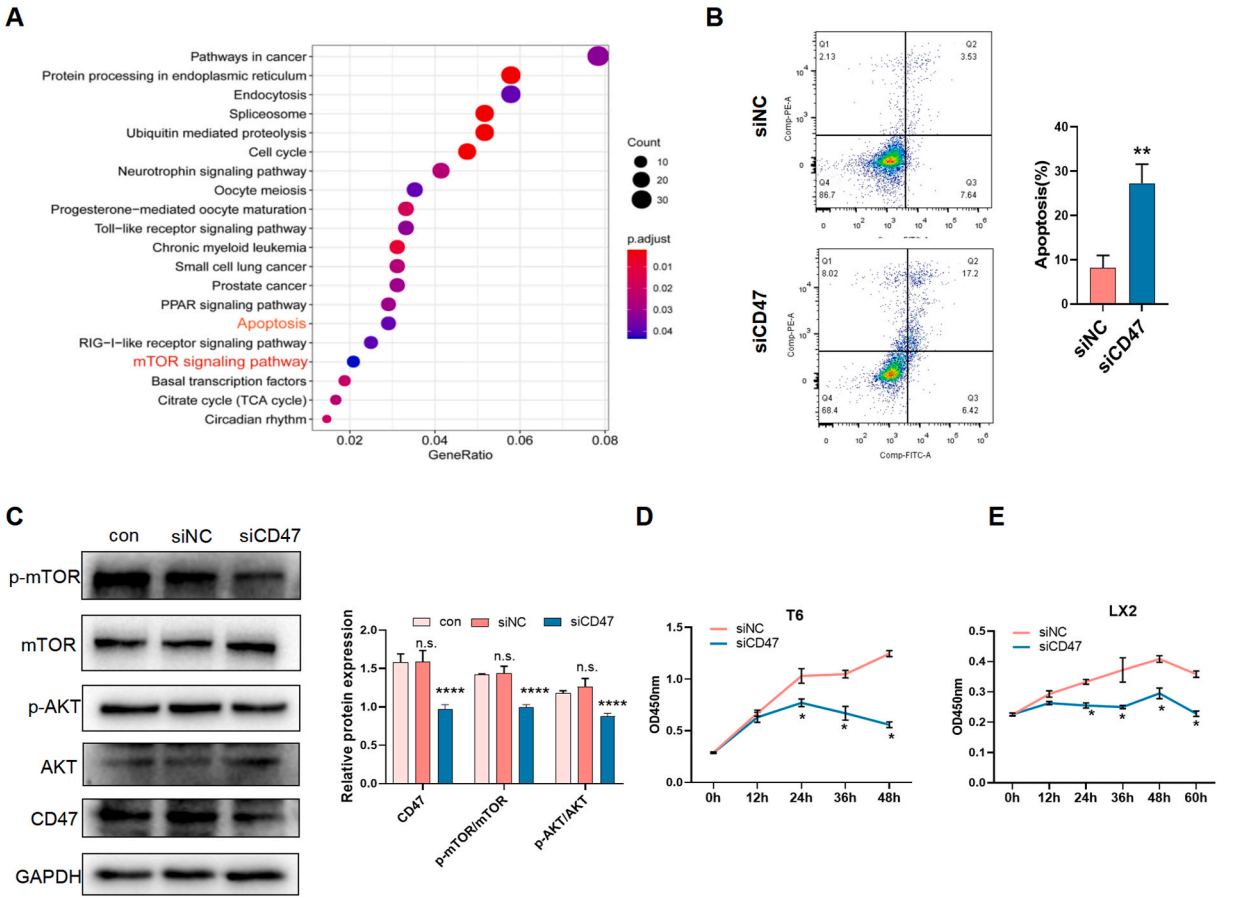

Fig. 3. Knockdown of CD47 promotes apoptosis in HSCs cells by inhibiting AKT/mTOR signaling activity. (A) The differentially altered genes was manifested through KEGG pathway enrichment analysis. **(B)** The ratio of apoptosis was detected by flow cytometry, n = 3. **(C)** The protein expression of AKT, p-AKT, mTOR, p-mTOR was analyzed by Western blot, n = 3(The original blot is provided in the Supplementary file). **(D, E)** The viability of T6 (D) and LX-2 (E) cells was measured using CCK-8 assay at designed time, n = 3.*$P < 0.05$, **$P < 0.01$.

4. Discussion

In this study, we aimed to investigate the role of CD47 in the development of liver fibrosis, with a particular focus on its regulation by the YAP signaling pathway and its effects on activated HSCs. Using single-cell RNA sequencing, we observed a significant upregulation of CD47 expression in activated HSCs induced by HFD. This finding suggests that CD47 may be involved in HSC activation and the progression of liver fibrosis. We further explored the functional implications of CD47 in HSCs and found that CD47 inhibited apoptosis and promoted cell proliferation in HSCs by activating the Akt/mTOR signaling pathway. This suggests that CD47 may contribute to the sustained activation and proliferation of HSCs, which are key drivers of liver fibrosis. , we revealed a novel regulatory mechanism linking CD47 and the YAP signaling pathway. We identified CD47 as a downstream target gene of the YAP/TEAD4 transcription complex. This provides mechanistic insights into the interplay between YAP signaling, CD47 expression, activated HSCs, and liver fibrosis. The present work shed light on the interrelationship between YAP signaling, CD47, activated HSCs, and liver fibrosis. These findings have important implications for the development of novel anti-fibrotic therapies in the liver. Targeting CD47 or modulating the YAP signaling pathway may hold promise as potential strategies to alleviate liver fibrosis and mitigate liver dysfunction.

Liver fibrosis is an outcome of excessive accumulation of extracellular matrix proteins resulting from different chronic liver injuries [16]. Activation of HSCs into fibrogenic myofibroblasts is the central driver of liver fibrosis and is now well established as a promising therapeutic target [17,18]. CD47, a widely expressed cellular receptor, plays a "don't eat me" signaling role in cellular regulation and its high expression on the surface of fibrotic cells [19]. Previous studies have provided evidence that mice lacking CD47 or its ligand thrombospondin1 (TSP1) exhibit a protective effect against lipid accumulation in the liver following HFD feeding [7,9,20]. Furthermore, liver inflammation and fibrosis in mice with experimental non-alcoholic steatohepatitis models were relieved after anti-CD47 antibody treatment [10,19]. Papadopoulos et al. have found that the expression of CD47 on the surface of erythrocytes were decreased in NAFLD patients and lead to erythrophagocytosis, which contributed to the pathogenesis of NAFLD [12]. In accordance with these findings, our study revealed a significant increase in CD47 expression in HFD-induced activated HSCs, while the silencing of

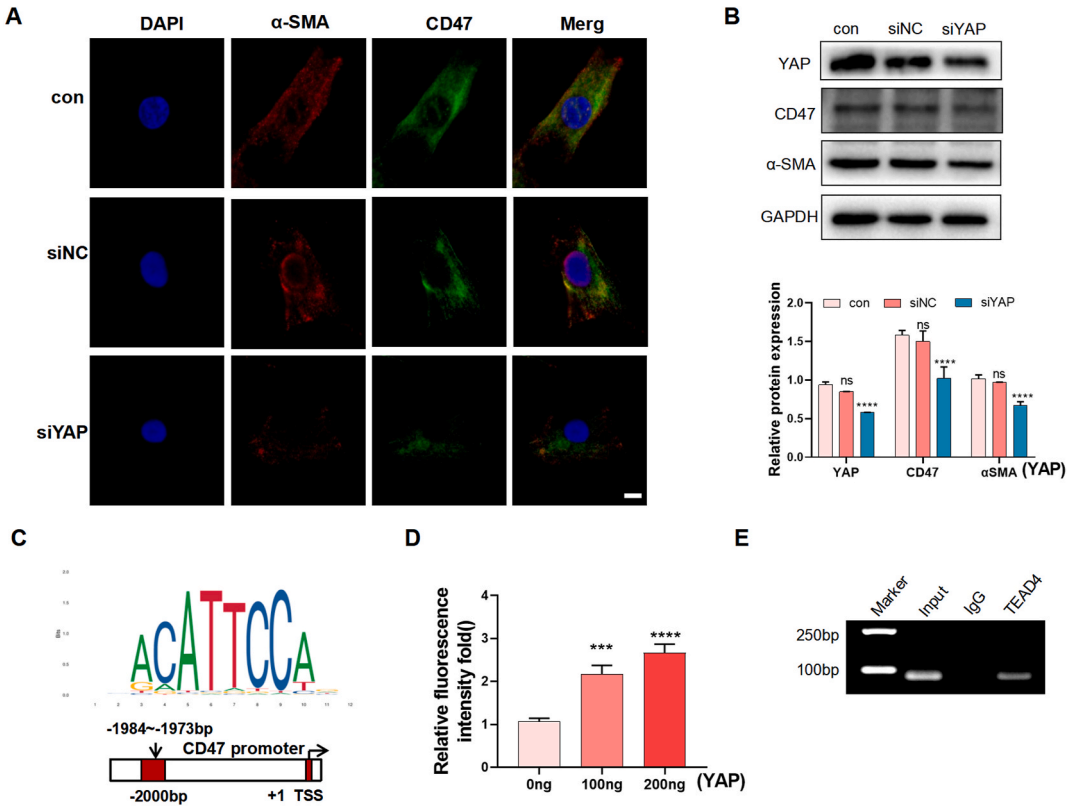

Fig. 4. YAP/TEAD4 regulates CD47 expression-mediated HSCs activation. **(A)**The representative illustrations of anti-CD47 and anti-α-SMA immunofluorescence staining. Scale bar: 50 μm. Nuclei were counterstained with DAPI (blue). **(B)** The expression of CD47 and α-SMA protein was detected by Western blot analysis after YAP knockdown (The original blot is provided in the Supplementary file). **(C)** A schematic of potential TEAD4 binding sites in the promoter region of CD47. **(D)** The expression of luciferase reporter gene was detected by dual luciferase reporter assay in T6 cells. **(E)** TEAD4 binding to the promoter of CD47 was detected by ChIP assay (The original blot is provided in the Supplementary file). The experiments were independently repeated three times. The ns refers to non-significant findings; $^{***}P < 0.001$, $^{****}P < 0.0001$.

CD47 in HSCs resulted in a significant reduction in the expression of fibrosis-related markers, α-SMA and COL1A1. Notably, a previous report demonstrated that mice lacking CD47 exhibited an exacerbation of diet-induced steatohepatitis (40 weeks of HFD) through the regulation of liver inflammation and lipid metabolism [21]. Multiple cell types are involved in pathogenesis of NAFLD, such as endothelial cells, hepatocytes, hepatic stellate cells or erythrocytes [22–24]. The effect of CD47 deficiency on liver diseases, such as steatohepatitis, could potentially be influenced by various factors, including the duration of feeding, the composition of different cell populations, and systemic or tissue-specific microenvironments. To gain a more comprehensive understanding of the role of CD47 in HSCs and liver diseases, it is essential to utilize mice with a specific knockout of CD47 in HSCs. This approach would allow for a more targeted investigation into the specific contributions of CD47 in HSCs and its impact on liver pathologies [25].

CD47 was originally reported to be involved in immune regulation and arterial plaque accumulation [19]. Subsequently, numerous studies have reported that the role of CD47 as a membrane receptor is involved in several cellular processes including proliferation, apoptosis, and adhesion by alternating various signaling pathways [26–28]. Liu et al. have reported CD47 overexpression enhanced cell proliferation and inhibited apoptosis in endometrial carcinoma cells via the PI3K/Akt/mTOR signaling pathway [29]. In this study, we performed KEGG analysis using transcriptome sequencing data from HSCs in both the normal diet (ND) and high-fat diet (HFD) groups. Our analysis revealed that the apoptosis, cell cycle, and mTOR signaling pathways were associated with HSC activation. Consistent with previous reports, we observed that knockdown of CD47 resulted in a significant reduction in the expression of p-AKT and p-mTOR in T6 cells, which are a type of HSCs. This suggests that CD47 may play a role in activating the Akt/mTOR pathway in HSCs. Furthermore, CD47 knockdown promoted apoptosis in HSCs, indicating that CD47 may have anti-apoptotic effects on these cells. In contrast, the disruption of the Akt/mTOR pathway using inhibitors targeting AKT or mTOR has been shown to suppress autophagy-mediated HSC activation and subsequently alleviate liver fibrosis. By inhibiting the Akt/mTOR pathway, these inhibitors can interfere with the signaling cascades that lead to HSC activation and fibrogenesis [30,31].

The Hippo pathway plays a vital role in liver growth and development, with its primary function being the regulation of cell proliferation, liver homeostasis, and regeneration following injury [32]. Previous studies have found out that expression of YAP is increased in patients with NAFLD, confirming its role in liver injury [33]. In parallel, pharmacological inhibition of YAP can improve liver fibrosis and block the progression of cirrhosis in mice [34,35]. Du et al. have reported that selectively depleting YAP in

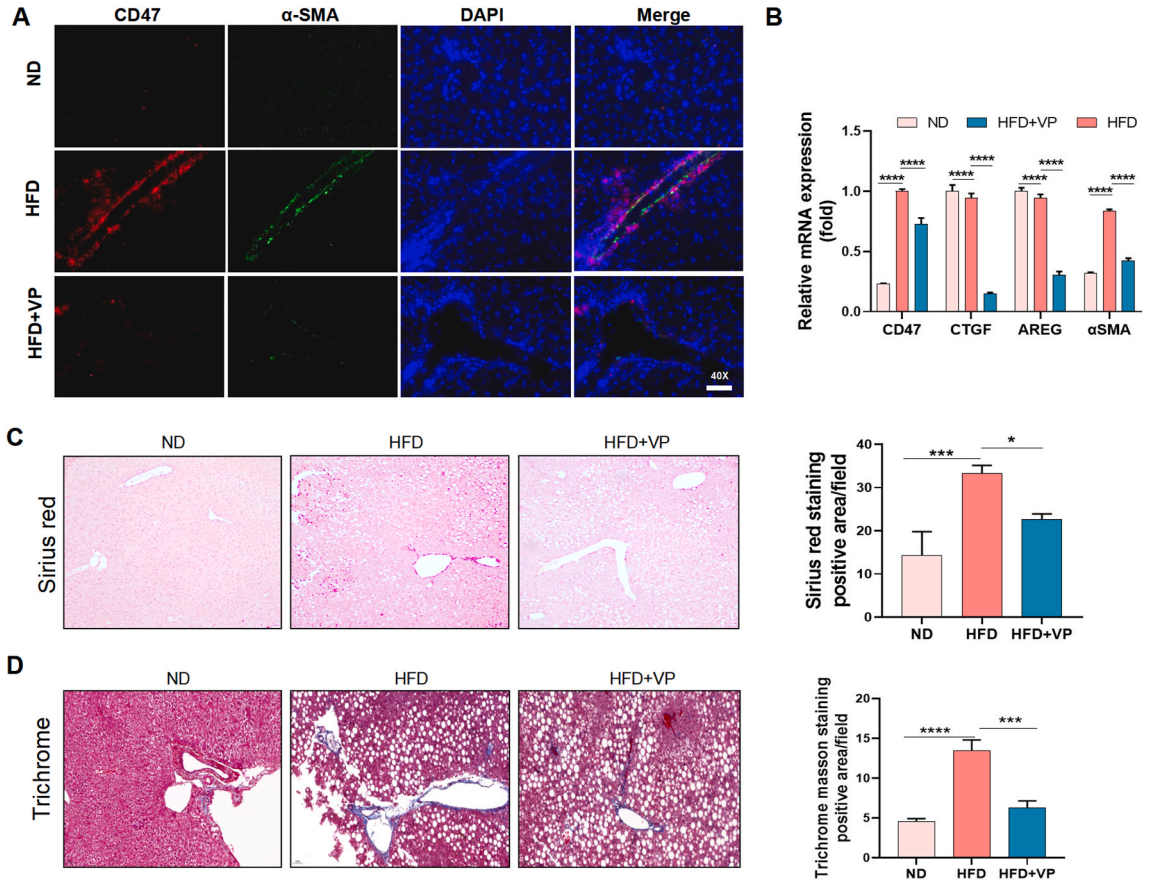

Fig. 5. Disruption of YAP/TEAD4 complex inhibited HSCs activation and attenuated hepatic fibrosis in experimental NASH. **(A)**C57BL/6 WT mice were fed HFD for 10 weeks, followed by treatment with Verteporfin every three day for 10 weeks. Immunofluorescence staining of α-SMA and CD47 expression in liver section, n = 3 mice/group. **(B)** Real-time quantitative PCR analysis of the mRNA expression of fibrotic genes α-SMA, CD47 and YAP target genes CTGF and AREG in primary HSCs, n = 3 mice/group. **(C)**Fibrosis was assessed by Sirius red and quantification of picrosirius red–positive area, n = 3 mice/group. **(D)** Representative histology of Masson staining and Quantification of positive staining areas was measured by software ImageJ, n = 3 mice/group. The ns refers to non-significant findings; $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

myofibroblastic HSCs induced senescence and decreased liver injury and fibrosis [15]. Our data highlights a significant reduction in cell viability and a decrease in the expression of the fibrosis-related marker α-SMA in HSCs following YAP knockdown or treatment with Verteporfin. Furthermore, we observed that both YAP and TEAD4 knockdown led to a notable inhibition of CD47 expression in HSCs. Intriguingly, we discovered that the YAP/TEAD4 complex directly binds to the promoter region of CD47 and promotes its expression. These findings suggest that the YAP/TEAD4/CD47 signaling pathway may serve as a potential mechanism for regulating HSC activation.

There are several limitations in our study that should be acknowledged. Firstly, although our single-cell RNA sequencing analysis demonstrated a significant upregulation of CD47 expression in HSCs of mice induced by a high-fat diet, the role of CD47 in HSC activation was primarily investigated at the cellular level in vitro. To obtain a more comprehensive understanding, the use of CD47 conditional knockout mice would be essential to validate our findings in an in vivo model. Moreover, while we have identified the transcriptional regulation of CD47 by the YAP/TEAD4 complex, it remains unclear if additional hierarchical regulation, such as regulation of CD47 protein stability, is involved in this process. Further investigations are warranted to elucidate the post-transcriptional mechanisms that may contribute to CD47 expression and function. Besides, to fully elucidate the role of the YAP/TEAD4/CD47 signaling axis in liver fibrosis, it would be valuable to employ HSC-specific YAP gene knockout mice. This approach would allow for a more targeted assessment of the specific contributions of YAP in HSCs and its interaction with CD47 in the context of liver fibrosis. Addressing these limitations through future studies will provide a more comprehensive understanding of the involvement of CD47 and the YAP/TEAD4/CD47 signaling axis in HSC activation and liver fibrosis.

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Data availability statement

Data will be made available upon reasonable request. All raw sequences of single-cell sequencing have been submitted to Genome Sequence Archive (Accession Number CRA013859).

CRediT authorship contribution statement

Ya Li: Writing – original draft, Software, Resources, Methodology. **Lin Dong:** Software, Resources, Methodology. **Xuecui Yin:** Resources, Methodology. **Xiaohan Wang:** Resources, Methodology. **Xiaohui Zhu:** Resources, Methodology. **Pengyuan Zheng:** Writing – review & editing, Conceptualization. **Youcai Tang:** Writing – review & editing, 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 to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31621.

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