

Original Article

Taraxasterol alleviates liver fibrosis by suppressing hepatic stellate cell activation via the HSD11B1-ERK MAPK signaling axis

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Abstract

Taraxasterol (TAR) exerts therapeutic effects on various liver diseases via its inherent hepatoprotective and anti-inflammatory properties. However, the mechanism by which TAR treats hepatic fibrosis remains unclear. This study aims to identify the potential targets and specific molecular mechanisms underlying the therapeutic effects of TAR on hepatic fibrosis. DDC- and CCl₄-induced mouse models of hepatic fibrosis are established. The human hepatic stellate cell (HSC) line LX-2 and primary mouse HSCs are used for *in vitro* experiments. Transcriptomics, network pharmacology, single-cell transcriptomics, and molecular docking are employed to identify potential therapeutic targets of TAR for hepatic fibrosis. Functional validation is performed via HSD11B1 overexpression and knockdown experiments, and the regulatory role of the ERK MAPK pathway is verified using the specific inhibitor U0126. Histological staining results show that TAR significantly alleviates DDC- and CCl₄-induced hepatic fibrosis in mice and reduces associated liver injury. *In vitro* assays reveal that TAR effectively reverses TGF- β -induced activation of LX-2 cells and primary mouse HSCs. Multi-omics and docking analyses identify HSD11B1 as a direct target of TAR, whose downregulation in activated HSCs is restored by TAR treatment. Functional experiments demonstrate that overexpression of HSD11B1 attenuates TGF- β -induced HSC activation, while *HSD11B1* knockdown abolishes the therapeutic effects of TAR. Subsequent transcriptomic analysis confirms that HSD11B1 suppresses the ERK MAPK pathway, and knockdown of *HSD11B1* compromises the therapeutic efficacy of the ERK MAPK inhibitor U0126. In summary, TAR alleviates liver fibrosis by inhibiting HSC activation through the HSD11B1-ERK MAPK axis.

Key words liver fibrosis, HSD11B1, taraxasterol, hepatic stellate cell, MAPK signaling pathway

Introduction

Liver fibrosis, a pathological wound-healing response characterized by the excessive deposition of extracellular matrix, represents a critical and common pathway in the progression of virtually all chronic liver diseases toward cirrhosis and hepatic failure [1–6]. Despite its significant global health burden, therapeutic options for reversing or halting liver fibrosis remain limited, highlighting an urgent need for the development of effective anti-fibrotic agents.

The activation of hepatic stellate cells (HSCs) is widely recognized as a central event in liver fibrosis [7–12]. Upon injury,

quiescent HSCs transdifferentiate into myofibroblast-like cells, which proliferate, migrate, and secrete large amounts of extracellular matrix (ECM) components such as collagens I and III. Targeting HSC activation and related signaling pathways has become a promising strategy for antifibrotic drug development [13–15].

Taraxasterol (TAR), a natural pentacyclic triterpene isolated from the traditional herb *Taraxacum*, has shown promising effects in models of acute liver injury, alcoholic liver disease, and even liver cancer [16–19]. TAR exerts anti-inflammatory and hepatoprotective

effects by activating the Nrf2/HO-1 pathway, downregulating the activities of TLR2, TLR4, and NF- κ B, increasing the anti-apoptotic protein Bcl-2, and reducing the pro-apoptotic protein Bax. Furthermore, a recent study demonstrated that TAR has a beneficial therapeutic effect in a CCl₄-induced mouse model of hepatic fibrosis, significantly reducing liver collagen deposition and injury [20]. Notably, despite this promising potential, it remains unclear whether TAR is effective in other models of hepatic fibrosis, and the specific targets and mechanisms involved are yet to be elucidated. 11 β -Hydroxysteroid dehydrogenase 1 (HSD11B1), a key tissue-specific regulator of glucocorticoid action, is abundantly expressed in the liver. Previous studies have shown that HSC specific, but not hepatocyte specific, knockout of *HSD11B1* significantly aggravates liver fibrosis in mice [21,22]; however, the underlying mechanism remains unclear.

In this study, by integrating network pharmacology, molecular docking, transcriptomic sequencing, and single-cell RNA sequencing, we identified HSD11B1 as a potential target of TAR. We demonstrated that TAR inhibits the progression of hepatic fibrosis by upregulating HSD11B1. Subsequent transcriptomic sequencing revealed that HSD11B1 suppresses ERK phosphorylation, thereby inhibiting HSC activation. Our findings confirm that TAR mitigates the progression of liver fibrosis through the HSD11B1-ERK axis, positioning TAR as a promising novel therapeutic strategy for hepatic fibrosis.

Materials and Methods

Establishment of mouse models

DDC diet-induced model

To induce cholestatic liver injury and subsequent fibrosis, 8-week-old male C57BL/6 mice (Guangdong Yikang Biotechnology, Guangzhou, China) were fed a diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC; Sigma-Aldrich, St Louis, USA) *ad libitum* for a period of 4 weeks. Control animals received an identical standard chow diet without DDC supplementation. In the TAR treatment group, mice fed the DDC diet concurrently received TAR (1059-14-9; MCE, Monmouth Junction, USA) at a dose of 10 mg/kg daily via oral gavage. Mice were housed under specific pathogen-free conditions with a 12/12-h light/dark cycle and free access to water. Body weight was monitored weekly. This model reliably induces portal inflammation, ductular reaction, and periportal fibrosis, mimicking aspects of human cholestatic liver disease. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Ethics Committee of South China Agricultural University (No. 2026H012).

CCl₄-induced model

Liver fibrosis was induced by intraperitoneal injection of carbon tetrachloride (CCl₄). Briefly, CCl₄ (Sigma-Aldrich) was mixed with olive oil (20% CCl₄). Male C57BL/6 mice (8 weeks old) received intraperitoneal injections of the CCl₄ mixture (5 ml/kg body weight) twice per week for 8 weeks. Control mice received equivalent volumes of olive oil vehicle. For therapeutic intervention, mice in the TAR treatment group were administered TAR (1059-14-9; MCE) orally at 10 mg/kg daily throughout the 8-week CCl₄ induction period. This chronic injury model leads to centrilobular necrosis, inflammatory infiltration, and progressive bridging fibrosis.

Sample collection and histological assessment

Mice were euthanized at the end of the modeling period. Liver

tissues were divided into two portions: one portion was fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned for histological staining; the other portion was snap-frozen in liquid nitrogen for protein extraction.

For histopathological evaluation, 4- μ m-thick paraffin sections were stained with hematoxylin & eosin (H&E) and Sirius Red (C0105M and C0190M, respectively; Beyotime, Shanghai, China) according to standard protocols. Fibrosis severity was quantified using digital analysis of the Sirius Red-positive area (%) with ImageJ software (NIH, Bethesda, USA).

Immunofluorescence staining was performed to assess the expression and localization of specific proteins. Briefly, after deparaffinization, rehydration, and antigen retrieval, sections were blocked with 5% bovine serum albumin and incubated overnight at 4°C with primary antibodies against α -SMA (1:200; ET1607-53; HUABIO, Hangzhou, China), DESMIN (1:200; ab6322; Abcam, Cambridge, UK), COL1A1 (1:200; 72026S; CST, Beverly, USA), or HSD11B1 (1:150; 10928-1-AP; Proteintech, Wuhan, China). After washing, the sections were incubated with appropriate fluorescent dye-conjugated secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI (C1006; Beyotime). Images were captured using a fluorescence microscope (Leica DMi8; Leica Microsystems, Wetzlar, Germany), and fluorescence intensity was quantified with ImageJ software.

Western blot analysis

Liver tissues or cultured cells were lysed using RIPA buffer supplemented with protease and phosphatase inhibitors (HY-K0010, HY-K0021; MCE). The protein concentration was quantified using a BCA assay kit (Beyotime). Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE and transferred onto NC membranes (10600001; Cytiva, Marlborough, USA). After blocking with 5% skim milk, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-HSD11B1 (1:1000; 10928-1-AP; Proteintech), anti-COL1A1 (1:1000; 72026S; CST), anti- α -SMA (1:1000; ET1607-53; HUABIO), anti-p-ERK (1:1000; 4695S; CST), anti-GAPDH (1:5000; 97166S; CST), and anti-COL4A1 (1:1000; 50273S; CST). Following incubation with HRP-conjugated secondary antibodies, protein bands were visualized using enhanced chemiluminescence reagent (P0018S; Beyotime). and quantified with ImageJ software.

Cell viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; C0037; Beyotime) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells per well and allowed to adhere overnight. After treatment with various concentrations of TAR or TGF- β as indicated, 10 μ L of CCK-8 solution was added to each well, followed by incubation at 37°C for 2 h. The absorbance at 450 nm was measured using a microplate reader (BioTek, Winooski, USA). Cell viability was expressed as a percentage relative to the control group.

Cell proliferation was assessed using the BeyoClick™ EdU-594 Cell Proliferation Detection Kit (C0078S; Beyotime) according to the manufacturer's instructions. In brief, following treatment, cells were incubated with 10 μ M EdU for 2 h, fixed, permeabilized, and subjected to the click reaction cocktail to label incorporated EdU with the Alexa Fluor 594 fluorophore. Nuclei were counterstained with Hoechst 33342. Fluorescence images were acquired using a

microscope (Leica DMi8), and the proliferation rate was calculated as the percentage of EdU-positive (red) nuclei relative to the total number of Hoechst-positive (blue) nuclei in three randomly selected fields per well.

Network pharmacology analysis

Prediction of TAR targets

The 2D and 3D structures of TAR were obtained from the PubChem database. These structures were subsequently submitted to the SwissTargetPrediction online platform (<https://swisstargetprediction.ch/>) for target prediction. The analysis was performed with the species parameter set to “*Homo sapiens*” and all predicted targets with a probability score of > 0.1 were retained for further analysis.

Collection of liver fibrosis-related targets

Disease targets associated with liver fibrosis were systematically retrieved from publicly available databases: the Online Mendelian Inheritance in Man (OMIM; <https://www.omim.org/>), the Therapeutic Target Database (TTD; <https://ttd.idrblab.cn/>), the Pharmacogenomics Knowledgebase (PharmGKB; <https://www.pharmgkb.org/>), and GeneCards (<https://www.genecards.org/>). The keyword “Liver Fibrosis” was used for the search across all platforms. The union of targets obtained from these databases constituted the comprehensive set of liver fibrosis-related genes.

Identification of potential therapeutic targets

To identify the putative targets through which TAR may exert its anti-fibrotic effects, the list of predicted TAR targets was intersected with the compiled list of liver fibrosis-related targets. This intersection, representing the common targets, was visualized using a Venn diagram and was defined as the potential therapeutic targets of TAR for liver fibrosis.

Protein-protein interaction (PPI) network construction and core target analysis

The potential therapeutic targets were imported into the STRING database (<https://string-db.org/>) to construct a PPI network. The analysis was restricted to “*Homo sapiens*” with a minimum required interaction confidence score set at > 0.4. The resulting network data were then downloaded and imported into Cytoscape software (version 3.9.1; <https://cytoscape.org/>) for further visualization and topological analysis. To identify the most crucial nodes within the network, the cytoHubba plugin was employed using the maximal clique centrality (MCC) algorithm. The top-ranked genes from this analysis were considered core targets. The size and color of the nodes are mapped to their degree values. A higher degree value results in a larger node size and darker color, indicating that the protein has more frequent interactions with other proteins in the network and may represent a key core target.

Cell culture and treatment

The human hepatic stellate cell line LX-2 (CL-0560; Procell, Wuhan, China) and the rat hepatic stellate cell line HSC-T6 (CL-0166; Procell) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) supplemented with 5% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin (HY-B1463; MCE), and 100 µg/mL streptomycin (HY-B1906; MCE). All cells were maintained in a humidified incubator at 37°C with 5% CO₂. For experiments, cells were seeded into culture plates and allowed to adhere overnight. To establish an *in vitro* model of activation, cells were treated with recombinant human TGF-β1 (20 ng/mL; HY-P70433; MCE). To investigate the role of the MAPK signaling

pathway, HSCs were treated with U0126 (HY-12031A; MCE) for 48 h. To investigate the inhibitory effects of TAR, cells were co-treated with TAR (20 µM) and TGF-β1. The treatment duration for all conditions was 48 h. Following this incubation period, cells were either harvested for protein extraction or processed for subsequent immunofluorescence staining. HSCs were exposed to vehicle, 500 nM corticosterone (HY-B1618; MCE), 500 nM 11-dehydrocorticosterone (HY-113447; MCE), and/or 10 µM UE2316 (HY-180415; MCE) for 6 days, with medium and all compounds refreshed daily. Cells were subsequently collected for protein analysis.

Cellular thermal shift assay (CETSA)

CETSA was performed using the human HSC line LX-2. LX-2 cells were harvested and resuspended in PBS. Whole-cell lysates were prepared by three freeze-thaw cycles (liquid nitrogen/37°C water bath) and cleared by centrifugation at 20,000 g for 10 min at 4°C. The supernatants were aliquoted and pre-incubated with either vehicle or the indicated compound at the desired concentration for 30 min at room temperature. Aliquots were then heated to a range of temperatures (e.g., 37°C–58°C in 3°C increments) for 3 min in a thermal cycler, followed by a 3-min incubation at room temperature to allow protein aggregation. After heating, samples were centrifuged at 20,000 g for 10 min at 4°C to separate soluble (non-denatured) proteins from insoluble aggregates. The soluble fractions were mixed with SDS loading buffer (HY-K1100; MCE), and immunoblotted using a specific antibody against the protein of interest.

Molecular docking analysis

Molecular docking was employed to preliminarily validate the direct interaction between TAR and its core predicted target, HSD11B1. The three-dimensional structure of the human HSD11B1 protein (UniProt ID: P28845) was retrieved as a predicted model from the AlphaFold database (<https://alphafold.ebi.ac.uk/>). The 3D chemical structure of TAR was obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Prior to docking, the protein structure was prepared using AutoDock Tools by removing water molecules and adding polar hydrogen atoms and Gasteiger charges. The TAR structure was likewise energy-minimized. The docking simulations were performed using AutoDock Vina 1.5.6 software (<https://vina.scripps.edu/>). The search space for the docking was defined to encompass the entire protein surface to identify the most likely binding site. The binding affinity (expressed in kcal/mol) was used as the key metric to evaluate the docking performance, where a more negative value indicates a stronger predicted binding interaction. A binding affinity of less than −5.0 kcal/mol was considered indicative of a reliable and stable binding interaction. The docking pose with the most favorable binding affinity was selected for further analysis, and the detailed molecular interactions within the binding pocket were visualized and analyzed using PyMOL (version 2.0; <https://pymol.org/>).

Transcriptomic sequencing and bioinformatic analysis

Bulk RNA sequencing and differential expression analysis

Differential expression analysis was conducted using the limma package (version 3.56.2) in R. Genes with an absolute log₂-fold change > 1 and an adjusted *P* value (FDR) < 0.05 were defined as significantly differentially expressed. The results were visualized using volcano plots and heatmaps.

Functional enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on the differentially expressed gene sets using the clusterProfiler package (version 4.8.3) in R. Terms with FDR < 0.05 were considered significantly enriched. The analyses covered Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) categories in GO, as well as signaling pathways in KEGG.

Single-cell RNA-sequencing analysis

Public single-cell RNA-seq data from mouse liver fibrosis models (e.g., dataset GSE171904) were downloaded and analyzed. Data processing, integration, and clustering were performed using Seurat (version 5.0.0) in R. Low-quality cells with high mitochondrial gene content (> 20%) or an extreme number of detected genes were filtered out. Data were normalized using the LogNormalize method, and the top 2000 highly variable features were identified for downstream analysis. Principal component analysis (PCA) was performed, followed by graph-based clustering using the top 30 principal components and a resolution parameter of 0.5. Cell clusters were visualized with UMAP. Cell types were annotated based on canonical marker genes (e.g., *Lrat* for HSCs, *Alb* for hepatocytes, *Cd68* for Kupffer cells). For the HSC subpopulation, cells were reclustered to distinguish activated HSC (aHSC) and quiescent HSC (qHSC) states using markers such as *Acta2* (α -SMA) and *Col1a1*. Differential expression analysis between conditions or clusters was performed using the FindMarkers function with the Wilcoxon rank-sum test.

Gene set enrichment analysis (GSEA)

GSEA was performed using R software (version 4.0) with the clusterProfiler package. Differentially expressed genes between two experimental conditions were ranked by a predefined metric (e.g., log2-fold change or signal-to-noise ratio) to generate a ranked gene list. The ranked list was then subjected to GSEA against a curated collection of gene sets obtained from the Molecular Signatures Database (MSigDB; <https://www.gsea-msigdb.org/gsea/msigdb>), including Hallmark, KEGG, GO, and custom pathway sets of interest. Enrichment scores (ESs) were calculated for each gene set, and statistical significance was assessed by 10,000 phenotype-based permutations (or the number of permutations recommended by the package). The normalized enrichment score (NES) and FDR were computed to correct for multiple hypothesis testing. A gene set was considered significantly enriched if the FDR q -value < 0.25 and the nominal P -value < 0.05. Representative enriched pathways were visualized using enrichment plots generated by the ggplot2 or enrichplot packages.

Plasmid and siRNA transfection

Cell seeding

The human hepatic stellate cell line LX-2 was cultured in DMEM supplemented with 5% FBS as described above. For transfection experiments, cells in the logarithmic growth phase were seeded into 6-well culture plates at an appropriate density to achieve approximately 70% confluence at the time of transfection.

Plasmid and siRNA preparation

For gain-of-function studies, a human HSD11B1 overexpression plasmid was utilized (Supplementary Table S1). For loss-of-function studies, a validated siRNA targeting *HSD11B1* and a corresponding non-targeting scrambled siRNA (si-NC; Guangzhou Youming Biotechnology, Guangzhou, China) were used (Supple-

mentary Table S2). All nucleic acids were diluted to the required working concentrations using the provided buffer.

Transfection procedure

Transfections were performed using the jetPRIME transfection reagent (Polyplus, Strasbourg, France) according to the manufacturer's protocol. Briefly, for each well of a 6-well plate, 0.5 μ g of plasmid DNA or 3 μ L of siRNA (at 1 OD concentration) was diluted in 200 μ L of the provided jetPRIME buffer. Subsequently, 2 μ L of jetPRIME reagent was added to the diluted nucleic acid solution, vortexed immediately for 10 s, and incubated at room temperature for 15 min to allow complex formation. The entire DNA/RNA-jetPRIME complex mixture was then added dropwise to the respective well containing cells and fresh medium. The plate was gently rocked to ensure even distribution.

Post-transfection processing

Six hours after transfection, the medium containing the transfection complexes was aspirated and replaced with fresh complete growth medium. Cells were then cultured for a total of 48 h post-transfection to allow sufficient time for gene expression or knock-down. Finally, cells were harvested for protein extraction and subsequent western blot analysis to verify transfection efficiency and assess downstream molecular changes.

Isolation and culture of primary HSCs

Primary hepatic stellate cells were isolated from C57BL/6 mice using a modified *in situ* collagenase perfusion protocol followed by density gradient centrifugation, adapted from established methods.

Briefly, mice were anesthetized, and the liver was perfused via the portal vein with calcium- and magnesium-free Hank's balanced salt solution (HBSS) for 10 min to clear blood. This was followed by sequential perfusion with a solution containing 0.2% pronase (HY-114158; MCE) and subsequently with a solution containing 0.2% collagenase type IV (HY-E70005D; MCE) until the liver tissue became adequately digested. The softened liver was then gently dissected and minced in DMEM. The resulting cell suspension was filtered through a 100- μ m nylon mesh to remove tissue debris.

To enrich for HSCs, the filtered cell suspension was subjected to density gradient centrifugation using an 11% OptiPrep solution (Sigma-Aldrich). Centrifugation was performed at 1400 g for 20 min at 4°C. HSCs, which banded at the interface due to their characteristic lipid droplet content, were carefully collected. The isolated cells were washed twice with DMEM and seeded onto culture plates.

Measurement of cellular corticosterone levels

Corticosterone (Cort) levels were quantified using an ELISA kit (ml077243; Shanghai Enzyme-linked Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, 50 μ L of standards or pretreated samples were added to each well along with 50 μ L of primary antibody, and the plate was incubated at 37°C for 30 min. After incubation, the wells were washed three times with 300 μ L of 1 $\times$ wash buffer. Subsequently, 100 μ L of enzyme-labeled antibody working solution was added to each well, followed by incubation at 37°C for 30 min in the dark and then washing four times. For color development, 50 μ L of substrate A and 50 μ L of substrate B were added to each well and incubated at 37°C for 15 min in the dark. The reaction was terminated by adding 50 μ L of

stop solution, and the absorbance was measured at 450 nm within 5 min using a pre-warmed microplate reader.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.01 software). All quantitative data are expressed as the mean $\pm$ standard error of the mean (SEM) following confirmation of a normal distribution. For comparisons between two independent groups, Student's *t* test was employed, whereas one-way analysis of variance (ANOVA) was utilized for multiple group comparisons (three or more groups). A two-tailed *P* value < 0.05 was defined as statistically significant to denote meaningful differences between groups.

Results

TAR significantly alleviates DDC- and CCl₄-induced hepatic fibrosis in mice

To investigate the effect of TAR on mouse hepatic fibrosis, we established models using both DDC and CCl₄ induction and treated the mice with TAR (10 mg/kg) (Figure 1A) [20]. Sirius Red and H&E staining revealed that both DDC and CCl₄ significantly exacerbated fibrotic scarring and liver injury in mice. In contrast, TAR treatment markedly alleviated the degree of liver fibrosis and reduced hepatic injury (Figure 1B and Supplementary Figure S1A,B). Consistent with these findings, western blot analysis and immunofluorescence staining results demonstrated that TAR significantly suppressed the DDC- and CCl₄-induced upregulation of COL1A1, COL4A1, and α -SMA (Figure 1C,D). Collectively, these results indicate that TAR exerts therapeutic effects against liver fibrosis in multiple experimental models.

TAR significantly inhibits the activation and proliferation of HSCs

To investigate the impact of TAR on HSCs, we conducted analyses using the human HSC line LX-2, the rat HSC line HSC-T6, and primary mouse HSCs. CCK-8 proliferation assays revealed that TGF- β significantly enhanced the proliferative capacity of both LX-2 and HSC-T6 cells. Conversely, TAR inhibited HSC proliferation in a dose-dependent manner, with a significant suppressive effect observed at a concentration of 20 μ M (Figure 2A). Furthermore, TAR (20 μ M) exhibited no significant cytotoxic effects on human or mouse hepatocytes (Supplementary Figure S2A,B). Subsequently, LX-2 and HSC-T6 cells were treated with 20 μ M TAR. Immunofluorescence staining demonstrated that TAR treatment attenuated the TGF- β -induced upregulation of α -SMA and Ki67 (Figure 2B). We then isolated primary HSCs from mice and treated them with TGF- β and TAR. Consistent with the findings in LX-2 cells, TAR significantly suppressed the activation of primary mouse HSCs and reduced collagen release (Figure 2C). Furthermore, EdU proliferation assays confirmed the potent inhibitory effect of TAR on HSC proliferation (Figure 2D).

Network pharmacology and transcriptomics reveal potential targets of TAR in inhibiting HSC activation

To identify potential therapeutic targets of TAR against hepatic fibrosis, we first retrieved 8741 liver fibrosis-related targets from the GeneCards, PharmGkb, TTD, and OMIM databases using the keyword "Liver Fibrosis" (Figure 3A). The 2D and 3D structures of TAR were obtained from PubChem and submitted to the Swiss

Target Prediction and PharmMapper databases. After quality control, a total of 219 potential TAR-binding targets were collected by merging the results from both platforms. The intersection between these drug targets and the disease-related targets yielded 182 potential targets through which TAR may exert its antifibrotic effects (Figure 3B). A PPI network of these core targets was constructed using the STRING database and visualized with Cytoscape (Figure 3E). GO and KEGG enrichment analyses indicated that these targets were significantly associated with biological processes such as cellular response to chemical stimulus and key signaling pathways, including the PI3K-Akt and Ras signaling pathways (Figure 3C,D).

To further refine key targets involved in TAR-mediated inhibition of HSC activation, we analyzed a public dataset (GSE119606) to identify critical regulators during LX-2 cell activation. Volcano and heatmaps illustrated the differentially expressed genes between the control and TGF- β -treated groups (Figure 3F,G). By intersecting the 828 differentially expressed genes with the 182 key targets, we identified 13 candidate targets (Figure 3H). Transcriptomic data analysis revealed that the majority of these candidate targets were downregulated following TGF- β treatment, suggesting that TAR likely attenuates fibrotic progression by upregulating these targets to suppress HSC activation (Figure 3I).

Single-cell transcriptome sequencing identifies HSD11B1 as a potential key target for TAR-mediated inhibition of HSC activation

To elucidate the relationship between the identified targets and murine hepatic fibrosis, we analyzed single-cell transcriptomic data from mouse models of liver fibrosis induced by CCl₄ and bile duct ligation (BDL; GSE171904). Freshly isolated HSCs were loaded onto the 10 $\times$ Genomics Chromium single-cell platform. Unsupervised clustering partitioned the cells into six distinct clusters, which were annotated as HSCs, endothelial cells, Kupffer cells, cholangiocytes, portal fibroblasts, and hepatocytes based on established key markers. A heatmap displays the highly variable genes across these cell populations (Figure 4A–C). Compared to the vehicle (oil)-treated control group, both CCl₄ and BDL models exhibited a significant increase in the HSC population. Bar plots further illustrate the proportional distribution of different cell types across experimental groups (Figure 4D,E). We next examined the expression of the 13 candidate targets within the single-cell dataset and found that only HSD11B1 expression was significantly enriched specifically within the HSC cluster (Figure 4F). Subsequently, we extracted the HSC subpopulation for deeper analysis. Based on established markers, HSCs were categorized into activated (aHSCs) and quiescent (qHSCs) states. UMAP visualization confirmed a marked expansion of the aHSC population in both CCl₄- and BDL-treated groups compared to controls (Figure 4G–I). Importantly, violin plots revealed that HSD11B1 expression was significantly downregulated in aHSCs compared to qHSCs, suggesting a strong correlation between HSD11B1 levels and HSC activation status (Figure 4J).

To investigate the interaction between TAR and HSD11B1, we performed molecular docking using AutoDock Vina. The results demonstrated that TAR forms a stable complex with HSD11B1, with a calculated binding energy of -6.9 kcal/mol (Figure 4K). In summary, these findings indicate that TAR inhibits HSC activation and attenuates the progression of liver fibrosis by mediating

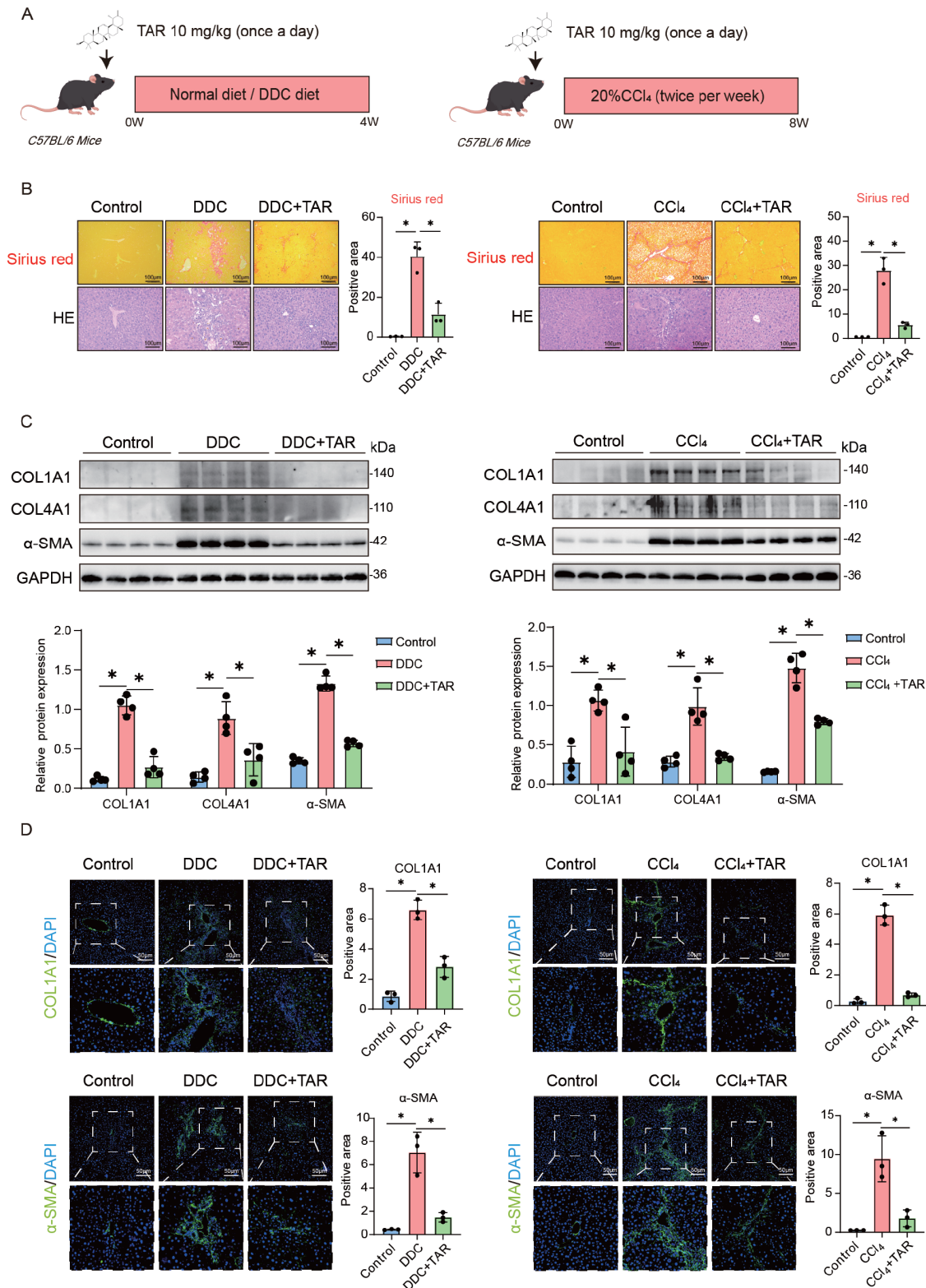


Figure 1. TAR attenuates DDC- and CCl₄-induced hepatic fibrosis in mice (A) Schematic diagram of the mouse model. (B) H&E and Sirius Red staining shows that TAR alleviates liver fibrosis and injury (scale bar: 100 μ m). Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the DDC or CCl₄ group. (C) Western blot analysis of α -SMA, COL1A1, and COL4A1 expression in liver tissues from different treatment groups. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 4$ per group). * $P < 0.05$ versus the DDC or CCl₄ group. (D) Immunofluorescence staining of α -SMA and COL1A1 expression in liver tissues from different treatment groups (scale bar: 50 μ m). Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 4$ per group). * $P < 0.05$ versus the DDC or CCl₄ group.

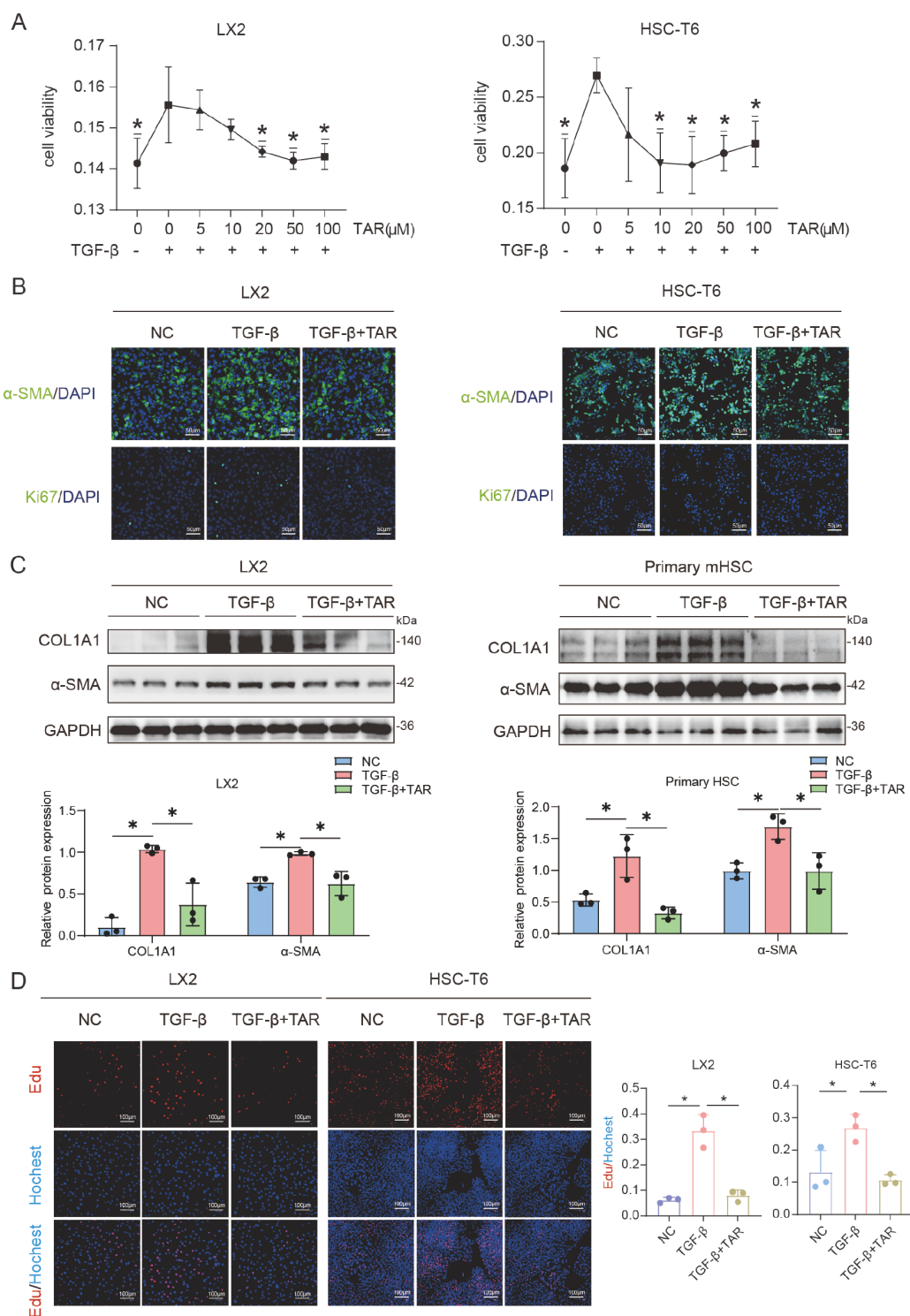
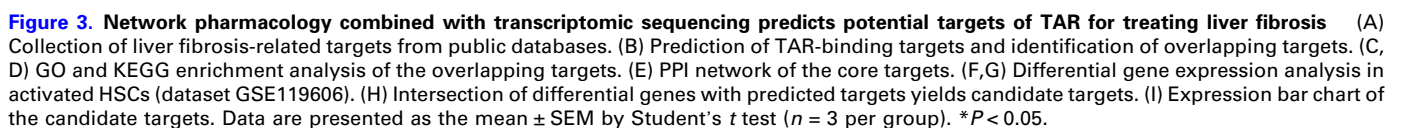


Figure 2. TAR inhibits HSC proliferation and activation (A) LX-2 and HSC-T6 cells were treated with various concentrations of TAR (0–100 μM) in the presence of TGF-β (20 ng/mL) for 48 h. The CCK-8 assay showed that TAR significantly inhibited the proliferation of LX-2 and HSC-T6 cells. Data are presented as the mean ± SEM by one-way ANOVA ($n = 5$ per group). * $P < 0.05$ versus the TGF-β group. (B) LX-2 and HSC-T6 cells were treated with TAR (20 μM) in the presence of TGF-β (20 ng/mL) for 48 h, and the expression of α-SMA and Ki67 in the cells was detected by immunofluorescence staining (scale bar: 50 μm). (C) LX-2 and mouse primary hepatic stellate cells were treated with TAR (20 μM) in the presence of TGF-β (20 ng/mL) for 48 h, and the expression of COL1A1 and α-SMA was detected by western blot analysis. Data are presented as the mean ± SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF-β group. (D) LX-2 and HSC-T6 cells were treated with TAR (20 μM) in the presence of TGF-β (20 ng/mL) for 48 h, and cell proliferation was subsequently assessed using an EdU assay (scale bar: 100 μm). Data are presented as the mean ± SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF-β group.



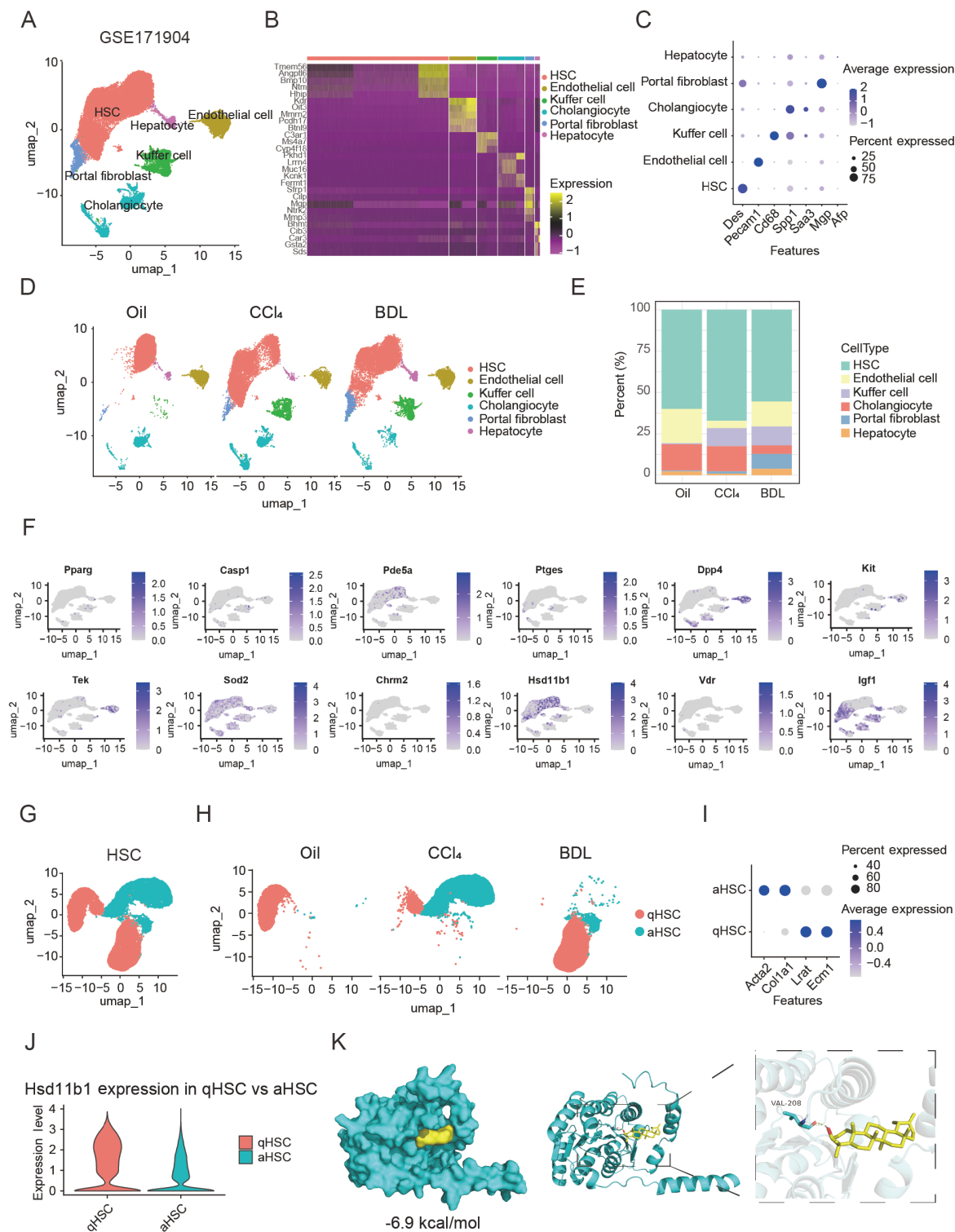


Figure 4. Identification of HSD11B1 as the key target of TAR via single-cell transcriptomics and molecular docking (A–C) Unsupervised clustering and annotation of major liver cell types from single-cell RNA-seq data of fibrotic mice. (D) UMAP visualization showing the distribution of cell populations from different experimental groups. (E) Proportional bar chart showing the percentage of each cell population in different experimental groups. (F) Expression screening of candidate targets across cell clusters, showing specific enrichment of HSD11B1 in HSCs. (G–I) Reclustering of HSCs and identification of activated HSC (aHSC) and quiescent HSC (qHSC) subpopulations using canonical marker genes. (J) Violin plot showing downregulation of HSD11B1 in aHSCs. (K) Molecular docking model depicting the stable binding between TAR and the HSD11B1 protein, with the calculated binding affinity.

HSD11B1 expression.

TAR elevates HSD11B1 expression in HSCs

Immunofluorescence co-localization analysis revealed that HSD11B1 is predominantly localized in HSCs. TAR treatment markedly increased HSD11B1 expression levels in mouse HSCs (Figure 5A). Western blot analysis of both LX-2 cells and primary mouse HSCs demonstrated that TAR significantly reversed the TGF- β -induced downregulation of HSD11B1 (Figure 5B). Consistently, TAR administration substantially elevated HSD11B1 protein levels in mouse liver tissues compared to the DDC- and CCl₄-induced fibrosis models (Figure 5C). To validate the interaction between TAR and HSD11B1, we designed a mutant plasmid of HSD11B1 (V208K). The CETSA results showed that TAR significantly increased the thermal stability of HSD11B1 but had no effect on the HSD11B1 mutant (Supplementary Figure S3). These findings suggest that TAR interacts with HSD11B1 via the VAL-208 residue.

To further investigate the functional role of HSD11B1, we established LX-2 cell models with either knockdown or overexpression of HSD11B1. The results showed that HSD11B1 overexpression significantly attenuated HSC activation and reduced collagen production. Conversely, *HSD11B1* knockdown promoted HSC activation and effectively abolished the therapeutic effects of TAR (Figure 5D,E). These findings collectively demonstrate that TAR regulates HSD11B1 expression and that the anti-fibrotic efficacy of TAR is critically dependent on HSD11B1.

HSD11B1 suppresses HSC activation through inhibition of the ERK MAPK signaling pathway

To further investigate the mechanism by which HSD11B1 inhibits HSC activation, we established an *HSD11B1*-knockdown LX-2 cell model and performed transcriptomic sequencing analysis. Volcano and heatmaps revealed a markedly altered gene expression profile in the *HSD11B1*-knockdown group (si*HSD11B1* group) compared to the control group (siNC group) (Figure 6A,B). KEGG pathway enrichment analysis indicated that *HSD11B1* knockdown was associated with pathways including smooth muscle contraction, TNFs binding their physiological receptors, MAPK family signaling cascades, RAF/MAP kinase cascade, and MAPK1/MAPK3 signaling (Figure 6C). GO functional enrichment analysis revealed that *HSD11B1* knockdown was linked to molecular functions such as cytokine receptor binding, cytokine binding, and extracellular matrix binding (Figure 6D). SEA demonstrated that HSC activation-related pathways, including the TGF- β signaling pathway and Hippo signaling pathway, were upregulated upon *HSD11B1* knockdown, suggesting that *HSD11B1* deficiency promotes HSC activation (Figure 6E).

Subsequent examination of the transcriptomic data showed that in the si*HSD11B1* group, markers of HSC activation—ACTA2 (α -SMA), COL1A1, COL4A1, and TGFBR1—were significantly upregulated. Furthermore, the upregulation of NFKB1 and CCL5 indicated enhanced activation of inflammatory pathways following *HSD11B1* knockdown, while the downregulation of MMP1 suggested impaired collagen clearance (Figure 6F). These results further corroborate that *HSD11B1* knockdown promotes HSC activation.

Given that multiple enrichment analyses pointed to an association between *HSD11B1* knockdown and the MAPK pathway (Figure 6C), we hypothesized that HSD11B1 might regulate HSC activation

by modulating MAPK signaling. To test this hypothesis, we established an HSD11B1-overexpressing LX-2 cell model. The results showed that TGF- β stimulation increased ERK phosphorylation levels, whereas HSD11B1 overexpression significantly suppressed this increase (Figure 7A). Consistently, TAR treatment markedly reversed the TGF- β -induced elevation of ERK phosphorylation (Figure 7B). To further verify whether TAR inhibits ERK MAPK activation specifically through HSD11B1, we performed a rescue experiment. Western blot analysis revealed that knockdown of *HSD11B1* abolished the inhibitory effect of TAR on the ERK MAPK pathway (Figure 7C). We then treated LX-2 cells with U0126, an ERK MAPK pathway inhibitor. As expected, U0126 significantly inhibited ERK phosphorylation, suppressed HSC activation, and reduced collagen secretion (Figure 7D). Notably, *HSD11B1* knockdown compromised the ability of U0126 to inhibit HSC activation (Figure 7E). Given the essential function of HSD11B1 in converting inactive cortisone into active cortisol in humans (or 11-dehydrocorticosterone into corticosterone in mice), we hypothesized that the inhibitory effect of HSD11B1 on the ERK MAPK pathway might be associated with this activity. To test this hypothesis, primary mouse hepatic stellate cells were incubated with corticosterone, the HSD11B1 inert substrate 11-dehydrocorticosterone, or the HSD11B1 inhibitor UE2316. The results showed that both corticosterone and 11-dehydrocorticosterone significantly reduced ERK phosphorylation levels in primary mouse hepatic stellate cells, while UE2316 reversed the therapeutic effect of 11-dehydrocorticosterone (Supplementary Figure S4). Furthermore, TAR treatment restored the reduction in corticosterone levels induced by TGF- β in primary mouse hepatic stellate cells (Supplementary Figure S5).

In summary, our findings demonstrate that TAR inhibits HSC activation by suppressing the ERK MAPK signaling pathway, which is mediated through HSD11B1.

Discussion

Liver fibrosis, as a reversible yet critical precursor to cirrhosis, presents a formidable therapeutic challenge due to the lack of targeted pharmacological agents [23]. In this study, we systematically demonstrated that TAR, a natural triterpenoid, exerts potent anti-fibrotic effects in multiple experimental models, and we delineated a molecular axis—TARHSD11B1-ERK MAPK—through which HSC activation is suppressed. Our results confirmed that TAR significantly attenuates hepatic fibrosis induced by both DDC and CCl₄, accompanied by reduced collagen deposition and improved histopathology. These findings extend previous reports on the hepatoprotective role of TAR and suggest its broad applicability across different fibrotic stimuli. We showed that TAR directly inhibits HSC activation and proliferation *in vitro*, indicating that its anti-fibrotic action is at least partly mediated through modulating HSC behavior.

Through network pharmacology and transcriptomic sequencing analysis, we identified 13 potential targets of TAR for the treatment of liver fibrosis, most of which are associated with metabolic pathways. Alcohol dehydrogenase 1B (ADH1B), a metabolic enzyme that accelerates ethanol oxidation, has been reported to correlate with clinical outcomes from liver fibrosis to hepatocellular carcinoma [24,25]. Hemeda *et al.* [26] demonstrated that specific SNPs in the superoxide dismutase-2 (SOD2)-encoding gene are significantly associated with the severity of fibrosis. Additionally, the vitamin D receptor (VDR) has been shown to alleviate

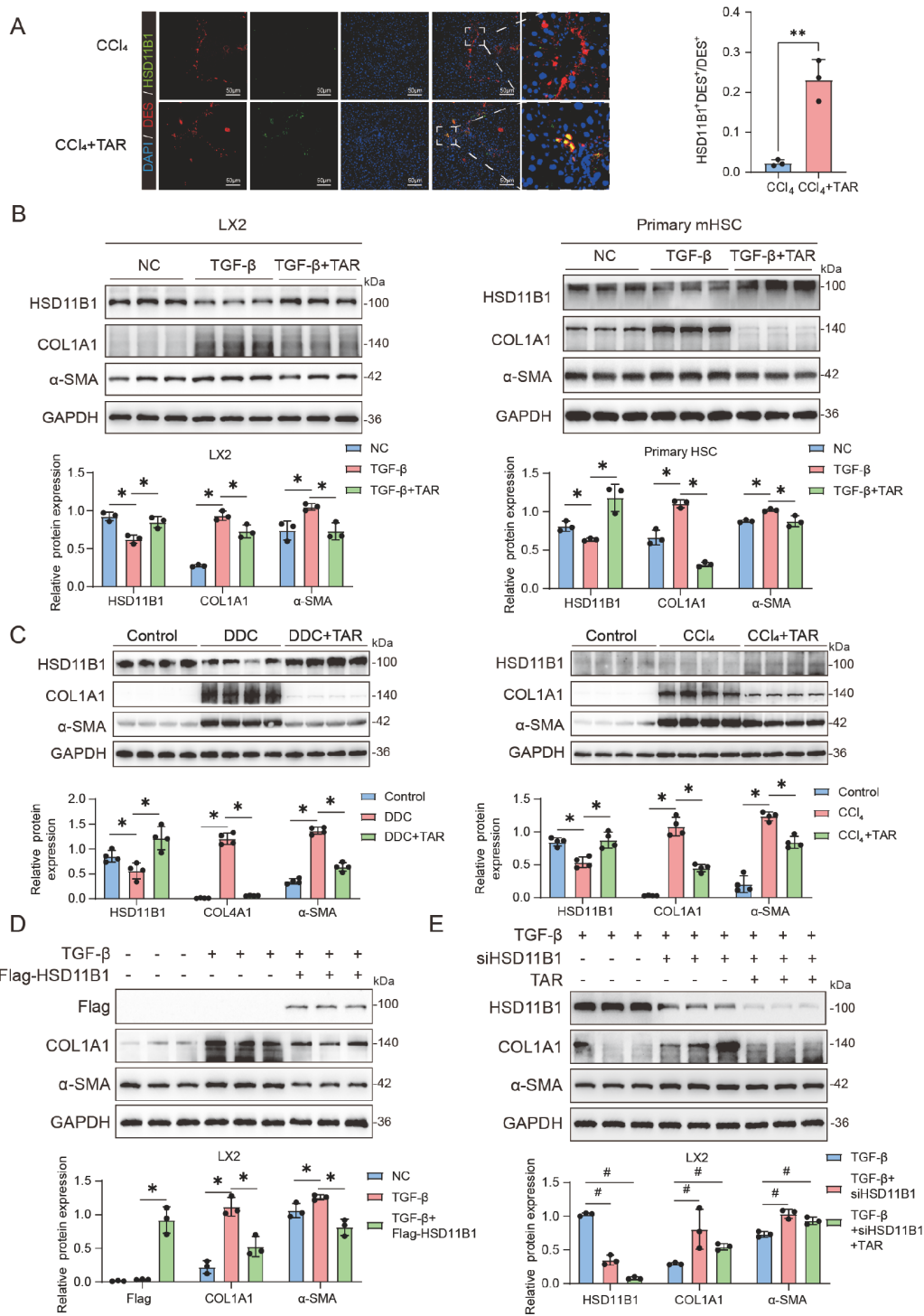


Figure 5. TAR inhibits HSC activation by promoting HSD11B1 expression (A) Immunofluorescence co-localization shows HSD11B1 expression in HSCs and its increase upon TAR treatment *in vivo* (scale bar: 50 μ m). Data are presented as the mean $\pm$ SEM by Student's *t* test ($n = 3$ per group). * $P < 0.05$. (B) LX-2 and mouse primary hepatic stellate cells were treated with TAR (20 μ M) in the presence of TGF- β (20 ng/mL) for 48 h. Western blot analysis confirms that TAR reverses TGF- β -induced downregulation of HSD11B1 in LX-2 and primary mouse HSCs. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF- β group. (C) TAR administration elevates HSD11B1 protein levels in the livers of DDC- and CCl₄-induced fibrotic mice. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 4$ per group). * $P < 0.05$ versus the DDC or CCl₄ group. (D,E) LX-2 cells were transfected with the Flag-HSD11B1 plasmid and stimulated with TGF- β (20 ng/mL), and protein expression was detected after 48 h. Separately, LX-2 cells were transfected with siHSD11B1 and then treated with TAR (20 μ M) in the presence of TGF- β (20 ng/mL), and protein expression was detected after 48 h. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF- β group; # $P < 0.05$ versus the TGF- β group.

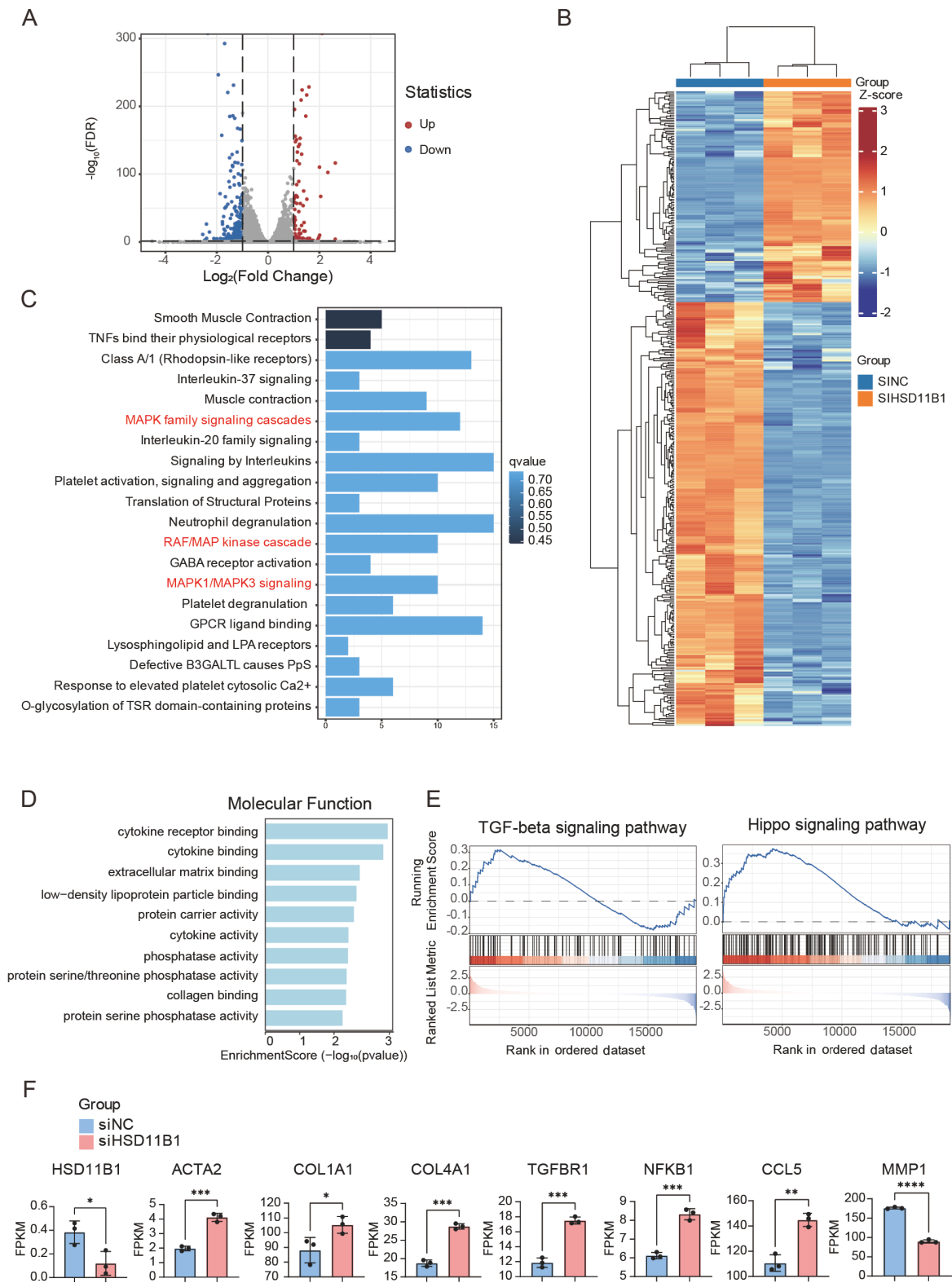


Figure 6. Transcriptomic sequencing analysis reveals that knockdown of *HSD11B1* promotes HSC activation (A,B) Volcano plot and heatmap of differentially expressed genes in *HSD11B1*-knockdown (si*HSD11B1*) LX-2 cells versus control (siNC) cells. (C) KEGG pathway enrichment analysis of upregulated genes in si*HSD11B1* cells, highlighting association with MAPK signaling cascades. (D) GO functional enrichment analysis. (E) GSEA demonstrates upregulation of HSC activation-related pathways, including TGF- β and Hippo signaling. (F) Bar chart showing the expression of HSC activation-related genes. Data are presented as the mean $\pm$ SEM by Student's *t* test (*n* = 3 per group). **P* < 0.05.

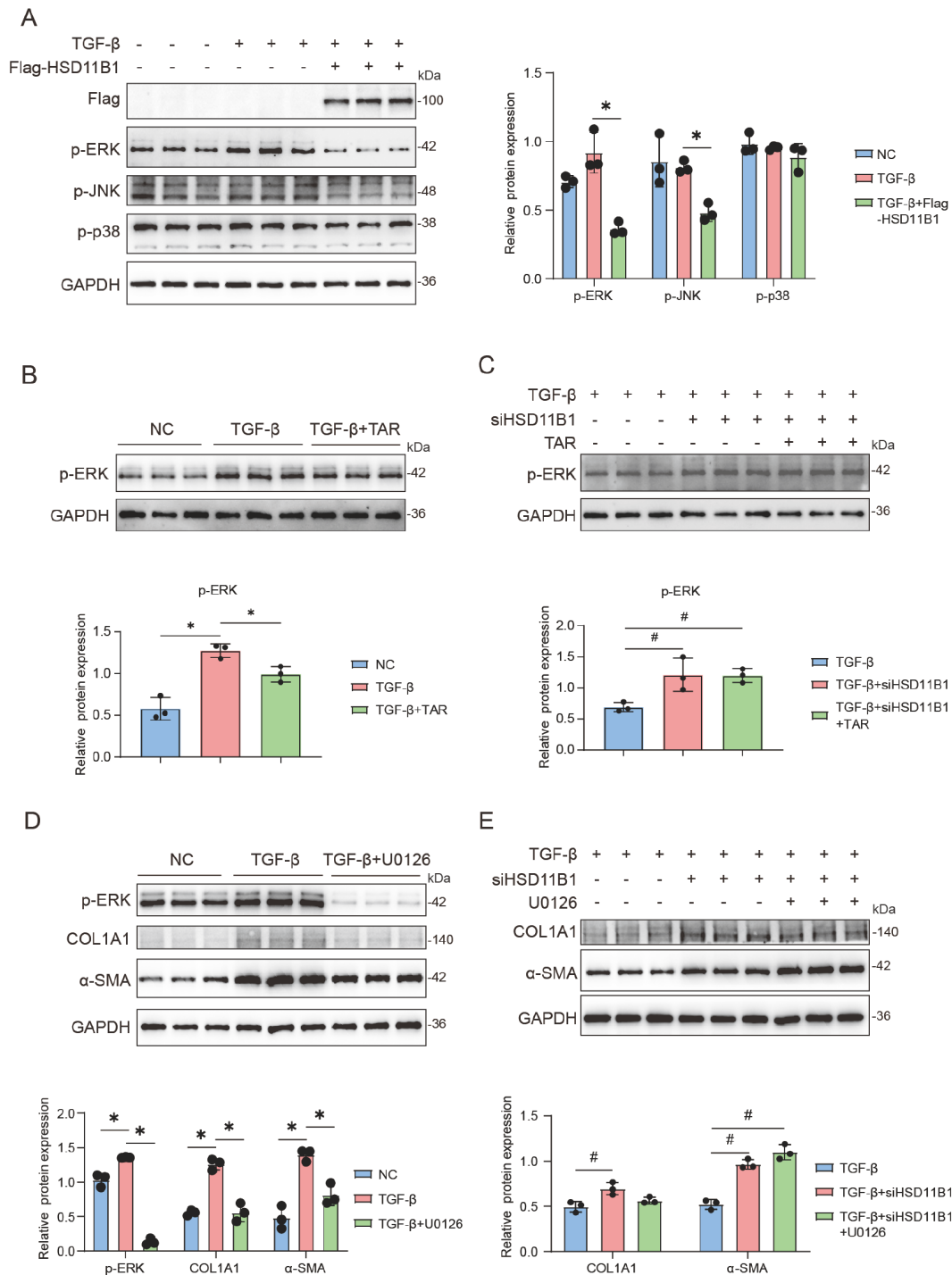


Figure 7. HSD11B1 inhibits HSC activation by suppressing the ERK MAPK signaling pathway (A) LX-2 cells were transfected with the Flag-HSD11B1 plasmid and subsequently stimulated with TGF- β (20 ng/mL). After 48 h, the phosphorylation levels of ERK, JNK, and p38 were detected by western blot analysis. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF- β group. (B) LX-2 cells were treated with TGF- β (20 ng/mL) and TAR (20 μ M) for 48 h, and the phosphorylation level of ERK was detected by western blot analysis. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF- β group. (C) LX-2 cells were transfected with siHSD11B1 and then treated with TGF- β (20 ng/mL) and TAR (20 μ M) for 48 h. The phosphorylation level of ERK was subsequently detected by western blot analysis. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). # $P < 0.05$ versus the TGF- β group. (D) LX-2 cells were treated with TGF- β (20 ng/mL) and U0126 (10 μ M) for 48 h, and protein expression was subsequently detected by western blot analysis. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). * $P < 0.05$ versus the TGF- β group. (E) LX-2 cells were transfected with siHSD11B1 and then treated with TGF- β (20 ng/mL) and U0126 (10 μ M) for 48 h, after which protein expression was detected by western blot analysis. Data are presented as the mean $\pm$ SEM by one-way ANOVA ($n = 3$ per group). # $P < 0.05$ versus the TGF- β group.

CCl₄-induced liver fibrosis by downregulating YAP, and VDR agonists can impede fibrosis progression by disrupting the interaction between neutrophil extracellular traps and hepatic stellate cells [27,28]. Whether TAR can mediate liver fibrosis by influencing the expression of these targets remains to be further investigated.

Through further screening integrating single-cell transcriptomic sequencing and molecular docking, we confirmed that HSD11B1 is a key target of TAR. HSD11B1, a glucocorticoid-metabolizing enzyme highly expressed in the liver, has been implicated in metabolic diseases [29]. The role of HSD11B1 in liver fibrosis remains contentious. Zou *et al.* [21] demonstrated that two genetic knockout models of *HSD11B1*—specifically, HSC-specific (*Hsd11b1^{fl/fl}/Pdgfrb-cre*) and systemic (*Hsd11b1^{-/-}*) knockout mice—exhibited significantly aggravated HSC activation and liver fibrosis. Furthermore, the HSD11B1 inhibitor UE2316 produced effects consistent with those observed in genetically modified mice. Similarly, research by Larner *et al.* [22] indicated that systemic *HSD11B1* knockout led to upregulation of *COL1A1* mRNA levels. In contrast to these findings, some other studies showed that another HSD11B1 inhibitor, J2H-1702, significantly ameliorated NASH and thioacetamide (TAA)-induced liver fibrosis [30,31]. However, the mechanism of J2H-1702 appears to extend beyond direct action on HSCs; Kim's work suggested that J2H-1702 enhances natural killer (NK) cell-mediated immune responses, thereby promoting the clearance of activated HSCs by NK cells to alleviate fibrosis [30]. Moreover, whether HSD11B1 inhibitors act exclusively through HSD11B1 remains to be fully elucidated. In the present study, through a combination of *in vivo* and *in vitro* experiments integrated with multiomics analyses, we confirmed that HSD11B1 inhibits HSC activation by suppressing the ERK MAPK pathway. This conclusion aligns with the findings from *HSD11B1* genetic knockout models.

Accumulating evidence suggests that inhibiting the activation of the MAPK pathway to alleviate liver fibrosis represents a promising therapeutic strategy. For instance, carvacrol, an inhibitor of transient receptor potential melastatin 7 (TRPM7), suppresses HSC activation by blocking the TRPM7-MAPK axis [32]. In alcoholic hepatic fibrosis, inhibition of α 5 β 1 Integrin and Metalloproteinase 8 (ADAM8) effectively reduces LX-2 cell activation and the phosphorylation levels of ERK and p38 [33]. Furthermore, hepatocyte-derived exosomes enriched with H2AFJ activate the MAPK/STMN1 signaling pathway to promote fibrosis progression, while *in vivo* intervention targeting H2AFJ expression significantly mitigates liver fibrosis [34]. In our study, TAR was found to inhibit ERK phosphorylation, thereby suppressing MAPK pathway activation in HSCs. This effect is mediated by HSD11B1, as *in vitro* knockdown of *HSD11B1* impeded the inhibitory effect of TAR on the MAPK pathway. U0126, a potent and selective cell-permeable compound, acts as an allosteric inhibitor of MEK1 and MEK2 kinases, thereby inhibiting ERK MAPK pathway activation [35]. Our results demonstrate that U0126 suppresses HSC activation, and knockdown of *HSD11B1* compromises this inhibitory effect, indicating that HSD11B1 functions upstream to block signal transduction along the MEK-ERK axis. Through the series of experiments described above, we confirmed the impact of TAR on the HSD11B1-ERK MAPK axis, providing an alternative mechanistic basis for the therapeutic strategy of inhibiting MAPK signaling to alleviate liver fibrosis.

In summary, our study demonstrates that TAR inhibits HSC activation and alleviates liver fibrosis by upregulating HSD11B1

expression, which in turn suppresses the ERK MAPK signaling pathway. These findings provide new insights into targeting HSD11B1 with TAR for the treatment of hepatic fibrosis, supporting its potential as a novel clinical therapeutic strategy.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The HSD11B1 structure reported in this study was obtained from the AlphaFold database (<https://alphafold.ebi.ac.uk/>) with the accession number AF-P28845-F1-v6. The RNA-seq datasets in this paper have been deposited in the NCBI under accession number PRJNA1469363. All other data supporting the findings of this study, including raw data and original images, are available from the corresponding author upon reasonable request.

Author Contributions

P.W. and B.W. designed study. P.W. and H.Z. performed experiments and analyzed data. P.W. and Y.D. collected clinical samples and studied results. Y.D. and Y.C. conducted animal experiments and isolated primary cells. L.H. and J.J. provided technical assistance. B.W. supervised experiments. P.W. and B.W. generated figures and wrote the manuscript. All authors approved the final version of the manuscript.

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Conflict of Interest

The authors declare they have no conflict of interest.

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