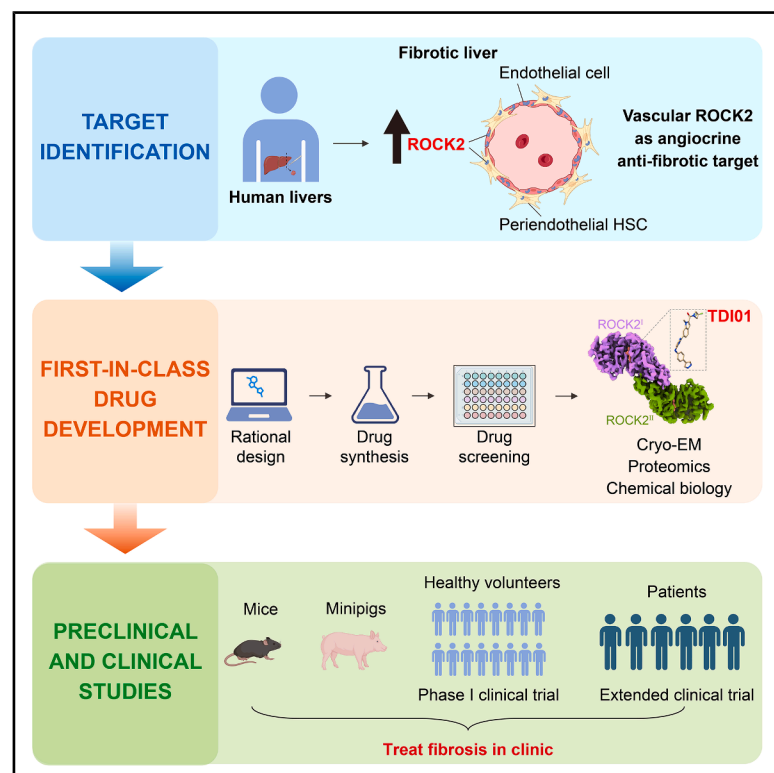


Selective targeting of endothelial and perivascular angiocrine ROCK2 treats liver fibrosis

Graphical abstract



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In brief

ROCK2 upregulation in endothelial and perivascular cells triggers fibrogenic vascular dysfunction in liver diseases. Targeting pro-fibrotic vascular ROCK2 by its selective inhibitor TDI01 exhibits anti-fibrotic potency in preclinical MASH models and clinical trials of human patients with liver fibrosis.

Highlights

- Identify vascular angiocrine ROCK2 as a druggable anti-fibrotic target in MASH
- Mechanism-based development of ROCK2-specific inhibitor as an anti-fibrotic therapeutic
- ROCK2-selective inhibitor TDI01 blocks liver fibrosis in rodent and minipig MASH models
- ROCK2 inhibitor TDI01 shows anti-fibrotic trend in human patients with liver fibrosis

Article

Selective targeting of endothelial and perivascular angiocrine ROCK2 treats liver fibrosis

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SUMMARY

Liver fibrosis is a prominent pathological process contributing to death from hepatic diseases, including metabolic dysfunction-associated steatohepatitis (MASH). There is limited treatment for liver fibrosis. Here, we find that upregulation of Rho-associated coiled-coil containing kinase 2 (ROCK2) in liver endothelial cells (ECs) and perivascular hepatic stellate cells (HSCs) causes vascular niche dysfunction and triggers pro-fibrotic angiocrine signaling. Based on the vascular druggable target ROCK2, we developed its selective inhibitor showing anti-fibrotic potency in preclinical models and human patients. The ROCK2-selective inhibitor TDI01 restored vascular phenotype and alleviated fibrosis in rodent and minipig MASH models. A phase 1 clinical trial (*ChiCTR2200058868*) of TDI01 demonstrated its favorable pharmacokinetics and safety in humans. An extended clinical trial (*ChiCTR2400082056*) showed a trend toward reducing liver fibrosis in five of six patients after TDI01 treatment. Thus, we discover vascular ROCK2 as a pro-fibrotic target, and development of an inhibitor selectively targeting angiocrine ROCK2 may provide a treatment of liver fibrosis in human patients.

INTRODUCTION

The liver undergoes self-repair after injury.^{1–12} Liver regeneration requires coordinated crosstalk between different cell types.^{13–16}

In this process, abnormally activated hepatic stellate cells (HSCs) differentiate to myofibroblast cells to cause liver fibrosis.^{17–27} Liver fibrosis is a histological feature found in many hepatic diseases, including metabolic dysfunction-associated

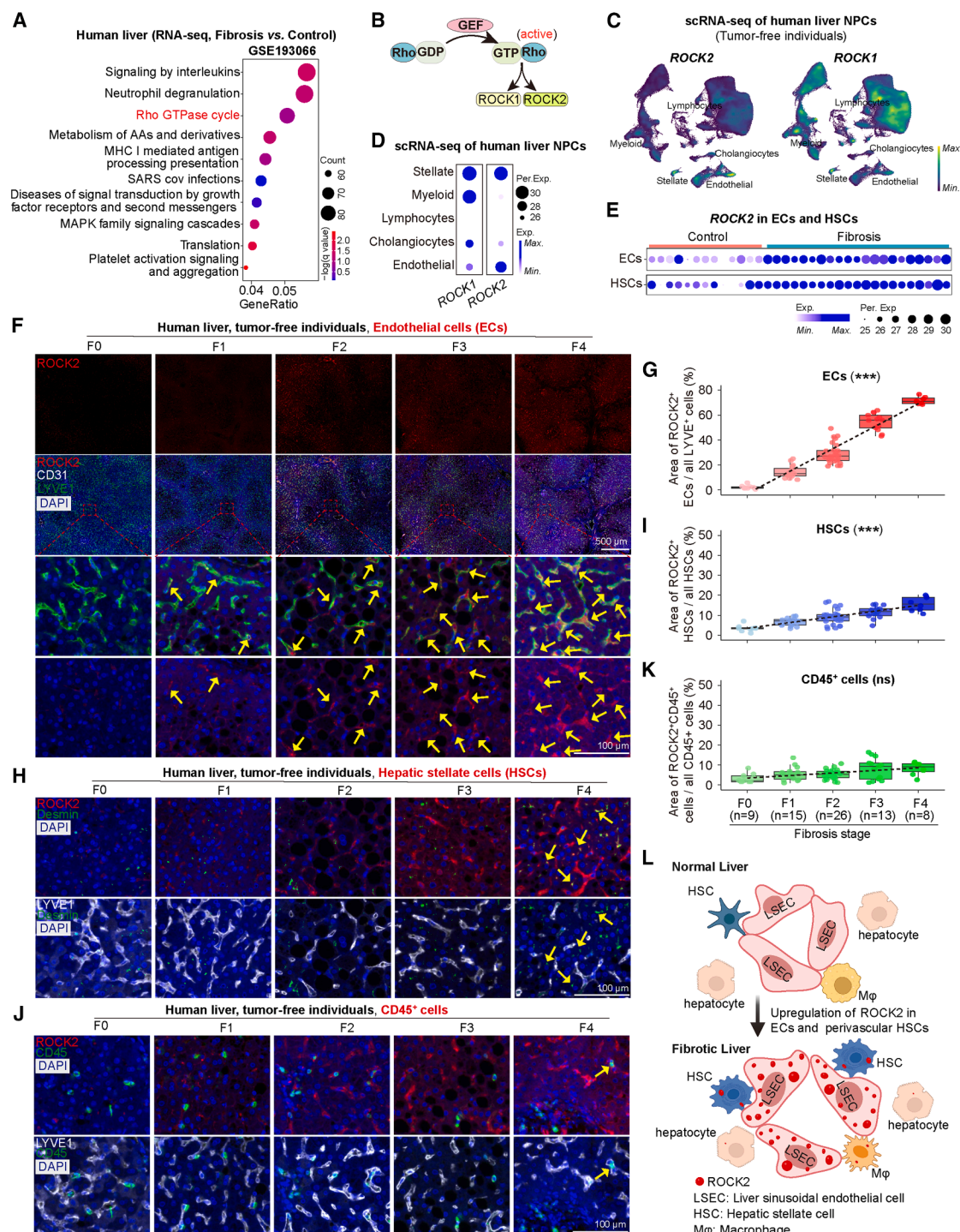


Figure 1. Upregulation of ROCK2 in vascular ECs and perivascular HSCs in human fibrotic livers

(A) Dot plot displaying the differentially enriched pathways in human fibrotic livers vs. normal livers, based on Reactome analysis ($n = 164$, GEO: GSE193066). (B) Schema demonstrating ROCK1 and ROCK2 as downstream effectors of Rho GTPase family, suggesting ROCKs as potential druggable targets to suppress the Rho GTPase pathway in fibrotic livers. (C) Dot plot showing the expression of ROCK1 and ROCK2 in indicated liver cell types, based on scRNA-seq analysis of NPCs from healthy and fibrotic human liver (tumor free). (D) Density plot of ROCK1 and ROCK2 expression across liver cell types from scRNA-seq data of healthy and fibrotic human livers (tumor free) (normal, $n = 12$; fibrotic, $n = 21$).

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steatohepatitis (MASH).^{19,28,29} Moreover, liver fibrosis can progress to deadly cirrhosis and liver cancer.^{19,28,30,31} While there are a series of clinical trials assessing the efficacy of anti-fibrotic candidates, liver fibrosis has limited treatment.^{32–40} Thus, there is an unmet need to identify druggable fibrogenic molecules in liver fibrosis.

HSC activation is modulated by surrounding cells.^{41–44} Immune cells have been shown to interact with HSCs to promote liver fibrosis.^{44–46} A growing list of therapeutic candidates have been tested in clinical trials of MASH patients with fibrosis.^{45,47–49} Metabolic regulators show beneficial effects in MASH patients, including the recently approved thyroid hormone receptor- β (THR β) and glucagon-like peptide-1 (GLP1) receptor agonists resmetirom and semaglutide.^{50,51} A mitogen-activated protein kinase kinase 4 (MKK4) inhibitor promotes liver regeneration in preclinical models.⁵² Nevertheless, very limited therapeutics show efficacy in reversing liver fibrosis in patients. Targeting pro-fibrotic molecules such as lysyl oxidase-2 (LOXL2) manifests suboptimal effects in liver fibrosis patients.^{53,54} Developing mechanism-based therapeutics is needed for the anti-fibrotic treatment of liver diseases.

Here, we identify that vascular upregulation of Rho-associated coiled-coil containing kinase 2 (ROCK2) triggers pro-fibrotic angiocrine signaling. A ROCK2-selective inhibitor TDI01 was developed with anti-fibrotic effects in preclinical and clinical studies.

RESULTS

Vascular upregulation of ROCK2 in human fibrotic livers

We conducted transcriptomic analyses of human livers. The RNA sequencing (RNA-seq) data of GEO: GSE193066 uncovered an enrichment of the “Rho GTPase cycle” in human MASH livers (Figure 1A). Integrative analysis of dataset GEO: GSE173735 similarly showed enrichment of “Rho GTPase effectors” and Rho GTPase cycle signals in liver fibrosis patients (Figure S1A). Both Rho GTPase effectors and the Rho GTPase cycle are linked to the ROCK family, which represents an ideal drug target to design a kinase inhibitor.^{55–62} ROCK consists of two isoforms, ROCK1 and ROCK2 (Figure 1B).⁶³ Thus, we delineated the expression of ROCKs in human livers, using single-cell RNA-seq (scRNA-seq) analysis (Figures S1B–S1G). ROCK2 is preferentially expressed in human liver endothelial cells (ECs) and HSCs. In contrast, ROCK1 is ubiquitously expressed (Figures 1C and 1D). Additionally, ROCK2 expression is upregulated in fibrotic liver ECs and HSCs compared with healthy livers, whereas ROCK1 expression shows no significant changes (Figures 1E and S1H). Therefore, ROCK2 expression and activity might be selectively increased in human fibrotic liver ECs and perivascular HSCs.

We then examined the expression of ROCK2 protein in human livers. There is a stage-dependent increase of ROCK2 in human fibrotic livers (Figures 1F, 1G, and S2A–S2E). Co-staining with lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) showed that ROCK2 and phosphorylated ROCK2 (pROCK2)⁶³ were predominantly localized in liver sinusoidal ECs (LSECs) expressing LYVE1. ROCK2 was also upregulated in the desmin⁺ perivascular HSCs of human fibrotic livers (Figures 1H and 1I). Other cell types exhibited lower levels of ROCK2, including CD45⁺ hematopoietic cells (HCs) and hepatocyte nuclear factor 4 α (HNF4 α)⁺ hepatocytes (Figures 1J, 1K, and S2F–S2H). These data show the selective upregulation of ROCK2 in endothelial and perivascular HSCs of human fibrotic livers, representing an anti-fibrotic target (Figure 1L).

The expression of transcription factors and receptors potentially upregulating ROCK2 were then assessed. Several reported transcription factors associated with ROCK2 upregulation were found to be induced in fibrotic liver ECs or HSCs, including ZNF143,⁶⁴ SMAD4,⁶⁵ SRF,⁶⁶ TEAD4, TEAD1, and YAP1.^{67,68} There is also upregulation of receptors that could upregulate ROCK2 expression in fibrotic liver ECs and HSCs, including EPHA4,⁶⁹ ESR1,⁷⁰ F2R,⁷¹ LPAR1,⁷² NRP1,^{73,74} P2RY12,^{75,76} and RTN4R^{77,78} (Figures S2I and S2J). Aberrant induction of these transcription factors and receptors might contribute to ROCK2 upregulation in fibrotic liver ECs and HSCs.

ROCK2 in ECs and HSCs promotes liver fibrosis in mouse models

ROCK2 expression was analyzed in a CCl₄-induced mouse liver fibrosis model. As compared with the vehicle-treated control group, ROCK2 was similarly upregulated in the mouse liver ECs and HSCs after CCl₄ injury (Figures S2K and S2L). We then tested mice lacking ROCK2 in different cell types, including ECs, HSCs, HCs, and hepatocytes. First, mice with inducible EC-specific knockout (KO) of ROCK2 (ROCK2 ^{Δ EC/ Δ EC}) were generated. Mice expressing floxed ROCK2 (ROCK2^{fl/fl}) were crossed with mice harboring EC-specific *Cdh5*/VE-Cadherin-Cre^{ERT2}. The resulting mice were treated with tamoxifen to generate ROCK2 ^{Δ EC/ Δ EC} mice (Figures 2A and S3A–S3E). ROCK2 ^{Δ EC/ Δ EC} and control ROCK2^{fl/fl} mice were tested in a CCl₄ injury model. ROCK2 ^{Δ EC/ Δ EC} mice showed lower levels of liver fibrosis and injury than controls, as evidenced by Sirius red staining, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and hepatic hydroxyproline (HYP) contents (Figures 2B–2E). These implicate that ROCK2 upregulation in ECs contributes to liver fibrosis after injury.

We then generated mice deficient in ROCK2 in HSCs (ROCK2 ^{Δ HSC/ Δ HSC})⁷⁹ using HSC-specific *Lrat*-Cre mice

(E) Dot plot showing selective ROCK2 expression in the ECs and HSCs of healthy and fibrotic human livers.

(F–K) Stage-dependent upregulation of ROCK2 in the ECs and HSCs of human fibrotic livers. ROCK2 expression was examined in ECs, LSECs, HSCs, and HCs across fibrosis stages. Co-staining was performed for ROCK2 with LSEC markers CD31 and LYVE1 (F), HSC marker desmin (H), or HC marker CD45 (J). ROCK2⁺ cell proportions were quantified relative to CD31⁺ and LYVE1⁺ LSECs (G), desmin⁺ HSCs (I), or CD45⁺ HCs (K). Statistical significance was assessed by one-way ANOVA (* p < 0.05 and *** p < 0.001). Sample sizes: F0, n = 9; F1, n = 15; F2, n = 26; F3, n = 13; F4, n = 8. Scale bars, 100 μ m.

(L) Schema showing selective ROCK2 upregulation in the ECs and peri-endothelial HSCs of fibrotic livers, suggesting endothelial/peri-endothelial ROCK2 as a druggable anti-fibrotic target.

See also Figures S1 and S2 and Table S6.

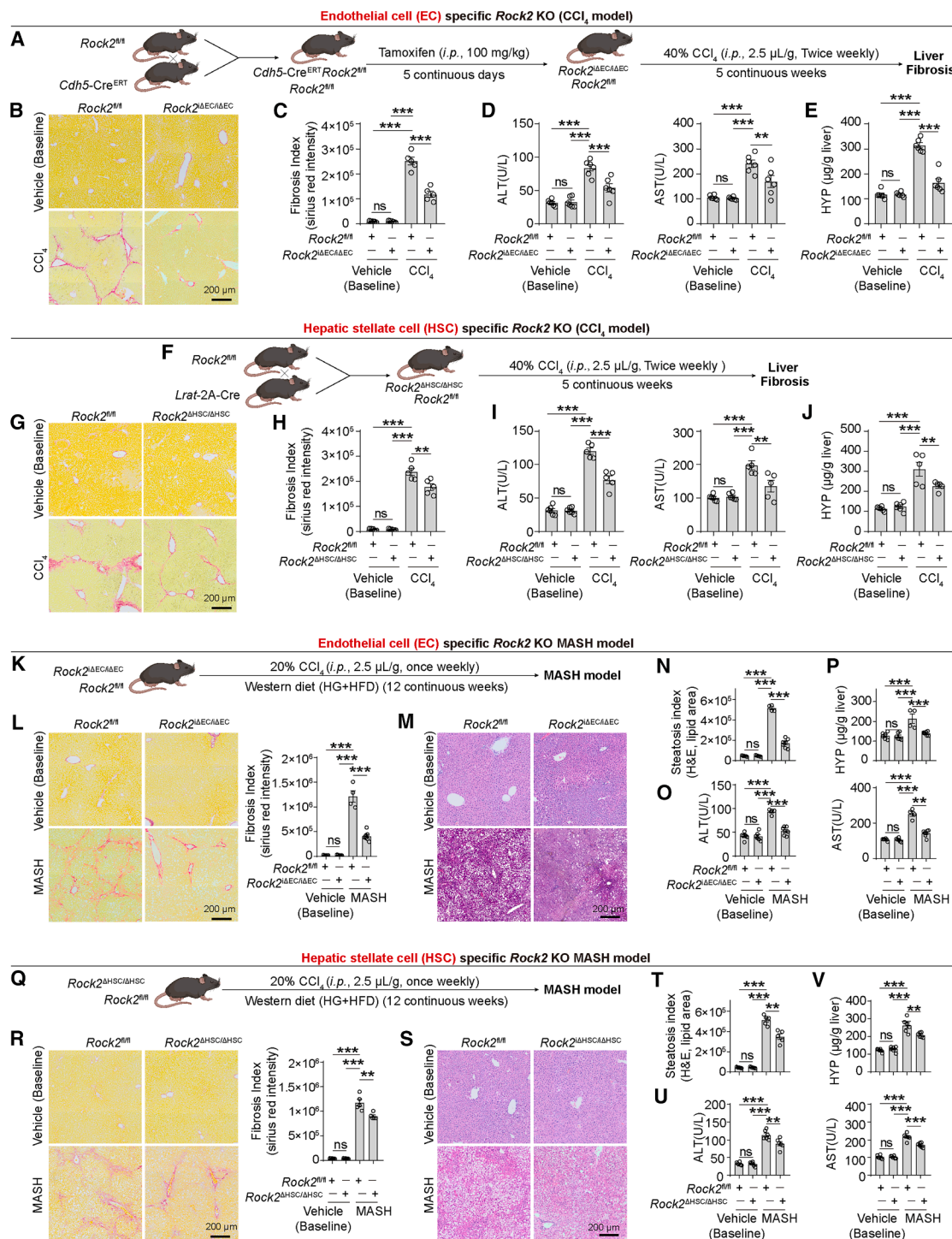


Figure 2. Genetic KO of *ROCK2* in ECs and HSCs attenuates liver fibrosis and injury in mouse models

(A) Experimental approach to test the contribution of endothelial *ROCK2* in mouse liver fibrosis model. EC-specific inducible *ROCK2* KO mice (*Rock2^{ΔEC/ΔEC}*) were generated by crossing floxed *ROCK2* (*Rock2^{fl/fl}*) mice with VE-cadherin/*Cdh5*-*Cre^{ERT2}* mice. Tamoxifen was administered to resulting mice induce Cre-mediated recombination of floxed *ROCK2*. *Rock2^{ΔEC/ΔEC}* and control *Rock2^{fl/fl}* mice were subjected to CCl₄ injection (2.5 μL/g) for 5 weeks to induce liver fibrosis.

(B–E) Liver injury and fibrosis were reduced in mice with inducible EC-specific *ROCK2* KO (*Rock2^{ΔEC/ΔEC}*) after repeated CCl₄ injections, as compared with control *Rock2^{fl/fl}* mice. Vehicle-treated groups were used to determine baseline levels. Fibrosis was assessed by quantifying Sirius red staining (B and C). Serum

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(Figures 2F and S3F–S3I). In the CCl₄-induced liver fibrosis model, *ROCK2*^{ΔHSC/ΔHSC} had reduced liver collagen deposition, lower serum levels of AST and ALT, and decreased HYP content compared with control mice (Figures 2G–2J). As compared with *ROCK2*^{ΔEC/ΔEC} mice, HSC-specific *ROCK2* deletion in mice reduced liver fibrosis to a lesser extent.

We also examined mice lacking *ROCK2* in HCs (*ROCK2*^{ΔHC/ΔHC}) with *Vav1*-2A-Cre mice (Figures S3J–S3N). As compared with control *ROCK2*^{fl/fl} group, *ROCK2*^{ΔHC/ΔHC} mice show little improvement in liver fibrosis (Figures S3O and S3P). Hepatocyte-specific knockdown of *ROCK2* in mice was performed with adeno-associated virus 8 (AAV8) expressing short hairpin RNA (shRNA) under the albumin (ALB) promoter (*Alb-shRock2*) (Figures S3Q–S3T). Mice were treated with *Alb-shRock2* AAV and tested in the liver fibrosis model. There was little difference in the degree of liver fibrosis in tested mice (Figures S3U and S3V).

The role of *ROCK2* was assessed in a mouse MASH model.⁸⁰ In this model, EC-specific KO of *ROCK2* in mice reduced liver fibrosis, compared with control mice (Figure 2K). *ROCK2*^{ΔEC/ΔEC} mice also exhibited attenuated liver steatosis, reduced serum levels of ALT and AST, and decreased liver HYP amounts (Figures 2L–2P). *ROCK2*^{ΔHSC/ΔHSC} mice displayed mitigated fibrosis, reduced hepatic steatosis, and lowered serum ALT and AST, as well as liver HYP levels, relative to control mice (Figures 2Q–2V). Hepatocyte-specific knockdown of *ROCK2* showed little effect on liver fibrosis in the MASH model (Figures S3W–S3Z). These results implicate that *ROCK2* upregulation in EC and HSC contributes to liver fibrosis in MASH.

These data implicate *ROCK2* kinase as a potential druggable target for anti-fibrotic therapy. First, it is challenging to pharmacologically target Rho GTPases because their globular structure lacks a well-defined deep binding pocket.⁸¹ By contrast, the downstream ROCK kinases are structurally tractable targets. ROCK inhibitors might directly block kinase activity in pathological processes.^{82,83} Among the two ROCK isoforms, *ROCK2* is upregulated in the ECs and HSCs of fibrotic livers, with its basal level being relatively low in normal livers. *ROCK2* has been shown to play a more prominent role in regulating vascular remodeling than *ROCK1*,^{84–88} which might be associated with the preferential effect of *ROCK2* on LSEC fenestration.⁷⁴ Therefore, *ROCK2*-selective inhibitors could effectively target the accessible liver vasculature with lower side effects. Third, current ROCK inhibitors exhibit suboptimal selectivity and po-

tency.⁸⁹ Thus, there is a need to develop a next-generation *ROCK2* inhibitor with higher selectivity and rigorously assessed anti-fibrotic efficacy.

Identification of TDI01 as a *ROCK2*-selective inhibitor

We then set out to identify a *ROCK2*-selective inhibitor (Figure 3A). Based on the crystallographic structure of *ROCK2* and known inhibitor scaffolds, we designed and synthesized 686 compounds. We employed homogeneous time-resolved fluorescence (HTRF) technology to identify compounds that specifically inhibit the activity of *ROCK2* but not *ROCK1*. The serine/threonine kinase (STK) substrate-biotin, a universal substrate for STK, was used to assess the inhibition of *ROCK1* and *ROCK2* activity, separately (Figure S4A). We identified 126 candidates that significantly inhibited *ROCK2* activity, with 47 candidates exhibiting a 50% inhibitory concentration (IC₅₀) for *ROCK2* below 45 nM (Figure 3B). Among these 126 candidates, 41 compounds showed an IC₅₀ ratio of *ROCK1* to *ROCK2* greater than 200 (Figure 3C), implying their more-specific potency in inhibiting *ROCK2* than *ROCK1*.

Through an intersection analysis, 37 compounds met both the criteria of an IC₅₀ for *ROCK2* < 45 nM and a selectivity index (SI) of *ROCK1* to *ROCK2* > 200, manifesting specificity in suppressing *ROCK2* activity (Figure 3D). Then, pharmacokinetic (PK) studies were conducted in mice. 8 *ROCK2*-selective inhibitors were confirmed with sufficient drug exposure (Figure 3E), which were tested for cellular toxicity using human umbilical cord vein ECs (HUVECs) and LSECs. Only TDI01470 demonstrated IC₅₀ values exceeding 20 μM in both cell types, indicating minimal cellular toxicity (Figure 3F). The cardiotoxicity of TDI01470 was assessed by evaluating the inhibition of human ether-a-go-go related gene (hERG), with the inhibitor dofetilide⁹⁰ as a positive control. TDI01470 manifested minimal cardiotoxicity (Figure 3G). PK studies of this candidate were also performed in rats. After oral administration at a dose of 15 mg/kg, T_{max} was observed at 4 h, with a maximum concentration (C_{max}) of 1,174.4 ng/mL and an area under the curve from time zero to infinity (AUC_(0–∞)) of 13,728.1 h*ng/mL. The half-life (T_{1/2}) was determined to be 3.2 h. Notably, the bioavailability (F%) of TDI01 was calculated to be 56% (Figure 3H). Hence TDI01470 (molecular formula, C₂₆H₂₁F₂N₇O), with its favorable drug exposure and oral bioavailability, was selected as the final candidate. This compound was named as TDI01, exhibiting high selectivity for *ROCK2* over *ROCK1*, with an IC₅₀ of 10.31 nM in a *ROCK2* kinase assay, and at least 598-fold selectivity over *ROCK1* (IC₅₀

levels of ALT and AST were determined to measure liver injury (D), and liver HYP amounts (E) were also assessed. Representative images and quantifications are shown. *n* = 5 per group. Throughout this figure, statistical significance was determined using one-way ANOVA. ns, not significant, *p* > 0.05; ***p* < 0.01; ****p* < 0.001. (F) Approach to test HSC-specific *ROCK2* KO (*ROCK2*^{ΔHSC/ΔHSC}) mice in liver fibrosis model. *ROCK2*^{ΔHSC/ΔHSC} mice were generated by crossing *Lrat*-2A-Cre mice with *ROCK2*^{fl/fl} mice. Resulting *ROCK2*^{ΔHSC/ΔHSC} mice and control *ROCK2*^{fl/fl} mice were then subjected to vehicle or CCl₄ treatment. (G–J) Relative to control groups, *ROCK2*^{ΔHSC/ΔHSC} mice exhibited reduced liver fibrosis and injury after CCl₄ treatment, as evidenced by Sirius red staining (G and H), serum levels of ALT and AST, and hepatic HYP amounts (I and J), *n* = 5 per group. (K–N) *ROCK2*^{ΔEC/ΔEC} and *ROCK2*^{fl/fl} mice were tested in a mouse MASH model. *ROCK2*^{ΔEC/ΔEC} and control *ROCK2*^{fl/fl} mice were subjected to weekly injections of CCl₄ and fed with a western diet to induce MASH phenotype, with vehicle-fed mice being baselines (K). *ROCK2*^{ΔEC/ΔEC} mice exhibited reduced collagen deposition and steatosis than control mice, as assessed by Sirius red (L), and H&E (M and N) staining. *n* = 5 per group. (O and P) Serum levels of AST and ALT (O) and hepatic HYP amounts (P) of *ROCK2*^{ΔEC/ΔEC} mice were lower than control group in the MASH model. *n* = 5 per group. (Q–V) Contribution of *ROCK2* in HSCs was tested in the mouse MASH model. In this model, *ROCK2*^{ΔHSC/ΔHSC} mice showed reduced fibrosis and steatosis (R–T), lower serum AST and ALT levels (U), and decreased hepatic HYP quantity (V) than controls (*n* = 5 per group).

See also Figure S3.

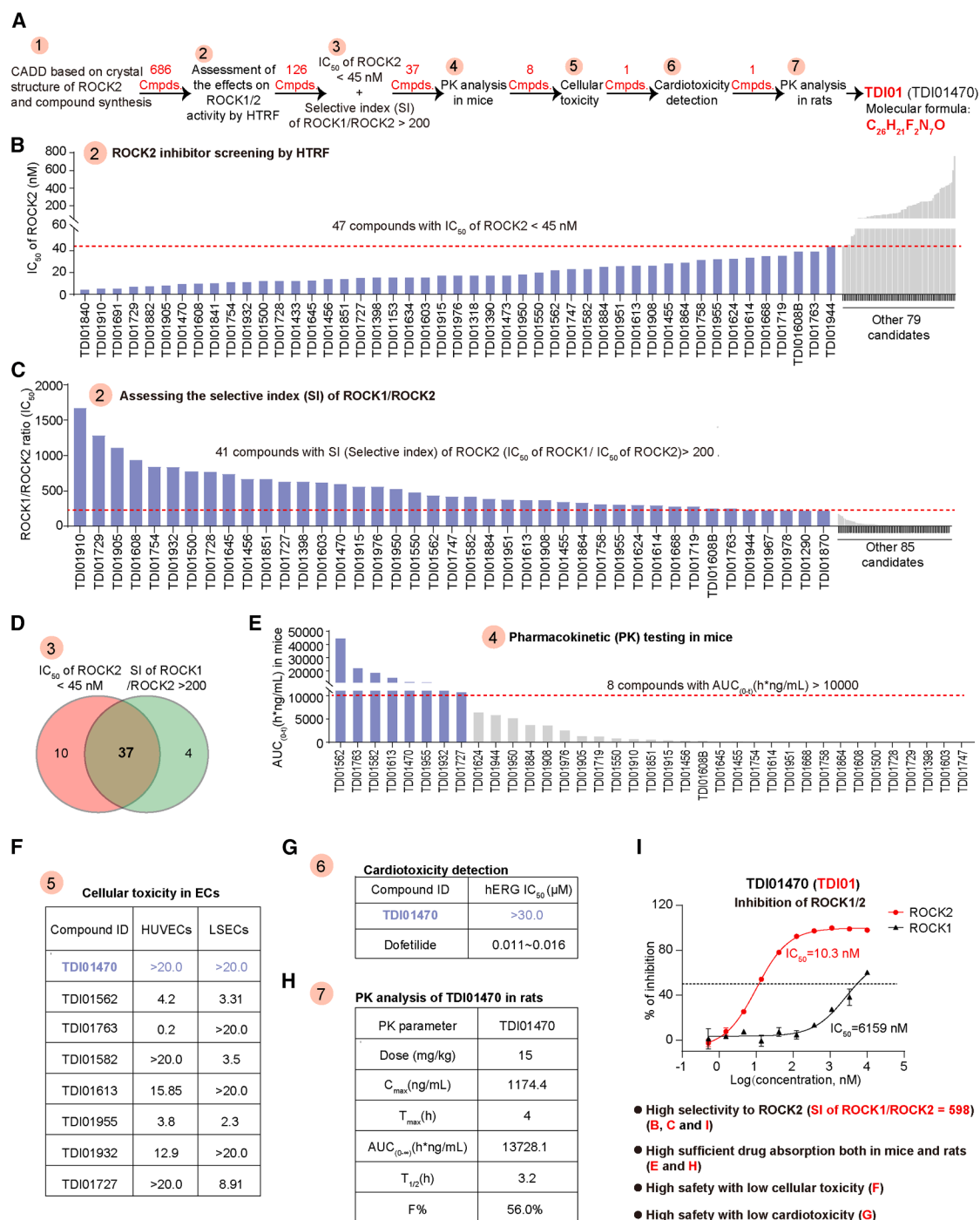


Figure 3. Identification of the ROCK2-selective inhibitor TDI01 with optimal PKs and safety

(A) Schematic illustrating the screening process for ROCK2-selective inhibitor. Based on ROCK2 crystal structures and known scaffolds, a computer-aided drug design (CADD) approach was used to yield 686 compounds, which were evaluated for ROCK1/ROCK2 activity by HTRF, followed by pharmacological safety and PKs analyses. Experiments were performed in triplicate.

(B) Identification of potent ROCK2 inhibitors. Among the 686 compounds, 129 compounds inhibited ROCK2, with 47 compounds showing IC_{50} < 45 nM.

(C) Identification of inhibitor with high ROCK2 selectivity. Among 129 ROCK2 inhibitors, 41 compounds showed IC_{50} ratio (ROCK1 to ROCK2) over 200.

(D) Venn diagram showing 37 compounds with IC_{50} of ROCK2 < 45 nM and IC_{50} ratio (ROCK1/ROCK2) > 200. These 37 inhibitors advanced to further screening.

(E) Selection of ROCK2 inhibitor with optimal PK in mice. PK screening of 37 ROCK2-selective inhibitors in mice (20 mg/kg oral). Based on the $AUC_{(0-\infty)}$, 8 compounds with exposure >10,000 were identified ($n = 3$ per compound).

(F) Cytotoxicity of 8 compounds was tested in HUVECs and LSECs. Only TDI01470 showed IC_{50} > 20 μ M in both cells and advanced to further evaluation.

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value of 6,159 nM) (Figure 3I). This ROCK2-selective inhibitor TDI01 holds potential as an anti-fibrotic therapeutic.

TDI01 directly binds to ROCK2 as its inhibitor

We next performed bio-layer interferometry (BLI) characterization of TDI01 with the purified kinase domains (KDs) of ROCK2 and ROCK1 (Figure S4B, left and middle). TDI01 exhibited high-affinity binding to ROCK2 ($K_D = 65.56$ nM) (Figure 4A), whereas it showed negligible binding to ROCK1 at concentrations up to 60 μ M (Figure 4B). A biotin-labeled derivative of TDI01 (Figure S4C) was incubated with human ECs and the human HSC line LX2. Quantitative proteomics of pulled down cellular lysates revealed ROCK2 as the highest-intensity protein interacting with biotin-TDI01 (Figures 4C and 4D). Western blot analysis confirmed the binding of TDI01 to ROCK2 but not ROCK1 (Figure S4D). Meanwhile, TDI01 exhibited dose-dependent inhibition of ROCK2 activity in ECs and LX2 cells, and the inhibitory effect of TDI01 on ROCK2 activity was enhanced in cells overexpressing ROCK2 (ROCK2 OE) (Figures 4E and 4F).

To elucidate the binding mode and inhibition mechanism of TDI01, we solved the structure of ROCK2 protein bound with TDI01 via cryo-electron microscopy (cryo-EM). Human ROCK2 KD was incubated with TDI01 (hereafter ROCK2-TDI01; Figures S4E and S4F). The cryo-EM structure of ROCK2-TDI01 was determined (Figure S4G), revealing that ROCK2 adopts a dimeric configuration (Figure 4G). The structure of ROCK2 KD consists of a small N-terminal lobe and a larger C-terminal lobe connected by a flexible hinge. Dimerization of the ROCK2 molecules is mediated by two α helices (α A2 and α A3) from the N-terminal lobe of each protomer (Figure S4H). The catalytic activity of ROCK2 relies on the binding of ATP into a conserved pocket located between the N- and C-terminal lobes (Figure S4I). TDI01 binds to a hydrophobic pocket within ROCK2 that partially overlaps the ATP-binding pocket (Figure 4H). Specifically, the 1H-pyrazole group of TDI01 engages the kinase hinge, and the pyrimidine ring of TDI01 forms a π - π stacking interaction with the side chain of ROCK2 residue F233 (Figure 4I). TDI01-bound ROCK2 adopts a “Asp-Phe-Gly motif (DFG)-out” conformation that is characteristic of the inactive state of kinase,⁹¹ whereas both ROCK1 and ROCK2 adopt the “DFG-in” conformation in the ROCK1-AMPPNP (adenylyl-imidodiphosphate, non-hydrolyzable ATP analog) complex (PDB: 2v55) and the ROCK2-AMPPNP complex (Figures S4J–S4L). The binding of TDI01 displaces the α C helix from the N-terminal lobe of ROCK2 to accommodate the difluoroazetidine group of TDI01 (Figure 4J). Mechanistic contrast analysis suggests that this displacement of α C helix is unlikely to occur in ROCK1, where the C-helix mobility is sterically hindered by the residue phenylalanine 194 (F194) in ROCK1, as compared to the residue leucine 210 (L210) in ROCK2. These data help to elucidate the selective binding of ROCK2 by TDI01 and its inhibitory activity.

To test the role of L210 in mediating the specific binding between ROCK2 and TDI01, we generated the ROCK2 KD with mutation of L210 to phenylalanine (L210F) (Figure S4B, right). TDI01-ROCK2 binding was abrogated by L210F point mutant in ROCK2 (Figure 4K). Biotin-TDI01 pulled down ROCK2 from control HUVECs and LX2 cells but not cells with ROCK2 KO. Complementing these ROCK2-deficient cells with wild type but not L210F mutant ROCK2 restored their binding with biotin-TDI01 (Figures 4L and 4M). TDI01 showed little inhibition of ROCK2 activity in ROCK2-deficient cells after reconstitution with ROCK2 L210F mutant. By contrast, complementing ROCK2-KO cells with wild-type ROCK2 reversed the inhibition of ROCK2 activity by TDI01 (Figures 4N and 4O). Hence ROCK2-specific interaction with TDI01 relies on leucine 210 in the ATP-binding pocket.

TDI01 attenuates liver fibrosis in mouse and minipig models

We then evaluated the *in vivo* effects of TDI01 on liver fibrosis in the CCl₄-induced mouse liver fibrosis model (Figure S5A). TDI01 reduced serum ALT and AST levels, decreased hepatic HYP content, and ameliorated liver fibrosis (Figures S5B–S5E). In the mouse MASH model, TDI01 decreased serum ALT and AST levels, lowered hepatic HYP content, alleviated liver collagen deposition, and reduced steatosis (Figures S5F–S5K).

As compared to mice, minipigs can closely mimic human metabolic disorders.^{92–94} Therefore, we first tested the efficacy of TDI01 in a minipig liver fibrosis model induced by intraperitoneal injection of CCl₄. After minipigs were subjected to injury, animals were orally treated with TDI01 (Figure S5L). TDI01 attenuated the extent of liver fibrosis in the injured minipigs, relative to the vehicle group (Figures S5M–S5O).

Then, we tested the effect of TDI01 in a minipig MASH model^{92–95} (Figure 5A). In the MASH minipigs, after single-dose oral administration of TDI01 at 5 mg/kg, PK analysis revealed non-linear dose-proportional characteristics, with drug concentrations rapidly increasing within 2 h post administration and reaching a peak of 543.65 ng/mL at 4 h (Figure 5B). This data suggests that once-daily oral dosing of TDI01 may achieve sufficient efficacy in the minipig MASH model.

In this minipig MASH model, a 3-month induction stage led to liver fibrosis with the formation of pseudolobules (Figures 5C and 5D). Then, minipigs were orally treated with TDI01 at 5 mg/kg. The therapeutic efficacy of TDI01 was compared with vehicle control and a comparable dose of THR β agonist resmetirom. A 9-month TDI01 treatment reduced liver stiffness relative to controls (Figure 5E). TDI01 also alleviated the collagen deposition in MASH minipig livers, approaching the levels lower than those before treatment. Post-biopsies from the vehicle group displayed increased collagen deposition, and the resmetirom group showed modest changes in fibrosis (Figures 5C and 5F). TDI01

(G) Cardiotoxicity of TDI01470 was assessed via hERG inhibition, using an automated patch-clamp system. Human embryonic kidney cells expressing hERG (hERG-HEK293 cells) were utilized.

(H) ROCK2-selective inhibitors with optimal PKs were tested in rats. PK of TDI01470 in rats was assessed ($n = 3$), with $AUC_{(0-t)}$ > 10,000 and bioavailability being 55.9%. T_{max} , time to maximum concentration; $t_{1/2}$, half-lives; C_{max} , maximum concentration; $AUC_{(0-\infty)}$, area under the curve from time zero to infinity.

(I) TDI01470 (designated as TDI01) selectively inhibits ROCK2, with an IC_{50} 10.31 nM for ROCK2 vs. 6,159 nM for ROCK1 ($n = 3$).

See also Figure S4.

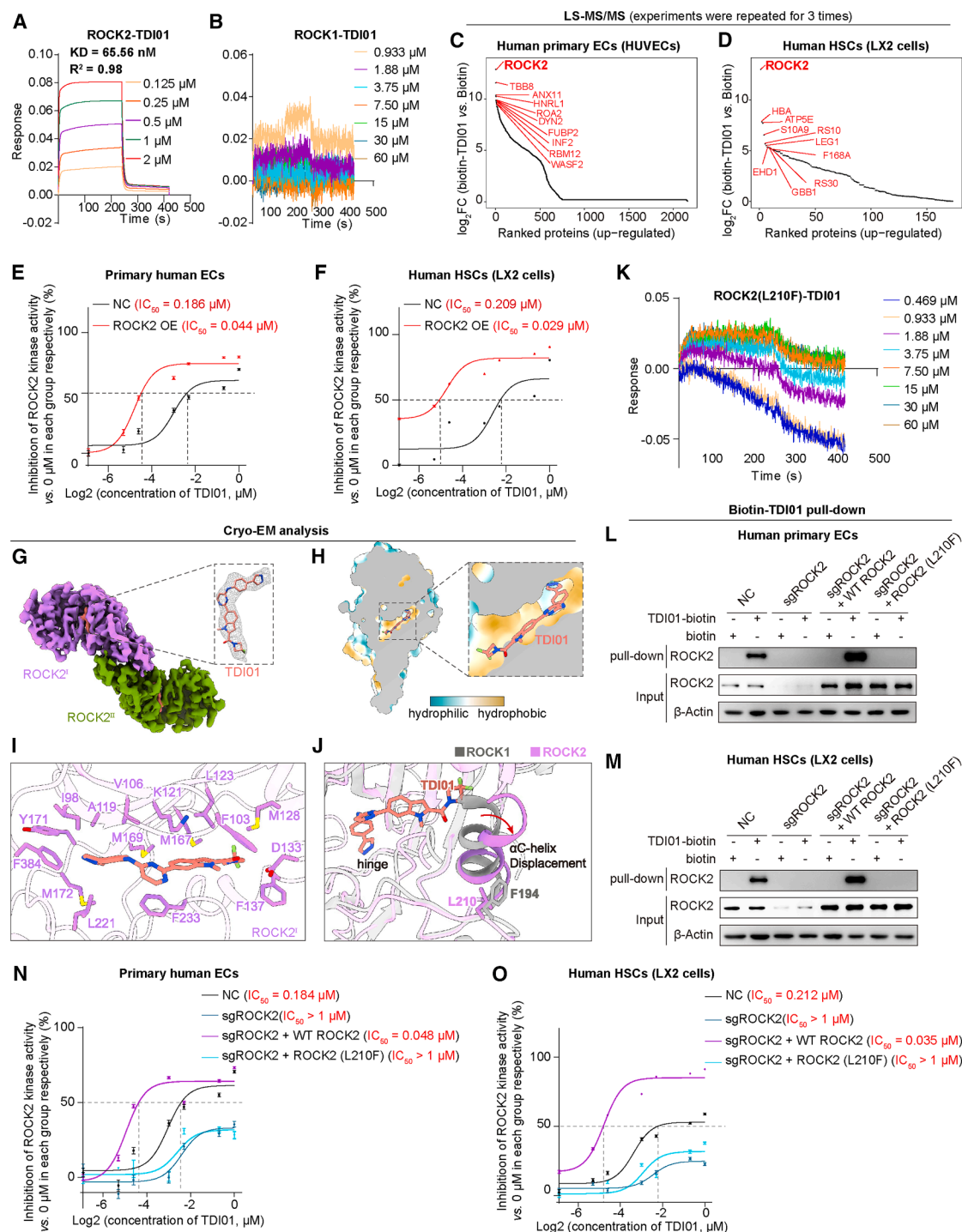


Figure 4. Characterization of TDI01 as a ROCK2-selective inhibitor

(A and B) BLI characterization of TDI01 binding to ROCK2 (A) and ROCK1 (B) KDs. TDI01 bound dose dependently to ROCK2 but showed negligible binding to ROCK1.

(C and D) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed selective binding of TDI01 to ROCK2 in HUVECs (C) and human HSC LX2 cells (D). Proteins pulled down with biotin-TDI01 were compared with biotin control and were subjected to LC-MS analysis. $n = 3$.

(E and F) Dose-dependent inhibition of ROCK2 activity by TDI01 in described ECs (E) and HSCs (F). ROCK2 was overexpressed in HUVECs (E) and LX2 cells (F). ROCK2-overexpressing (OE) and negative control (NC) cells were treated with TDI01. ROCK2 activity in the cell lysates was determined ($n = 3$ per concentration). (G) Cryo-EM density map of ROCK2-TDI01 complex. Each protomer of dimeric ROCK2 binds to TDI01.

(legend continued on next page)

treatment also reduced lobular inflammation and steatosis, as compared with the vehicle group. Resmetirom also decreased inflammation and steatosis in the MASH liver^{50,119–121} (Figures 5D, 5G, and 5H). Hepatic HYP content was also reduced after 9 months of TDI01 treatment (Figure 5I). These data show that TDI01 reverses liver fibrosis in minipig MASH model.

Formation of fenestration in LSECs is crucial for their functions. Loss of LSEC fenestration leads to capillarization in liver sinusoidal vasculature, a unique morphological feature that promotes fibrosis. In this minipig MASH model, animals showed a decreased fenestration number in LSECs and capillarization during vehicle treatment. By contrast, TDI01 treatment restored the number of fenestrations in minipig LSECs (Figures 5J and 5K). scRNA-seq analysis revealed an upregulation of genes related to fenestration formation, including *NOS3*,^{96–98} *PPARG*,^{99–101} *GATA4*,^{102,103} *KLF2*,¹⁰⁴ *SCARB1*,¹⁰⁵ *FLNA*,^{106,107} *VEGFA*,^{108,109} and *SIRT1*,¹¹⁰ accompanied by downregulation of capillarization-related genes such as *NRIH3*,¹¹¹ *MMP2*,^{112–114} *COL1A1*,^{112,115} *ANGPT2*,^{116,117} and *HIF1A*¹¹⁸ in pig liver ECs following TDI01 treatment (Figure 5L). These data suggest that inhibition of vascular ROCK2 by TDI01 restores LSEC function to block fibrosis.

TDI01 inhibits ROCK2 to restore the paracrine functions of LSECs in fibrotic livers

LSECs produce paracrine/angiocrine factors to influence perivascular cells.^{15,122–125} We then explored the influence of TDI01 on the angiocrine functions. Differentially expressed genes (DEGs) were identified between human fibrotic and healthy LSECs, as well as minipig MASH LSECs pre- and post-TDI01 treatment. DEGs encoding secreted proteins were revealed to discover the mechanism of how TDI01 modulates the angiocrine function of LSECs^{122,126–128} (Figures S6A and S6B). Comparison of human fibrotic and healthy LSECs revealed the 6 most upregulated pro-fibrotic angiocrine genes and 7 most downregulated anti-fibrotic angiocrine genes (Figure S6C). scRNA-seq analysis of minipig non-parenchymal cells (NPCs) before and after TDI01 treatment revealed concordant changes in angiocrine gene expression (Figure S6D). *LGALS3* was found to be the top upregulated gene in fibrotic human LSECs, which was suppressed by TDI01 in minipig LSECs (Figures S6E and S6F). Galectin-3, encoded by *LGALS3*, is a secreted protein that activates HSCs,¹²⁹ with its inhibitors showing anti-fibrotic effects.¹³⁰

We employed human precision-cut liver slices (hPCLSs) to define the pro-fibrotic effect of galectin-3 and its regulation by ROCK2. hPCLSs were derived from both healthy and fibrotic human livers (Figure S6G). In healthy hPCLSs, the addition of exog-

enous galectin-3 promoted collagen deposition (Figure S6H), induced HSC activation, and augmented collagen I production (Figures S6I and S6J). Cytokine array analysis of hPCLS culture supernatants demonstrated that galectin-3 increased the release of pro-inflammatory and pro-fibrotic cytokines (Figure S6K). In hPCLS from human fibrotic livers, treatment with TDI01 suppressed both HSC activation and cytokine release downstream of galectin-3. These effects of TDI01 were partially restored by galectin-3 supplementation (Figures S6L–S6O). These data imply that the anti-fibrotic effects of ROCK2 inhibition might be at least partially via inhibition of galectin-3.

Phase 1 clinical trial of TDI01 in humans

To assess the safety, tolerability, and PKs of TDI01, a placebo-controlled exploratory phase 1 clinical trial was performed (trial number, ChiCTR2200058868). A total of 62 healthy human volunteers were allocated to three treatment groups (Figure S7A; Table S1). Both single oral administration, multiple oral administrations, and food impact were tested in human volunteers. For single administration, TDI01 at the doses of 400, 800, and 1,200 mg were administered to healthy adults, respectively. Both fasting and postprandial states were tested. To test drug accumulation after multiple administrations, TDI01 at the doses of 200 and 400 mg TDI01 in suspension were administered to volunteers once daily for 7 days. Overall, TDI01 treatment did not cause severe adverse events in either group, suggesting its favorable safety profile (Table S2).

Single-dose administration of TDI01 revealed non-linear dose-proportional PK parameters, with a biphasic concentration-time curve observed post oral administration, and the first peak concentration was reached at 4 h (Figure S7B; Table S3). There was a dose-dependent increase in plasma exposure after a single administration, with C_{max} and AUC_{0-t} increasing as the dose increased (Figure S7B). Descriptive statistics of key PK parameters of plasma TDI01 revealed mean half-lives ($t_{1/2}$) of 12.8, 10.2, and 8.8 h for the 400, 800, and 1,200 mg doses, respectively (Table S3). Thus, oral administration of TDI01 once daily might achieve sufficient efficacy. Drug accumulation was then analyzed from the group that received multiple administrations of TDI01 with once-daily dosing for 7 days (Figure S7C; Table S3). The C_{max} and AUC_{0-t} increased with higher doses, with T_{max} being 4.5 and 3.2 h for the multiple administrations of 200 and 400 mg doses, respectively. The drug accumulation index, residual accumulation coefficient (Rac) for AUC_{0-t} were 1.96 ± 0.50 and 2.39 ± 1.52 for the 200 and 400 mg multiple groups, respectively. Rac values for C_{max} were 1.68 ± 0.34 and 2.18 ± 1.13 , respectively. These data show the optimal drug accumulation after multiple administrations. The half-life of

(H) TDI01 binds in the hydrophobic pocket of ROCK2. Protein in the cryo-EM structure is displayed according to hydrophilicity and hydrophobicity.

(I) Interactions between TDI01 and ROCK2. ROCK2 dimer (purple/green) with TDI01 (pink) and key interacting residues are highlighted.

(J) Binding of TDI01 leads to displacement of the αC helix of ROCK2 but not ROCK1.

(K) Leucine 210 to phenylalanine (L210F) mutation in ROCK2 KD abolished TDI01 binding, as revealed by BLI analysis.

(L and M) ROCK2 KO in HUVECs or LX2 cells abolished TDI01 binding, which was rescued by wild-type (WT) but not L210F ROCK2. ROCK2 was knocked out in HUVECs and LX2 cells (sgROCK2), and WT or L210F ROCK2 were introduced to sgROCK2 cells, respectively. ROCK2 bound with biotin-TDI01 was pulled down and analyzed by western blot.

(N and O) Dose-dependent inhibition of ROCK2 activity by TDI01 in HUVECs and LX2 with indicated treatments, including sgROCK2, sgROCK2 + WT ROCK2, and sgROCK2 + L210F. $n = 3$.

See also Figure S4.

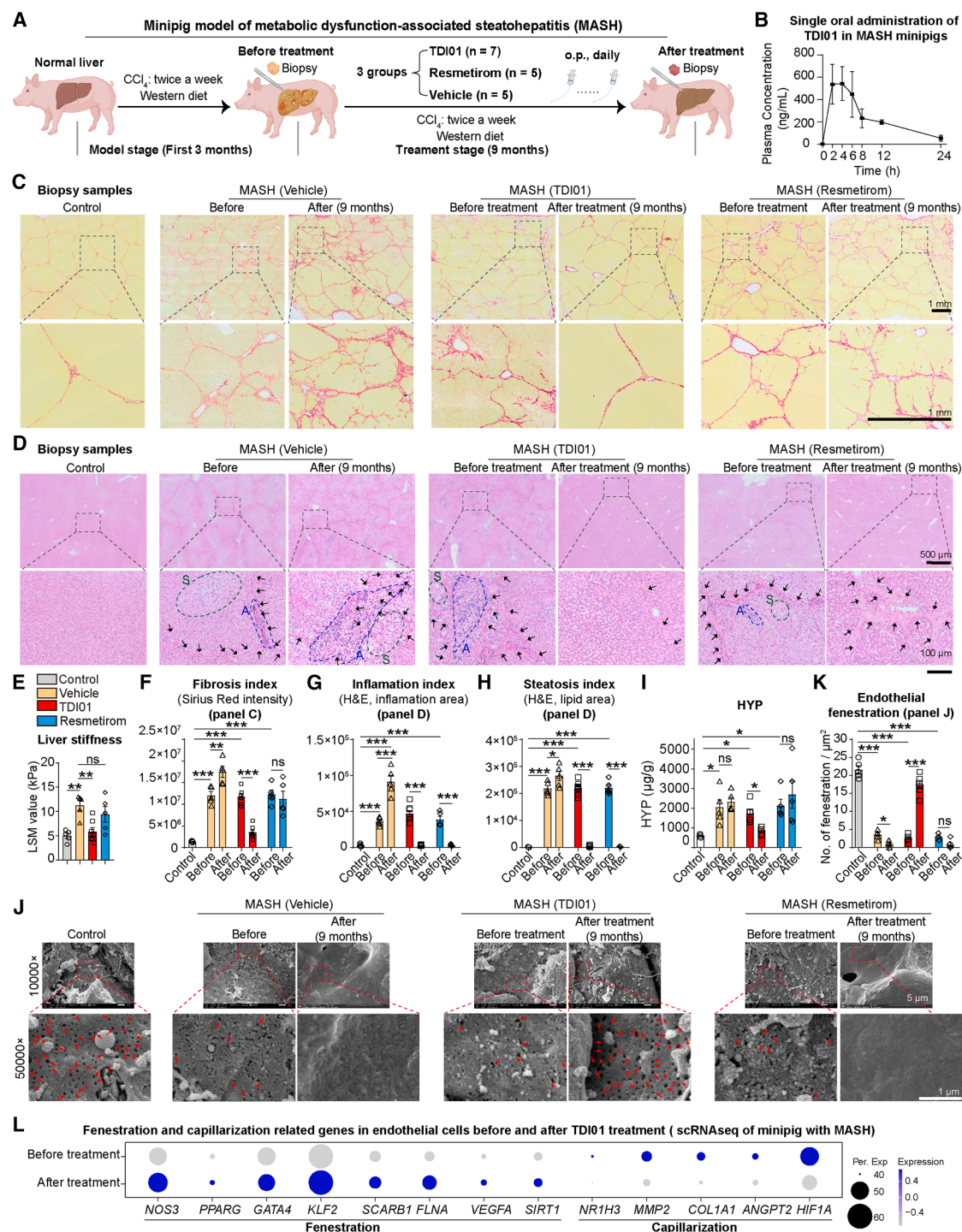


Figure 5. TDI01 attenuates fibrosis in a minipig MASH model recapitulating human pathology

(A) Strategy to test TDI01 in the minipig MASH model. Minipigs were fed a high-fat/high-cholesterol diet with a high-sugar solution and received biweekly CCl₄ to induce MASH, then treated with vehicle, TDI01, or resmetirom. Liver biopsies were performed before and after treatment to evaluate anti-fibrotic effects.

(B) Plasma TDI01 concentrations after single 5 mg/kg oral dose in the minipig MASH model; *n* = 4.

(C and D) TDI01 mitigated the established liver fibrosis, inflammation, and steatosis in the minipig MASH model. Collagen deposition was assessed by Sirius red staining (C), and tissue morphology, inflammation, and steatosis were examined by H&E staining (D). Collagen deposition is indicated by the arrows. "A" denotes inflammation, and "S" represents steatosis. Scale bar, 1 mm in this figure unless indicated.

(E) LSM was performed by ultrasound elastography in minipigs following indicated treatments.

(legend continued on next page)

TDI01 after multiple administrations was similar to that after single administration, ranging from 11.8 to 13.4 h. On the basis of the half-life and dosing interval, steady-state blood concentrations of TDI01 were achieved following consecutive dosing for 3–4 days.

We also performed food-effect studies in humans. High-fat meals delayed absorption (mean T_{max} 3.7 vs. 7.1 h) postprandially (Figure S7D; Table S3). Administration after meal intake also enhanced TDI01 exposure and bioavailability after the 400 mg dose, as shown by the increased C_{max} and AUC_{0-t} values following meal intake. Comparison of PK parameters (C_{max} and AUC_{0-t}) between fasting and high-fat meal groups revealed geometric mean ratios and 90% confidence intervals falling beyond the acceptable equivalence range (80.00%–125.00%), suggesting increased drug exposure and bioavailability with food. Thus, the phase 1 study of TDI01 demonstrated its favorable PK and safety profiles.

Extended clinical study suggests a trend toward alleviating liver fibrosis in patients by TDI01

We performed an extended clinical study to evaluate the safety and therapeutic efficacy of TDI01 in patients with liver fibrosis (trial number, ChiCTR2400082056, China). The study was designed as a dose-escalation trial, comprising an initial low-dose cohort (200 mg) followed by escalation to a higher-dose cohort (400 mg). To ensure the safety of enrolled patients, dose escalation to high dose (400 mg) would not be initiated until the completion and interim analysis of the low-dose cohort data. In total, six patients were enrolled in this low-dose cohort after pathological biopsy to determine liver fibrosis severity (Figures 6A and 6B; Table S4). To date, the low-dose TDI01 treatment cohort (200 mg) has been completed. After this low-dose cohort was completed, we followed the protocol to perform the interim analysis of all six patients. Here, we report the analysis results from all six patients in the low-dose cohort before the initiation of high-dose cohort.

Among this low-dose cohort, we did not observe severe adverse effects (SAEs) (Table S5). PK analysis of these 6 patients shows that the absorption and metabolism of TDI01 in liver fibrosis patients are comparable to those in healthy individuals (Figures S7E–S7J; Table S3). We then evaluated the therapeutic efficacy of TDI01 in this cohort via performing liver stiffness measurement (LSM) with ultrasound transient elastography. LSM values progressively declined in patients 1–5 (Figures 6C and 6D). Multiple noninvasive indices of liver fibrosis were evaluated, including the S index, γ -glutamyl transpeptidase-to-platelet ratio (GPR), red cell distribution width-to-platelet ratio (RPR), and

fibrosis-4 (FIB-4) index. Both the S index and GPR demonstrated marked reductions in patients 2, 4, and 5, whereas patients 1, 3, and 6 maintained stable low values throughout the treatment (Figures 6E and 6F). The RPR index decreased substantially in patient 4, with other patients exhibiting persistently low levels (Figure 6G). The FIB-4 index exhibited a notable decline in patients 1 and 4, while remaining consistently low in patients 2, 3, and 6 (Figure 6H). The serum levels of ALT and AST fluctuated during the treatment period; however, in patients 1, 2, 3, 4, and 6, these values remained nearly within the normal range. Patient 5 exhibited abnormally elevated baseline levels, which declined substantially after treatment and nearly returned to the normal range (Figures 6I and 6J). The serum level of albumin (ALB) remained stable in patients 1 and 3, whereas patients 2, 4, 5, and 6 demonstrated a modest elevation in ALB levels following TDI01 administration, suggesting a potential improvement in hepatic synthetic function (Figure 6K). Platelet (PLT) count, an important marker of liver function, remained relatively stable throughout the treatment (Figure 6L). An upward trend in serum total bilirubin (TBIL) levels was observed in patients 1, 2, 4, and 6, which declined 1 month after treatment cessation (Figure S7K). Collectively, these findings show a trend toward reducing liver fibrosis in these six patients who received low-dose TDI01 treatment, with a relatively favorable safety profile.

Following the 24-week treatment period, liver biopsies were obtained from all six patients in this cohort. Sirius red staining showed that TDI01 treatment reduced collagen deposition in biopsy specimens from patients 1–5 (Figures 7A and 7B). H&E staining demonstrated pronounced improvements in fibrosis staging in patients 1–5, as evaluated by the meta-analysis of viral hepatitis (METAVIR), non-alcoholic fatty liver disease (NAFLD), and Scheuer activity score systems (“F” denotes fibrosis, “S” steatosis, and “A” inflammation). Patients 4 and 5, who presented with cirrhosis at baseline, showed discernible histopathological improvement, highlighting the potential of TDI01 to ameliorate advanced fibrosis (Figures 7C and 7D). There is also a reduction in inflammation in patients 1–5. Notably, TDI01 treatment reduced the quantity of pROCK2 but not pROCK1 in the biopsy samples of all patients (Figures 7E, 7F, S7L, and S7M). These findings imply that TDI01 blocks liver fibrosis in these patients via selectively inhibiting ROCK2 activity.

During the treatment of these six patients, there were no treatment-emergent serious adverse events (TESAEs), treatment-related serious adverse events (TRESAEs), or treatment-emergent adverse events (TEAEs) leading to treatment discontinuation. Nor were any TEAEs of special concern observed. A total of six treatment-related adverse events were observed,

Throughout this figure, $n = 5$ (normal, vehicle, and resmetirom) and 7 (TDI01), before and after treatment, and statistical significance was determined using one-way ANOVA. ns, no significant difference, $p > 0.05$, *** $p < 0.001$, and * $p < 0.05$.

(F–H) Quantifications of Sirius red and H&E staining in indicated minipig groups. TDI01 alleviated collagen deposition (F), inflammation (G), and steatosis (H) in the minipig MASH livers.

(I) Hepatic HYP content in the biopsy samples before and after indicated treatment.

(J and K) TDI01 restored LSEC fenestrations in minipig MASH model. Scanning electron microscopy (SEM) was performed to examine the fenestration (red arrows) and capillarization of LSECs (H). Fenestration was quantified in (K).

(L) Dot plot showing the upregulation of genes related to LSEC fenestration^{96–110} and downregulation of capillarization-related genes^{111–118} in liver ECs after TDI01 treatment. Data are from scRNA-seq, $n = 6$ per group.

See also Figures S5 and S6.

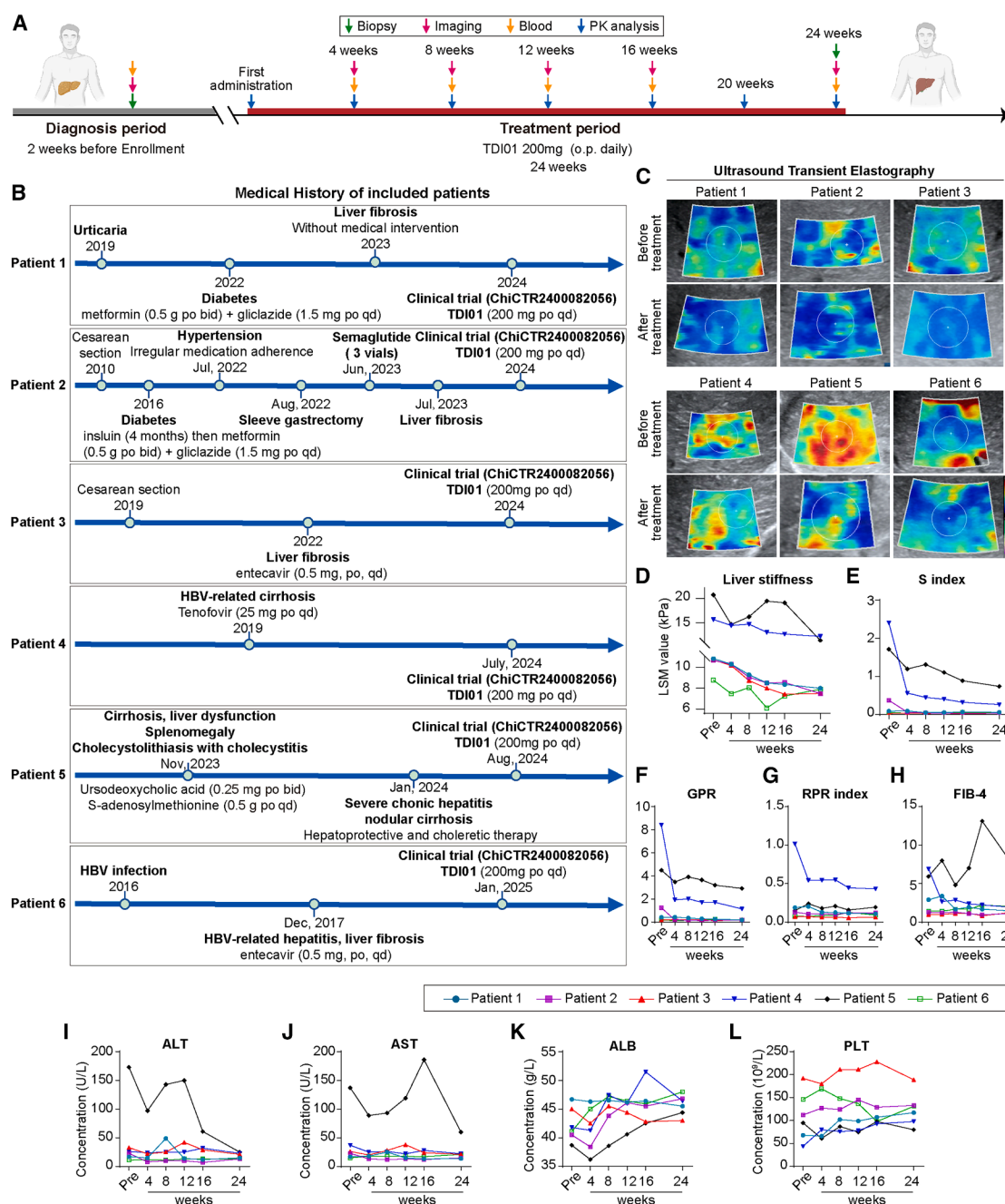


Figure 6. Extended clinical trial data implies that TDI01 alleviates liver fibrosis in 6 tested patients

(A) Study design is as follows: subjects underwent ultrasound, fibroscan, serum tests (AST, ALT, total bilirubin [TBIL], direct bilirubin [DBIL], indirect bilirubin [IBIL], α -fetoprotein [AFP], albumin [ALB]), and liver biopsy to assess fibrosis. Once subjects with liver fibrosis were enrolled and the treatment started, PK tests were conducted after drug administration at the initial, second, and third treatment courses. To evaluate the treatment strategy and sample size, an analysis was conducted after we finished the 24-week treatment of the sixth patient (trial number: ChiCTR2400082056). Both occurrences of adverse events and therapeutic efficacy in six patients were assessed.

(B) The detailed medical history of the six enrolled patients.

(C and D) Longitudinal liver elasticity assessment shows reduced fibrosis after TDI01 (C). LSM values were measured by ultrasound elastography pretreatment (pre) and 4, 8, 16, and 24 weeks post treatment (D).

(E–H) Longitudinal liver fibrosis indices indicate reduced fibrosis in patients after TDI01. S index (E), GPR (F), RPR (G), and FIB-4 (H) were measured at described time.

(I–L) Serum levels of ALT (I), AST (J), and ALB (K) and blood PLT counts (L) in patients at indicated times.

See also Figure S7 and Tables S3, S4, and S5.

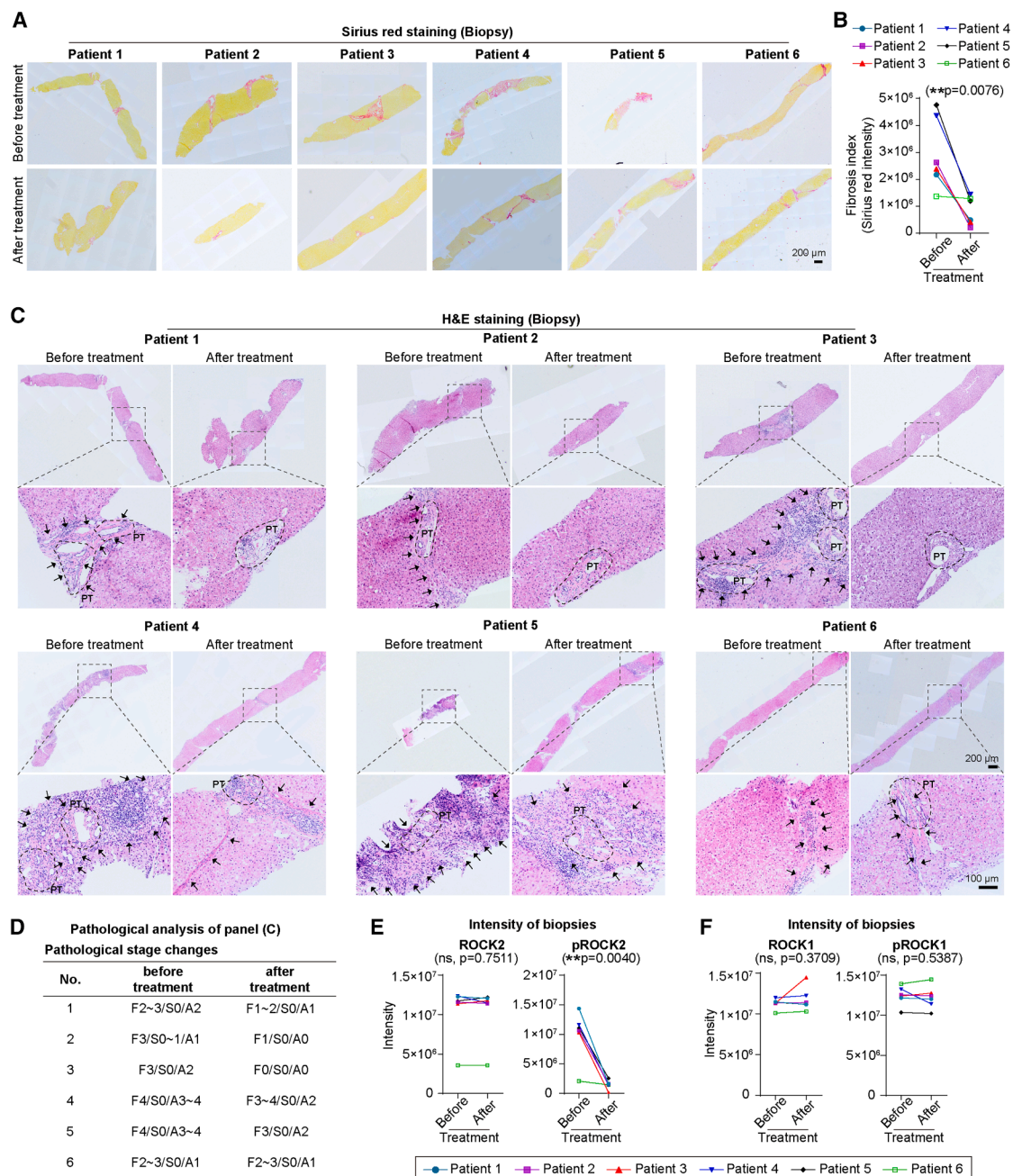


Figure 7. Biopsy samples from the extended clinical trial suggest that TDI01 ameliorates fibrosis via inhibiting ROCK2 activity

(A and B) Liver biopsies from six patients pre- and post-TDI01 were assessed by Sirius red staining (A), and quantification of staining is shown in (B). Throughout this figure, paired Student's *t* test (two-tailed) was conducted to evaluate the statistical differences before and after treatment. *n* = 6 patients; **p* < 0.05, ns (no significant difference), and *p* > 0.05.

(C and D) Tissue morphology and architecture changes in the liver biopsy specimens before and after treatment. H&E images (C) and pathological grading (D) are shown, with portal tract (PT), bridging fibrosis (black arrows), fibrosis (F), steatosis (S), and inflammation (A) indicated.

(E and F) TDI01 inhibits the phosphorylation of ROCK2 but not ROCK1 in patient livers. ROCK2 and pROCK2 in the biopsy specimens were quantified with multicolor immunohistochemistry staining (E). ROCK1 and pROCK1 amounts were also compared (F).

See also Figure S7 and Tables S4 and S5.

and adverse events were minimal (Table S5). In sum, data from this low-dose treatment cohort suggests that TDI01 might be a safe and efficacious therapeutic drug that potentially treats patients with fibrosis-related liver diseases.

DISCUSSION

Liver fibrosis is mediated by HSCs and modulated by their interaction with adjacent cells such as LSECs.^{41–44,131–136} Here, we find a selective upregulation of ROCK2 in the vascular cells of human fibrotic livers. A group of transcription factors^{64–68} and receptors^{69–78} have been reported to upregulate ROCK2. scRNA-seq analysis revealed the induction of several reported transcription factors and receptors involved in ROCK2 upregulation. Among these genes, *SMAD4* and *YAP-TEAD* have been shown to more selectively interact with the promoter region of *ROCK2* than *ROCK1*.^{65,66} Since *YAP1* has been shown to promote liver fibrosis,^{95,137–140} it is conceivable that an increase of these transcription factors and receptors at least partially contributes to ROCK2 upregulation in fibrotic livers. ROCK2 is also reported to be expressed in immune cells and hepatocytes.^{62,141,142} Our analysis suggests that at least in normal and fibrotic livers at an early stage, ROCK2 is expressed at a relatively low level in hepatocytes and immune cells. In mouse models, ROCK2 expression and upregulation in immune and hepatocytes are also lower than those of ECs and HSCs. Together with the results from mice with *ROCK2* knockdown/KO in hepatocytes and HCs, these ROCK2 expression data imply that while ROCK2 might be expressed by immune or hepatocytes at several circumstances, hepatocyte and immune cell ROCK2 play a lesser contribution to liver fibrosis, as compared with ROCK2 in ECs and HSCs. Future studies will be needed to reveal the mechanisms governing ROCK2 expression in different liver cell types in various hepatic diseases.

In the minipig MASH model, TDI01 treatment attenuates LSEC capillarization. ROCK2 can directly modulate the cytoskeleton to cause defenestration in LSECs.¹⁴³ Phosphorylation of myosin light chain (MLC) by ROCK2¹⁴⁴ stabilizes the actin cytoskeleton and reduces fenestrations in LSECs.¹⁴⁵ ROCK2 can also phosphorylate and inhibit the myosin phosphatase targeting subunit (MYPT1) of MLC phosphatase (MLCP), sustaining MLC phosphorylation.¹⁴⁶ Thus, ROCK2 promotes actomyosin contractility via directly phosphorylating MLC and suppressing its phosphatase to reinforce MLC phosphorylation. Compared with ROCK1, ROCK2 preferentially facilitates the phosphorylation and inactivation of cofilin by Lin-11, Isl-1, and Mec-3 domain (LIM) kinase 1.^{74,147–150} Cofilin inactivation further increases the contraction of fenestrations,¹⁵¹ which culminates in sinusoidal capillarization.¹⁵² We find that ROCK2 also influences the expression of genes related to fenestration (e.g., *NOS3* and *PPARG*)^{96–101} and capillarization (e.g., *MMP2*)^{112–114} in LSECs. Thus, ROCK2 can directly promote actin polymerization and cellular hypercontractility, disintegrating LSEC fenestrations. ROCK2 might also regulate genes related to LSEC fenestration. This ROCK2-mediated transcriptional regulation might be mediated by its influence on cytoskeletal dynamics altering downstream events.¹⁵³ Indeed, we find TDI01 selectively suppresses the expression

and pro-fibrotic function of galectin-3,^{129,130,154} which might promote liver fibrosis via stimulating HSC activation and immune responses. Upregulation of ROCK2 has also been shown to trigger release of cytokines.^{155,156} Therefore, the observed *in vivo* effects of TDI01 might involve layered mechanisms, including inhibition of HSC activation, restoration of impaired LSEC fenestration, as well as paracrine modulation of immune responses via suppressing the release of downstream fibrogenic and inflammatory factors (e.g., galectin-3). The mechanism by which ROCK2 upregulates its downstream paracrine factors in liver ECs needs to be delineated in future studies.

In summary, we identify ROCK2 as a druggable target promoting a pro-fibrotic liver vascular phenotype. Compared with current ROCK inhibitors,^{56,82,157–159} the selectivity index (SI) of ROCK2 inhibitor TDI01 is substantially higher.^{61,160–167} Therefore, this ROCK2-selective inhibitor with optimal safety and PK profiles holds unique clinical potential to treat liver fibrosis, a major histological feature of chronic liver diseases linked to clinical outcomes.

Limitations of the study

In the future, the safety and efficacy of TDI01 will be further evaluated in a high-dose TDI01 treatment (400 mg) cohort. A larger patient cohort with broader etiology, with a randomized, placebo-controlled clinical trial will also be needed for this ROCK2-selective inhibitor. Technically, we use pROCK to examine ROCK2 activity.⁶³ In future studies, we might utilize more comprehensive assays to characterize ROCK2 activation. Last, the mechanism(s) underlying ROCK2 upregulation and downstream pro-fibrotic effects need to be further elucidated.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Bi-Sen Ding (dingbisen@scu.edu.cn).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

- The scRNA-seq data supporting the findings of this study have been deposited in NCBI Gene Expression Omnibus (GEO: GSE290642). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository with the dataset identifier PXD: 069697. In addition, the cryo-EM structure of the ROCK2 KD in complex with TDI01 has been deposited in the Protein Data Bank (PDB) under accession number PDB: 9HJQ.
- This paper did not report original code.
- Additional information, including clinical trial data (e.g., de-identified participant data necessary to reanalyze the results reported), is available from the lead contact upon request.
- Data sharing statement of clinical trials: individual participant data that underlie the reported results will be shared after de-identification. Data will be publicly available at ResMan (<http://www.medresman.org.cn/login.aspx>) 1 year after completion, including metadata and statistical analysis plan. Study protocols are available in Data S1. Researchers who provide a methodologically sound proposal can receive data to achieve the aims of an approved proposal; proposals should be directed to the lead contact.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, B.-S.D., Z.C., and C.W.; methodology, investigation, and visualization (including target identification, mouse and pig studies, and mechanistic studies of ROCK2 inhibitor), Y.H., C.X., X.Y., H.Z., P.Y., Y.T., Y. Chen, Z.G., and J. Chen; compound synthesis and screening, H.W., Y.Z., D.Z., and W.Z.; clinical trials, C.Y., Y. Cai, B.Y., X.Y., Y.H., Y.T., B.L., N.B., D.C., Y.C., and Z.G.; resources, X.D., G.Z., and D.W.; cryo-EM, Q.L. and H.D.; multiplex immunohistochemistry, C.Z.; formal analysis, writing, review and editing, B.-S.D., Z.C., Y.H., and S.L.F.; and advisory support and expert input, T.Y., S.R., H.H., J. Chang, X.L., X.Q., J.G., and L.P.

DECLARATION OF INTERESTS

B.-S.D., Z.C., Y.H., X.Y., B.Y., C.X., and H.Z. are inventors of patents and have a patent application on discovering therapeutics targeting vascular pro-fibrotic pathways. Y.Z., H.W., and W.Z. are co-inventors of a patent covering the synthesis of kinase inhibitors. Y.Z., H.W., D.Z., W.Z., and D.C. are employees of Beijing Tide Pharmaceutical Co. Ltd.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cell.2026.02.001>.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-ROCK2 antibody	Abcam	Cat# ab125025; RRID:AB_10972853
CD31 Mouse Monoclonal Antibody	HUABIO	Cat# M1511-8; RRID:AB_3073158
Anti-Desmin antibody	Abcam	Cat# ab15200; RRID:AB_301744
Anti-LYVE1 antibody	Abcam	Cat# ab219556; RRID:AB_2884014
CD45 Recombinant Rabbit Monoclonal Antibody	HUABIO	Cat# IRS025; RRID:AB_3739963
Anti-ROCK2 (phospho S1366) antibody	Abcam	Cat# ab228008; RRID: AB_3739962
ROCK1 (C8F7) Rabbit Monoclonal Antibody	Cell Signaling Technology	Cat# 4035S; RRID:AB_2238679
phospho-ROCK1 (Thr455+Ser456) Rabbit pAb	Bioss	Cat# bs-4630R; RRID:AB_11047086
ROCK2 Polyclonal Antibody	Fisher scientific	Cat# PA5-78290; RRID:AB_2736534
Anti-CD31 antibody	Abcam	Cat# ab182981; RRID:AB_2920881
Anti-Galectin 3 antibody	Abcam	Cat# ab76245; RRID:AB_2265782
COL1A1 Recombinant Rabbit Monoclonal Antibody	HUABIO	Cat# HA722517; RRID:AB_3714533
alpha smooth muscle Actin Recombinant Rabbit Monoclonal Antibody	HUABIO	Cat# ET1607-53; RRID:AB_3069772
ROCK2 (D1B1) Rabbit Monoclonal Antibody	Cell Signaling Technology	Cat# 9029; RRID:AB_11127802
Anti-β-Actin (ACTB) Antibody	Sigma-Aldrich	Cat# A5441; RRID:AB_476744
Anti-ROCK2 antibody - C-terminal	Abcam	Cat# ab228000; RRID:AB_3739966
TGF Beta 1 Polyclonal antibody	Proteintech	Cat# 18978-1-AP; RRID:AB_11182379
Desmin Polyclonal antibody	Proteintech	Cat# 16520-1-AP; RRID:AB_2292918
Anti-alpha smooth muscle Actin antibody	Abcam	Cat# EPR5368; RRID:AB_3739968
ROCK2 (D1B1) Rabbit Monoclonal Antibody	Cell Signaling Technology	Cat# 9029S; RRID:AB_3739969
HNF-4-alpha Recombinant Rabbit Monoclonal Antibody [JE63-17]	HUABIO	Cat# HA721006; RRID:AB_3072131
CD31 Recombinant Antibody [7-A1-R] - Rat IgG1 (Chimeric)	HUABIO	Cat# HA601522; RRID:AB_3739970
Anti-Galectin 3 antibody [A3A12]	Abcam	Cat# ab2785; RRID:AB_303298
Recombinant Anti-CD45 Antibody [RM1007]	Abcam	Cat# ab281586; RRID:AB_3697165
Anti-CD31 antibody [JC/70A]	Abcam	Cat# ab9498; RRID:AB_307284
Anti-Collagen I antibody	Abcam	Cat# ab6308; RRID:AB_305411
Anti-ROCK2 antibody	Abcam	Cat# ab125025; RRID:AB_10972853
Bacterial and virus strains		
pFastBac1 vector	Invitrogen	N/A
pLV3-U6-ROCK2(human)-sgRNA1-Cas9-Puro	MIAOLING PLASMID	Cat# P67155
pCMV-ROCK2(human)-HA-Myc-6×His-Neo	MIAOLING PLASMID	Cat# P56844
pCMV-ROCK2 WT (silent mutations within the sgRNA target site to prevent degradation)	Sangon Biotech	Modified based on pCMV-ROCK2(human)-HA-Myc-6×His-Neo
pCMV-ROCK2 L210 to F210 mutation	Sangon Biotech	Modified based on pCMV-ROCK2 WT
HBAAV2/8-alb-mir30-m-Rock2-ZsGreen	HANBIO	N/A
Biological samples		
Human liver surgical or biopsy samples	West China Hospital	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Galectin 3	Sino Biological	Cat# 10289-HNAE
CCl ₄	Sigma-Aldrich	Cat# 319961
SIM SF Medium	Sino Biological Inc.	Cat# MSF-AF
EDTA-free protease inhibitor cocktail	MedChemExpress	Cat# HY-K0010
NI-NTA agarose	MedChemExpress	Cat# HY-K0210
Superdex 200 Increase 10/300 GL	GE Healthcare	Cat# 28990944
EDTA-free protease inhibitor cocktail	MedChemExpress	Cat# HY-K0010
Biotin-TDI01 (Compound ID: TDI01-IMP-11)	BioDuro Beijing Co., Ltd.	N/A
TDI01	TIDE Pharmaceutical Co., Ltd.	N/A
Resmetirom	Bidepharm Co., Ltd.	Cat# BD764733
Critical commercial assays		
HTRF KinEASE-STK S2 kit	Kinases	Cat# 62ST2PEB
ADP-Glo Kinase Assay Kit	Promega	Cat# V6930
IRISKit HyperView multiplex immunostaining kit	LUMINIRIS	Cat# MH010101
IRISKit® HyperView Advanced Ab-Stripping Kit	LUMINIRIS	Cat# MH900402
LUMINIRIS Fluorescence Quenching System	LUMINIRIS	Cat# MH030101
IRISKit® HyperView mTSA Kit	LUMINIRIS	Cat# 20T-4C
Pierce™ Classic Magnetic IP/Co-IP Kit	ThermoFisher	Cat# 88804
Human IL-2 ELISA Kit	MULTI SCIENCES	Cat# EK102 - 96
Human IL-17A ELISA Kit	MULTI SCIENCES	Cat# EK117/2 - 96
Human IL-1 beta Uncoated ELISA	Invitrogen	Cat# 88-7261
Human IL-6 Uncoated ELISA	Invitrogen	Cat# 88-7066
Human IL-8 Uncoated ELISA	Invitrogen	Cat# 88-8086
Human TNF alpha Uncoated ELISA	Invitrogen	Cat# 88-7346
Human IL-4 Uncoated ELISA	Invitrogen	Cat# 88-7046
Human IL-10 Uncoated ELISA	Invitrogen	Cat# 88-7106
Human IL-18 ELISA Kit	MULTI SCIENCES	Cat# EK118/2-96
Human/Mouse TGF beta 1 Uncoated ELISA	Invitrogen	Cat# 88-8350
ELISA Kit for Monocyte Chemotactic Protein 1 (MCP1/CCL2)	Cloud-Clone Corp.	Cat# SEA087Hu
GEXSCOPE Single Cell RNA Library Kit	Singleron Biotechnologies	N/A
Hydroxyproline (HYP) Content Assay Kit	solarbio	Cat# BC0255
Deposited data		
Single-cell RNA sequencing (scRNA-seq) of human and minipig liver NPCs	NCBI GEO	GEO: GSE290642
Mass spectrometry proteomics data	ProteomeXchange Consortium	ProteomeXchange: PXD: 069697
Cryo-EM structure of the ROCK2 kinase domain (KD)	Protein Data Bank (PDB)	PDB: 9HJQ
Experimental models: Cell lines		
HUVECs	This paper	N/A
DH10Bac competent cell	Invitrogen	Cat# 10361012
<i>E. coli</i> DH5a Competent Cells	Invitrogen	Cat# 18263012
LX2	Sigma-Aldrich	Cat# SCC06
Sf9 insect cells	Invitrogen	Cat# 711043

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Male Bama minipigs	Chengdu Dossy Biological Technology Co. Ltd.	N/A
C57BL/6J mice	GemPharmatech Co., Ltd	N/A
<i>Rock2^{fl/fl}</i> mice	GemPharmatech Co., Ltd	N/A
Cdh5/VE-Cadherin-Cre ^{ERT2} mice	Dr. Ralf H. Adams.	N/A
Vav1-2A-Cre mice	Shanghai Model Organisms Center	NO. NM-KI-233228
Lrat-2A-Cre mice	Shanghai Model Organisms Center	NO. NM-KI-190097
Oligonucleotides		
Mouse <i>Rock2</i> shRNA GCATCTCTTGAAGAAACAAAT	This paper	N/A
Human ROCK2-sh1 CCTTGATGTCTGTCTATTATT	This paper	N/A
Human ROCK2-sh2 CCTTTCAAGATGATAGGTATC	This paper	N/A
Human ROCK2—sh3 CAGATGACATTGGACAGTAAA	This paper	N/A
HBAAV2/8-alb-mir30-m-Rock2-ZsGreen GCATCTCTTGAAGAAACAAAT	This paper	N/A
Human ROCK2 sgRNA TAGTAGGTAAATCCGATGAA	This paper	N/A
Primer: Mouse <i>Gapdh</i> Forward: GTGCCGCCTGGAGAAACCT; Reverse: TGAAGTCGCAGGAGACAACC	This paper	N/A
Primer: <i>Rock2</i> Forward: GGCAATGAACCTCTTGTGAAG; Reverse: CTCTCCTCTGAGGTTCTAACTCC	This paper	N/A
Primer: Vav1-2A-cre Forward: GGTGTGTAGTTGTCCCACT; Reverse: CAGGTTTTGGTGCACAGTCA	This paper	N/A
Primer: Lrat-cre-mutant Forward: CGGGTGGACACAGAACAATCG; Reverse: ACCGACGATGAAGCATGTTTAGCTG	This paper	N/A
Primer: Ve-Cad/ <i>Cdh5</i> -cre Forward: GCCTGCATTACCGGTCGATGCAACGA Reverse: GTGGCAGATGGCGCGGCAACACCATT	This paper	N/A
Recombinant DNA		
pFastBac1-His-Rock2(18-417)	This paper	N/A
pFastBac1-His-Rock2 L210(18-417)	This paper	N/A
pFastBac1-His-Rock1(1-415)	This paper	N/A
Software and algorithms		
ImageJ	National Institutes of Health (NIH)	Version 1.53t; https://imagej.nih.gov/ij/ ; RRID:SCR_003070
QuPath	University of Edinburgh	Version 0.4.3; https://qupath.github.io/ ; RRID:SCR_018257
GraphPad Prism	GraphPad Software	Version 8.4.3; https://www.graphpad.com/ ; RRID:SCR_002798
OLY / Olympus imaging software	Olympus Corporation	N/A
SerialEM	Mastronarde	http://bio3d.colorado.edu/SerialEM/Cat#18263012
CryoSPARC	Punjani et al. ¹¹⁷	https://cryosparc.com/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
UCSF ChimeraX	Pettersen et al. ¹⁶⁸	https://www.cgl.ucsf.edu/chimerax/
MolProbity	Williams et al. ¹⁶⁹	http://molprobity.biochem.duke.edu/
AutoDock Vina	Eberhardt et al. ¹⁷⁰	https://vina.scripps.edu/
AlphaFold Server	N/A	https://alphafoldserver.com/
Phenix	Adams et al. ¹⁷¹	https://www.phenix-online.org/
CTFFIND4	Rohou and Grigorieff ¹⁷²	https://grigoriefflab.umassmed.edu/ctffind4
COOT	Emsley and Cowtan ¹⁷³	https://www2.mrc-lmb.cam.ac.uk/personal/
SAS software	SAS Institute Inc.	version 9.4; https://www.sas.com/
Relion4	Kimanius et al. ¹⁷⁴	https://www2.mrc-lmb.cam.ac.uk/relion
CeleScope	Singleron R&D	v1.15.0; https://github.com/singleron-RD/CeleScope

Other

25mM D-Glucose	Sigma-Aldrich	Cat# G8270
amphotericin B	Gibco™	Cat# 15290026
Ni-NTA resin	MedChemExpress	Cat# HY-K0210
M-PER buffer	Thermo Scientific	Cat# 78501
Pierce™ Streptavidin Magnetic Beads	Thermo Scientific	Cat# 88817
Binding/Wash Buffer	Thermo Scientific	Cat# 28379
CD45 MicroBeads	Miltenyi Biotec	Cat# 130-052-301
Western Diet (21.1% fat, 41% sucrose, and 1.25% cholesterol)	Beijing HFK Bioscience CO., LTD	N/A
D-(+)-Glucose	sigma	Cat# G8270-5KG
HTRF 96-well low volume plate	Cisbio	Cat# 66PL96001
ATP	Sigma	Cat# A7699
IRISKit® HyperView Quench Buffer	LUMINIRIS	Cat# MH900102
Pierce™ Streptavidin-Coated Magnetic Beads	ThermoFisher	Cat# 88816
cComplete™ Mini Protease Inhibitor Cocktail	Roche	Cat# 11836153001
Electron Microscopy Fixative	Servicebio	Cat# G1102-100mL
Mouse CD31 Beads	Miltenyi Biotec	Cat# 130-097-418
RIPA lysis buffer	Beyotime Biotechnology	Cat# P0013B
protease inhibitor cocktail	Bimake	Cat# B14011
Percoll	cytiva	Cat# 17089101
Gibco William's E medium	Gibco™	Cat# A1217601
Primary Hepatocyte Maintenance Supplements	Gibco™	Cat# CM4000
Agarose (Low Melting Point)	Beyotime	Cat# ST105
DMEM basic	Gibco	Cat# C11995500BT
Fetal Bovine Serum	Gibco	Cat# 10099141
LS Columns	Miltenyi Biotec	Cat# 130-042-401
CD45 MicroBeads	Miltenyi Biotec	Cat# 130-052-301
HBSS without Ca ²⁺ /Mg ²⁺ or phenol red	Gibco	Cat# 14175095
HBSS with Ca ²⁺ /Mg ²⁺	Gibco	Cat# 24020117
Collagenase IV	Gibco	Cat# 17104019
Collagenase I	Gibco	Cat# 17100017
Collagenase II	Gibco	Cat# 17101015
Dispase II	Sigma-Aldrich	Cat# D4693
DNase I	Sigma-Aldrich	Cat# 11284932001
IRISKit HyperView quench buffer	LUMINIRIS	Cat# MH010301

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Endothelial Cell Growth Supplement	Sciencell	Cat# 1052
Endothelial Cell Medium	Sciencell	Cat# 1001
Freezing medium	CELLSAVINGTM	Cat# C40100
DPBS	Gibco,	Cat# 14190144
GlutaMAX Supplement	Gibco	Cat# 35050061
Nycodenz	Serumwerk Bernburg AG	Cat# 18003
0.25% trypsin-EDTA	Gibco	Cat# 25200072
phosphatase inhibitor cocktail	Bimake	Cat# B15002

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human participants

Healthy volunteers for phase I clinical trial of TDI01 (ChiCTR2200058868)

The study involved 62 healthy volunteers, comprising 30 females and 32 males. All volunteers were from China and of Asian descent. Demographic characteristics of the healthy volunteers are listed in [Table S1](#), including age, gender, ethnicity, and race. All volunteers were strictly screened in accordance with ethical and safety procedures approved by the Clinical Ethics Committee of the National Medical Products Administration and the Chinese PLA General Hospital, Beijing (Ethics Approval Number: 2020LP00635). Written informed consents were obtained from all participants prior to enrollment.

Sample size estimation

As a first-in-human study in the Chinese population, the sample size was determined based on conventional Phase I trial design and safety observation requirements. The planned size of 10 subjects per dose group (single and multiple ascending dose) and 12 subjects in the food-impact cohort was considered sufficient for the preliminary assessment of safety, tolerability, and PK characteristics. Due to the limited sample size, this study was not designed or powered to assess the potential influence or association of sex or gender on Phase I outcomes. The influence or association of sex- and gender-related covariates on PKs will be further evaluated in subsequent studies with larger sample sizes through population PK (PopPK) analyses.

Subject allocation and grouping:

The single ascending dose cohorts (400 mg, 800 mg, and 1200 mg). Progression to a higher dose cohort was permitted only after the safety data from the preceding cohort had been reviewed. This design is a standard safety-driven approach in Phase I trials. Eligible subjects were recruited into the specific dose group or study phase (single dose, multiple doses, or food impact) that was open at the time of their enrollment. Within each dose cohort, subjects were randomized in an 8:2 ratio to receive either the investigational drug or placebo. For the food-impact assessment, subjects were randomized in a 1:1 ratio into two sequence groups and studied under a two-period crossover design.

This study was designed as a randomized, double-blind, placebo-controlled trial. Randomization was performed by unblinded statisticians using SAS software (version 9.4) to generate the random allocation sequence.

Extended clinical trial of TDI01 treatment for patients with liver fibrosis (ChiCTR2400082056)

The study included six patients with varying degrees of liver fibrosis: two males (ages 48 and 42, fibrosis stages F2–F3 and F4) and four females (ages 36, 42, 54, and 46, fibrosis stages F3, F3, F4, and F2–F3, respectively). All patients were from China and of Asian descent. All patients were strictly screened according to the protocol included in [Data S1](#) and in accordance with the Biomedical Ethics Committee of West China Hospital, Sichuan University (Ethics Approval Number: 238, year 2024). Written informed consent was obtained from all participants prior to enrollment. Prior treatment histories are shown in [Figure 6B](#). The patients' information of demographic and baseline clinical characteristics is summarized in [Table S4](#), including age, gender, ethnicity and race.

Sample size estimation and subject allocation:

Participants were sequentially assigned according to a standard 3 + 3 rule-based dose-escalation design. An initial cohort of three patients was enrolled in the low-dose group (200 mg). No dose-limiting toxicities (DLTs), defined as clinically significant adverse events of Grade 3 or higher according to the Common Terminology Criteria for Adverse Events (CTCAE), were observed during the first treatment cycle. To further ensure patient safety, particularly in individuals with liver fibrosis, the cohort was expanded to include three additional patients at the same 200 mg dose prior to dose escalation. The resulting sample size of six patients in the low-dose group was considered sufficient for a preliminary assessment of safety, pharmacokinetic profiles, and trends in therapeutic effectiveness in the treatment of liver fibrosis. In this clinical trial, the influence or association of sex, gender, race, ethnicity or ancestry on the results could not be determined due to the small sample size and the early-phase design. This limitation will be considered when interpreting the current findings to broader populations.

The study employed an open-label design. No additional randomization or stratification procedures were implemented.

Human Samples

Human liver surgical or biopsy samples were obtained from patients at West China Hospital, Sichuan University, Chengdu, China, with informed consents. Control healthy liver tissues (without fibrosis) were obtained either from a small portion ($\sim 1 \times 1$ cm) of liver routinely collected during donor liver resection for pathological examination, which was included in the study after confirmation as normal liver tissue, or from liver samples isolated from patients undergoing cholecystectomy. Fibrotic liver tissues with different fibrosis stages were obtained from patients diagnosed with liver fibrosis or MASH who underwent sleeve gastrectomy. Besides, liver tissues from cirrhotic patients were sourced from individuals with liver cirrhosis who were histologically diagnosed with liver fibrosis at stage F4, and partly from patients requiring liver transplantation. Informed consent for tissue collection was obtained from all patients. This study was approved by the Medical Ethics Committee of West China Hospital of Sichuan University and adhered to the principles outlined in the Declaration of Helsinki regarding research involving human subjects.

This study cohort included 120 individuals, comprising 19 healthy controls and 101 patients with tumor-free liver fibrosis of varying severity. Patient demographics and the related clinical information are detailed in Table S6, including age, sex, race and ethnicity. Fibrosis staging was determined by the Department of Pathology at West China Hospital and stratified by fibrosis stage (F0–F4) and body mass index (BMI), defined using Asian-specific criteria as normal (18.5–27.5) or obese (>27.5). Within the normal BMI group, samples included F0 (12), F1 (4), F2 (5), F3 (6), and F4 (10); within the obese group, F0 (7), F1 (24), F2 (27), F3 (17), and F4 (8). Each fibrosis stage included at least 10 samples in total, and at least 3 samples per BMI-stratified subgroup. Given the limited sample size, the study was not designed or powered to systematically assess sex- or gender-related influences. Accordingly, sex or gender was not incorporated into the stratification scheme or analytical framework.

Animals

Minipigs

Bama minipigs (*Sus scrofa domestica*) were obtained from Chengdu Dossy Biological Technology Co., Ltd. (China). All minipigs were male, aged 6–8 months at enrollment, weighing 25–30 kg. Health status was confirmed prior to enrollment, all minipigs were immunocompetent and test-naïve, with no prior involvement in experimental procedures. The minipigs were individually housed in separate cages equipped with multiple air supply and exhaust vents to ensure adequate ventilation. Environmental conditions were maintained under a standard light-dark cycle (light period: 7:00 to 19:00), with temperature controlled at $22 \pm 2^\circ\text{C}$ and relative humidity at 40%–70%. Animals had ad libitum access to water, and daily feed allowance was provided at 2%–3% of body weight, distributed in three portions during the morning, noon, and evening. All procedures were approved by the Laboratory Animal Ethics Committee of West China Second University Hospital, Sichuan University, and Chengdu Dossy Biological Technology Co., Ltd., and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (2011). Minipigs were randomly assigned to experimental groups, each containing at least five animals (exact group sizes are indicated in figures and legends). Gender does not influence the results of the used liver fibrosis models in this study.

Mice

All mouse procedures were conducted in accordance with the ARRIVE guidelines and were approved by the Laboratory Animal Ethics Committee of West China Second University Hospital, Sichuan University. All mice used in this study were enrolled at age of 6–8 weeks old. The mice were healthy, immunocompetent, and naïve to previous procedures or tests. They were housed under specific pathogen-free (SPF) conditions in a barrier facility with a controlled environment ($22 \pm 2^\circ\text{C}$, 40–70% humidity, and a 12/12-hour light/dark cycle). All mice had ad libitum access to chow and water. Both male and female mice were randomly assigned to experimental groups with balanced sex distribution as evenly as possible within each group. Group sizes ($n \geq 5$) are specified in the respective figure legends. Gender does not influence the results of the used liver fibrosis models in this study.

Wild type C57BL/6 mice and *Rock2*^{fl/fl} mice were from GemPharmatech Co., Ltd. Cdh5/VE-Cadherin-Cre^{ERT2} mice expressing EC-specific inducible Cre were from Taconic Biosciences (Cat# 13073). The Vav1-2A-Cre mice (Cat. NO. NM-KI-233228)¹⁷⁵ and Lrat-2A-Cre mice (Cat. NO. NM-KI-190097)⁷⁹ mice were purchased from Shanghai Model Organisms Center, Inc. *Rock2*^{fl/fl} mice were bred with Cdh5/VE-Cadherin-Cre^{ERT2} mice, which express tamoxifen-inducible Cre^{ERT2} driven by the EC-specific VE-Cadherin promoter. The deletion of *Rock2* in ECs was induced by tamoxifen injection to generate *Rock2*^{ΔEC/ΔEC} mice, as verified by the described method.^{176,177} Lrat-2A-Cre mice were crossed with *Rock2*^{fl/fl} mice to specifically delete *Rock2* in HSCs (*Rock2*^{ΔHSC/ΔHSC}). Vav1-2A-Cre mice were also crossed with *Rock2*^{fl/fl} mice to delete *Rock2* in hematopoietic cells (*Rock2*^{ΔHC/ΔHC}).^{79,175}

Cell lines

Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were isolated as follows. The umbilical vein was thoroughly washed with $1 \times$ PBS. Subsequently, 0.2% collagenase solution was injected into the vein, clamped tightly with a surgical clamp, and incubated at 37°C for 10 minutes while protecting both cord extremities with clean aluminum foil. Cells were then collected by washing the vein with EndoGRO-VEGF Complete Culture Medium (Millipore, SCME002). The cells were spinned down at 750 g for 10 minutes and resuspended in EndoGRO-VEGF Complete Culture Medium. Cell dissociation was performed by gentle aspiration and repulsing three times with a 30 ml syringe equipped with a needle (inner diameter 0.7 mm, length 30 mm). The isolated HUVECs were verified based on the expression of specific surface markers.

HUVECs were cultured in Sciencell Endothelial Cell Medium (Sciencell, Cat# 1001), supplemented with Sciencell Endothelial Cell Growth Supplement (Sciencell, Cat# 1052), 5% FBS (Gibco, Cat# 10099141) and 1% penicillin-streptomycin (Gibco, Cat#15140122).

Cells were cultured at 37°C with 5% CO₂ and passaged at 80–90% confluence using 0.25% trypsin-EDTA (Gibco, Cat# 25200072). All experiments utilized HUVECs within passage 10 to maintain functional integrity.

For subculturing, cells were rinsed with DPBS (Gibco, Cat# 14190144), digested with 0.25% trypsin-EDTA at 37°C for 1–2 min, and neutralized with complete medium. After centrifugation at 220 × g for 5 min, cells were resuspended and seeded at a 1:2 to 1:3 ratio into freshly coated vessels. Medium was replaced every 48 h. For cryopreservation, cells were suspended in freezing medium (CELLSAVINGTM, Cat# C40100), stored at -80°C overnight, and transferred to liquid nitrogen for long-term storage.

Human Hepatic Stellate Cell Line (LX-2)

LX2 (human hepatic stellate cell line) was purchased from Sigma-Aldrich (catalog number SCC064) and cultured as per vendor's instruction. Briefly, LX2 was cultured in high-glucose DMEM (Gibco, Cat. #C11995500BT) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% GlutaMAX Supplement (Gibco, Cat. # 35050061). Cells were maintained at 37°C with 5% CO₂ and passed at 80% confluence. Cells were used within passages 5–15 to prevent activation or phenotypic drift.

For subculturing, cells were rinsed with PBS, briefly exposed to 500 μL trypsin-EDTA for 10 sec, aspirated, and incubated at 37°C for 2 min before neutralization with pre-warmed medium. Cells were split at a 1:2 to 1:3 ratio. Medium changes were performed every 2–3 days using pre-warmed solutions to avoid temperature shock. Cryopreservation followed the same protocol as HUVECs.

METHOD DETAILS

Compound synthesis

The TDI01 and biotin-labeled TDI01 compounds used in this study were provided by Tide Pharmaceutical Co., Ltd. Resmetirom was synthesized by Bidepharm Co., Ltd. TDI01 was administered orally in both mice and minipigs, with doses of 100 mg/kg and 5 mg/kg, respectively. Resmetirom was administered orally in minipigs at a dose of 1 mg/kg.

Human ex vivo PCLSs

Human PCLS was prepared following previously established protocols.^{178,179} Briefly, fresh liver specimens with fibrosis were promptly placed on ice in a sterile D-hanks solution upon harvesting. Liver specimens were generated using 8 mm diameter biopsy punches, embedded in 5% low-gelling-temperature agarose (Sigma-Aldrich), and mounted in a tissue slicer (automated vibrating blade microtome, WPI, VF-310-0Z) filled with Hanks Balanced Salt Solution supplemented with 25mM D-Glucose (Sigma-Aldrich), 100 μg/mL streptomycin, and 1 μg/mL amphotericin B (Gibco™). PCLSs with a diameter of 8 mm and thickness of 250 μm were obtained using the following slicing parameters: speed 0.5 mm/s, thickness 250 μm, amplitude 3mm. Human PCLSs were transferred onto 8 μm PET tissue culture inserts (ThinCert™, Greiner bio-one) in six-well plates and pre-incubated in William's E Medium (Gibco™) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 1 μg/mL amphotericin B (Gibco™), and 25mM D-Glucose (Sigma-Aldrich). After 1 hour of pre-incubation, fresh culture medium containing 50 ng/ml Galectin-3 (Sino Biological, 10289-HNAE), 50 ng/ml Galectin-3 + 0.3 μM TDI01, or vehicle (0.01% DMSO) was added, and PCLSs were cultivated at 37°C, 5% CO₂, under continuous orbital agitation (70 rpm). The culture medium was renewed daily. After 72 hours of culture, PCLS were fixed in 10% formalin for 24 hours for subsequent immunohistochemistry experiments.

Liver fibrosis models

The MASH model was induced in male Bama minipigs as previously reported.^{92,95} These minipigs were fed with diet containing 2% cholesterol and 30% fat, supplemented with a high sugar solution (23.1 g/L d-fructose and 18.9 g/L d-glucose), and intraperitoneally injected with 0.1 ml/kg (0.16 g/kg) CCl₄ twice a week throughout the entire procedure. For the chronic liver injury model with fibrosis, minipigs were intraperitoneally injected with 0.1 ml/kg (0.16 g/kg) CCl₄ twice weekly for 6 months. All minipigs in control group received injections of vehicle (corn oil). Liver tissues were surgically biopsied at the first 3 months for MASH model and at 6 months for the chronic liver injury model with fibrosis and subsequently biopsied every three months.

The mouse MASH model was induced as previously described.^{80,92} 8-week-old wild-type control and *Rock2*^{ΔEC/ΔEC} mice were fed a diet containing 21.1% fat, 41% sucrose, and 1.25% cholesterol, supplemented with a fructose (23.1 g/L) and glucose (18.9 g/L) solution, and intraperitoneally injected with 20% CCl₄ (2.5 μL/g) every week for 12 weeks. Mice were sacrificed, and serum and liver samples were collected two days after the last injection of CCl₄.

The chronic liver injury mouse model was induced by intraperitoneal injection of 40% CCl₄ (2.5 μL/g) twice a week for 5 continuous weeks. Mice were sacrificed, and serum and liver samples were collected two days after the last injection of CCl₄.

Fibrosis index was quantified using ImageJ (National Institutes of Health, USA) by measuring the integrated intensity of Sirius red stain-positive area within each tissue section, following the ImageJ official workflow for "Quantifying Stained Liver Tissue" (<https://imagej.net/ij/docs/examples/stained-sections/>), as previously described.¹⁸⁰ This quantification was used to determine the collagen deposition, a major parameter of fibrosis. For quantification of steatosis index, lipid droplet-positive areas were manually outlined as regions of interest (ROIs) on H&E-stained liver sections using ImageJ (NIH, USA). The "Measure" function was then used to obtain the area of each ROI, providing a precise quantitative measure of lipid accumulation.¹⁸¹ Within each representative ROI, the areas of all individual lipid droplet regions were summed to calculate the steatosis index.

Isolation of human and minipig NPCs

Liver tissues from human and minipigs were minced, and incubated in a digestive mixture (1 mg/ml collagenase I (Gibco, 17100017), 1 mg/ml collagenase II (Gibco, 17101015), 1mg/ml dispase II (Sigma-Aldrich, D4693) and 10 U/ml DNase I (Sigma-Aldrich, 11284932001) in DMEM) on an orbital shaker at 37 °C, for 40 min. After filtration through a 70 μ m cell strainer, the cell suspensions were centrifuged at 300 g for 10 min and resuspended in ACK lysis buffer for 8 min to remove red blood cells. Following centrifugation, the cell pellet was resuspended in PBS, and NPCs were then isolated from the supernatant by an additional centrifugation step at 50 g for 2 min.

Isolation of hepatocytes, CD45⁺ hematopoietic cells, endothelial cells (ECs), and hepatic stellate cells (HSCs) from mouse liver s

Hepatic cell populations, including hepatocytes, CD45⁺ hematopoietic cells, ECs, and HSCs, were isolated from related C57BL/6 mice in this study, using a combination of in situ perfusion, enzymatic digestion, magnetic-activated cell sorting (MACS), and density gradient centrifugation. All procedures were approved by the Institutional Animal Care and Use Committee. Briefly, perfusion buffer (HBSS without Ca²⁺/Mg²⁺ or phenol red [Gibco, 14175095], supplemented with 0.5 mM EDTA and 25 mM HEPES) and digestion buffer (HBSS with Ca²⁺/Mg²⁺ [Gibco, 24020117], containing 1 mg/ml collagenase IV [Gibco, 17104019] and 25 mM HEPES) was pre-warmed to 42°C. Mice were anesthetized, and the liver was perfused via the portal vein using a peristaltic pump (5 mL/min). Perfusion was initiated with pre-warmed perfusion buffer for 2–3 min until the liver turned pale, followed by digestion buffer for 15–20 min until tissue fissures appeared. The liver was excised, minced, and filtered through a 70- μ m cell strainer. The filtrate was centrifuged at 50 $\times$ g for 3 min to pellet hepatocytes, which were washed and reserved for downstream applications. The supernatant was further centrifuged at 580 $\times$ g for 10 min at 4°C to collect non-parenchymal cells (NPCs). NPCs were resuspended in MACS buffer (PBS with 0.5% BSA and 2 mM EDTA, pH 7.2) at a density of 10⁷ cells per 90 μ L, incubated with CD45 MicroBeads (Miltenyi Biotec, 130-052-301) for 15 min at 2–8°C, and subjected to positive selection using LS Columns (Miltenyi Biotec, 130-042-401) to isolate CD45⁺ hematopoietic cells. The flow-through was collected and processed for CD31⁺ EC separation with CD31 MicroBeads (Miltenyi Biotec, 130-097-418), following the same protocol. The resulting CD45⁺CD31⁺ cells were resuspended in 20 mL GBSS, mixed with 10 mL of pre-cooled Nycodenz medium (28.7% w/v Nycodenz [Serumwerk Bernburg AG, Cat# 18003] in GBSS), and aliquoted into three 15-mL tubes. The samples were overlaid with 1.5 mL GBSS and centrifuged at 1,380 $\times$ g for 17 min at 4°C without brake. The intermediate white layer containing HSCs was collected, washed, and used for subsequent experiments.

Homogeneous time-resolved fluorescence (HTRF)

The HTRF assay for screening ROCK2-selective inhibitors without ROCK1 inhibition was performed following the instructions provided in the HTRF KinEASE-STK S2 kit (Kinases, Cat #62ST2PEB). The detailed procedure is illustrated in [Figure S6A](#). The kinase domain of ROCK1 or ROCK2 was purified by Sino Biological Ltd.

Phase I clinical trial (ChiCTR2200058868)

The Phase I clinical trial was conducted in China (ChiCTR2200058868) to assess the safety and tolerability of TDI01. Healthy adults received single oral doses of 400 mg, 800 mg, and 1200 mg in both fasting and postprandial states, as well as multiple doses of 200 mg and 400 mg TDI01 suspension. Additionally, the pharmacokinetics of TDI01 were explored as an endpoint. The study design adhered to the recommendations of the National Medical Products Administration (NMPA) guidelines on mitigating risks in early clinical trials. These single-center trials were conducted in a randomized, double-blind, placebo-controlled manner, involving 42 healthy subjects. Within each dose step, 6 healthy male subjects were randomized to receive either HRX215 (8 subjects) or a respective placebo (2 subjects). A 40 mg/mL TDI01 suspension was prepared for administration. The specific implementation process and group allocation are detailed in [Figure S7A](#). To evaluate the pharmacokinetics of TDI01, serial blood samples were collected at various time points: 30 min before administration, and 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, and 48 h after single dose administration. Before advancing to the next dose level, safety and pharmacokinetic data were reviewed by a safety assessment team. Protocols are provided in [Data S1](#).

Extended clinical study (ChiCTR2400082056)

Extended clinical study was conducted in China (ChiCTR2400082056) to evaluate the efficacy, safety, and pharmacokinetics of TDI01 in liver fibrosis patients. This cohort study was approved by the Local Ethics Committee of West China Hospital, Sichuan University. A written informed consent was obtained from each patient after being informed of the purpose and investigational nature of the present study. This study was conducted according to the Declaration of Helsinki, and the study design adhered to the recommendations of the NMPA guidelines on mitigating risks in early clinical trials. In this extended Phase I study, patients underwent imaging and ultrasound examination, ultrasound transient elastography (UTE), serum evaluation of AST, ALT, TBIL, PLT, ALB, and liver biopsy with corresponding pathological analyses to determine the stage of liver fibrosis for final enrollment. Once each patient with liver fibrosis was enrolled and the treatment period started, pharmacokinetics test was conducted after the patient's initial drug administration and completion of every treatment cycle (4 weeks/cycle). Additionally, all included patients were re-evaluated for liver fibrosis grade after every cycle. To evaluate the pharmacokinetics of TDI01, serial blood samples were collected at various time points: 0, 2, 4, 5, 6, 8, and 24 hours after the first administration of 200 mg TDI01, and after the final administration of 200 mg

TDI01 in the first 4 weeks (first cycle). Afterwards, blood samples were collected 0 hour and 4 hours after the final administration of each cycle. Liver biopsy was performed after 24-week treatment of TDI01 to assess histological features and anti-fibrotic efficacy. The protocols are provided in [Data S1](#).

scRNA-seq of human and minipig NPCs

Human and minipig NPCs were resuspended in PBS for scRNA-seq. scRNA-Seq was performed through Singleron Matrix single cell processing system using the GEXSCOPE Single Cell RNA Library Kit. Single cells were processed in the following steps: GEM generation & Barcoding, RNA reverse transcription, cDNA amplification and library construction, and finally were sequenced on an Illumina HiSeq X with 150 bp paired end reads. The resulting raw reads were processed using CeleScope software.

Processing of scRNA-seq data

R (v.4.3.3) and Seurat (v.5.0.1) were used for processing the single-cell transcriptomics data. Unsupervised clustering and gene expression analysis were performed according to the Seurat guidance. We omitted genes and cells in the data based on the following criteria: (1) genes detected in fewer than 5 cells; (2) cells with fewer than 500 or more than 6000 genes; and (3) cells with more than 10% mitochondrial genes. In human single-cell data, 13 healthy and 21 fibrosis used for downstream analysis. The NPC data were integrated using harmony to remove batch effects. Following linear dimensionality reduction with principal component analysis (PCA), the first 40 principal components were selected for UMAP projection analysis, with a final resolution set to 1. The “FindAllMarkers” function was employed to identify specific marker genes for each cell cluster to aid in cellular identification. For the identification of protein types associated with genes, we first used the “FindMarkers” function to obtain significant differential genes between liver Cirrhosis and Control groups in human and pig liver endothelial cells. The protein types from the Human Protein Atlas website were used to categorize gene types, and ggplot2 was employed for graph drawing.

Reuse of public data

We utilized the GEO (Gene Expression Omnibus) datasets GEO: GSE173735, containing RNA-seq data from whole liver samples of MASH and healthy individuals, and GEO: GSE193066, comprising of RNA-seq data from human fibrotic and non-fibrotic livers. Enrichment analysis of significantly differentially expressed genes was conducted using the clusterProfiler package, comparing MASH vs. healthy livers (GEO: GSE173735) and fibrotic vs. non-fibrotic livers (GEO: GSE193066), with pathway enrichment performed using the REACTOME database. Additionally, four healthy hepatic NPC scRNA-seq samples from GEO: GSE136103 and six healthy samples from ArrayExpress under accession number E-MTAB-10553 were integrated for single-cell RNA seq analysis.

Scanning electron microscopy (TEM) sample preparation and observation

Liver biopsy samples were collected from normal control and MASH minipigs from different groups. Immediately after surgical resection, the tissues were fixed in 2.5% glutaraldehyde, followed by further fixation in 1% osmium tetroxide. Dehydration was performed using a graded acetone series with concentrations of 30%, 50%, 70%, 80%, 90%, 95%, and 100%, with three changes in 100% acetone to ensure complete dehydration. After dehydration, samples were infiltrated with acetone and Epon-812 embedding resin at ratios of 3:1, 1:1, and 1:3, followed by embedding in pure Epon-812 resin. Ultrathin sections (60–90 nm) were prepared using an ultramicrotome and mounted onto copper grids. The sections were stained with uranyl acetate for 10–15 minutes and lead citrate for 1–2 minutes at room temperature before observation. Transmission electron microscopy (TEM) was performed using a JEM-1400FLASH transmission electron microscope (JEOL, Japan). Low-magnification imaging was conducted to examine the entire tissue on each copper grid, after which regions of interest were selected for high-resolution imaging to analyze pathological changes.

Multiplex immunofluorescence analysis

Multiplex immunofluorescence staining for all tissue sections was performed using the CmTSA technology provided by LUMINIRIS.

For human liver section staining, paraffin sections were first dewaxed, rehydrated, and underwent antigen retrieval. Background fluorescence from the tissue was eliminated using the LUMINIRIS Fluorescence Quenching System (Cat#MH030101) and the IRISKit HyperView quench buffer (Cat#MH010301). Fluorescent labeling of the antigens was performed using the IRISKit HyperView multiplex immunostaining kit (Cat#MH010101). In the first round, ROCK2 (Abcam, Cat#ab125025, 1:200, 1h), CD31 (HUABIO, Cat#M1511-8, 1:1000, 1h), were sequentially labeled, with corresponding fluorophores Cyclic-480, Cyclic-550. After acquiring images of the whole slide, fluorescence signals, except for DAPI, were quenched using the LUMINIRIS Fluorescence Quenching System (Cat#MH030101) and IRISKit HyperView quench buffer (Cat#MH010301). In the second round, Desmin (Abcam, Cat#ab15200, 1:800, 1h), and LYVE1 (Abcam, Cat#ab219556, 1:1000, 20min), and CD45 (HUABIO, Cat#IRS025, 1:1000, 20min) were sequentially labeled, with corresponding fluorophores Cyclic-480, Cyclic-550, and Cyclic-630. After staining, images were acquired. Unless otherwise specified, all procedures were conducted at room temperature. For image registration, the imaging results from both rounds were aligned into a single 8-bit TIFF multichannel image, facilitating subsequent colocalization analysis. In addition, pROCK2 (Abcam, Cat# ab228008, 1:1000, 20 min; Cyclic-480), ROCK2 (Abcam, Cat# ab125025, 1:1000, 20 min; Cyclic-480), ROCK1 (CST, Cat#4035S, 1:1000, 1h; Cyclic-480) and pROCK1 (Bioss, Cat# bs-4630R, 1:1000, 20 min; Cyclic-550) were used to assess the activity of ROCK2 and ROCK1 in human patient liver biopsy tissues, respectively.

For mouse liver section staining, the procedure was similarly performed with different antibodies. In the first round, ROCK2 (Fisher scientific, Cat#PA5-78290, 1:500, 1h), CD31 (Abcam, Cat#ab182981, 1:1000, 1h), and Galectin-3 (Abcam, Cat#ab76245, 1:200, 4°C overnight) were sequentially labeled. In the second round, LYVE1 (Abcam, Cat#ab219556, 1:1000), Collagen I (HUABIO, Cat#HA722517, 1:10000), and α SMA (HUABIO, Cat#ET1607-53, 1:5000) were labeled.

Constructs, expression, and purification of ROCK2-TDI01 complex

The truncated human ROCK2 (18–417) protein was optimized and cloned into the pFastBac1 vector (Invitrogen), with an 8×His tag appended to the C-terminus. The recombinant protein was expressed in Sf9 insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Sf9 cells were cultured in SIM SF Medium (Sino Biological Inc.) at 27°C and infected at a density of $1.5\sim 2.0\times 10^6$ cells/mL. After 48 hours post-infection, the cells were harvested by centrifugation and lysed with a buffer containing 50 mM HEPES (pH 7.5), 300 mM NaCl, and 10% glycerol, supplemented with an EDTA-free protease inhibitor cocktail (MedChemExpress). The lysate was clarified by centrifugation at 18,000 rpm for 30 minutes, and the supernatant was incubated with Ni-NTA resin (MedChemExpress) for 2 hours at 4°C. The resin was washed with 30 column volumes of buffer containing 50 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, and 30 mM imidazole, and the protein was eluted with the same buffer containing 250 mM imidazole. The eluted ROCK2(18–417) was concentrated using a 30-kDa molecular weight cut-off concentrator (Millipore) and buffer-exchanged into 50 mM HEPES (pH 7.5), 300 mM NaCl, and 10% glycerol using a desalting column (Bio-Rad). The ROCK2 (18–417) and TDI01 were incubated at a 1:2 molar ratio at 4°C for 1 hour. Unbound TDI01 was removed by size-exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column (Cytiva), pre-equilibrated with 50 mM HEPES (pH 7.5) and 150 mM NaCl. The protein fractions were collected and concentrated to 2 mg/mL for Cryo-EM analysis.

Cryo-EM sample preparation, data collection, data processing and model building

For cryo-EM data collection, 3 μ L of the purified ROCK2-TDI01 complex was applied to glow-discharged Quantifoil R1.2/1.3 holey-carbon grids (300 mesh, copper). The grids were briefly blotted for 4 s and immediately plunged into liquid ethane using a Vitrobot Mark IV (Thermo Fisher) operating at 100% humidity. Data acquisition was performed using a Titan Krios electron microscope (Thermo Fisher) equipped with a Falcon4 detector. Cryo-EM images were collected in counting mode at a nominal magnification of 165,000 $\times$. Each micrograph was acquired with an exposure time of 3s, and dose fractionation was employed with a dose rate of approximately 10 counts per pixel per second.

To process the acquired data, beam-induced motion correction was performed using MotionCor2¹⁸² to align the image stacks and generate dose-weighted micrographs. The resulting micrographs had a pixel size of 0.73 Å. Contrast transfer function (CTF) correction was performed with Gctf,¹⁸³ and particle picking was conducted using Gautomatch. For the initial stages of data analysis, 2D classification was carried out, followed by ab initio model generation, and 3D classification in CryoSPARC.¹⁸⁴ 3D refinement was subsequently performed using relion4.¹⁷⁴ The final 3D reconstruction map was obtained at a resolution of 3.8 Å, utilizing a B-factor of -114 Å^2 . A complete data processing workflow is depicted in Figures S6E–S6G. The overall resolution was determined using the Fourier shell correlation (FSC) criterion, with a cutoff of 0.143.

The Cryo-EM map of the ROCK2-TDI01 complex was resolved at a final resolution of 3.8 Å, which enabled the initiation of model building. The model of ROCK2 kinase domain (18–417) predicted by AlphaFold3.¹⁸⁵ TDI01 coordinates and geometry restraints were generated using phenix.elbow.¹⁸⁶ The models were fitted to the EM density map using UCSF Chimera,¹⁶⁸ then subjected to iterative manual building in Coot.¹⁸⁷ The coordinates were refined using phenix.real_space_refine in Phenix.¹⁸⁸ The model geometry was validated using MolProbity.¹⁶⁹ Structural representations were prepared in UCSF ChimeraX.¹⁸⁹ The atomic coordinates and structure factors for the ROCK2-TDI inhibitor complex have been deposited in the Protein Data Bank (PDB) and assigned an accession number.

Molecular Docking of ROCK2 with AMP-PNP using AutoDock Vina

The molecular docking was performed using AutoDock Vina 1.1.2. The ROCK2(18–417) protein structure was predicted with the AlphaFold3 and prepared by removing water molecules, adding polar hydrogens, and assigning Gasteiger charges via AutoDockTools. The AMP-PNP ligand (PubChem CID: 33113) was energy-minimized using OpenBabel and converted to PDBQT format. The docking grid box (center: $x=-8.317$, $y=-1.804$, $z=0.36$; dimensions: $20\times 20\times 20\text{ Å}$) encompassed the ATP-binding site identified from homologous kinase structures. Vina parameters included an exhaustiveness of 32 and 20 binding poses. Top-ranked conformations were selected based on binding affinity ($\Delta G \leq -8.0\text{ kcal/mol}$) and visual validation of key interactions using PyMOL.

Evaluation of ROCK2 activity in ECs and HSCs

ROCK2 activity was evaluated by immunoprecipitation followed by an *in vitro* kinase assay, as previously reported.¹⁹⁰ Experimental conditions included the following groups in human primary ECs and the human HSC cell line LX2: negative control (NC), ROCK2 knockout (sgROCK2), ROCK2 overexpression (ROCK2 OE), and two rescue groups in which the sgRNA was co-expressed with either wild-type ROCK2 (bearing silent mutations within the sgRNA target site to prevent degradation) or the L210F mutant ROCK2 (derived from the wild-type construct described above).

Cells were treated with increasing concentrations of TDI01 (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 μ M) overnight, lysed on ice in M-PER buffer (Thermo Scientific, Cat. #78501) supplemented with protease and phosphatase inhibitor cocktails, and centrifuged at

18,000 × *g* to remove insoluble debris. The resulting supernatants were incubated with 5 μg of anti-ROCK2 antibody (Cell Signaling Technology, Cat. #9029) for 1 h at 4°C, followed by overnight incubation with Protein A/G agarose beads at 4°C to capture immune complexes. The beads were collected by centrifugation, washed extensively, and resuspended in kinase reaction buffer (40 mM Tris, pH 7.5; 20 mM MgCl₂; 0.1 mg/mL BSA; 2 mM DTT; 2 mM MnCl₂) containing 2.5 μM STK substrate (Kinase, Cat. #62ST2PEB) and 100 μM ATP in a final reaction volume of 5 μL. The reaction mixture was incubated for 30 min at room temperature.

Kinase activity was quantified using the ADP-Glo Kinase Assay Kit (Promega, Cat. #V6930). In brief, 5 μL of ADP-Glo reagent was added to terminate the kinase reaction and deplete residual ATP, followed by a 40-min incubation at room temperature. Subsequently, 10 μL of Kinase Detection Reagent was added to convert ADP to ATP and introduce a luciferase/luciferin system for ATP detection. After a 30-min incubation, luminescence was measured using a plate-reading luminometer (PE EnVision 2105). Relative ROCK2 kinase activity was calculated based on luminescence intensity.

Pull-down assay

HUVECs and the human HSC (LX2 cells) were lysed in M-PER buffer (Thermo Scientific, Cat. #78501), and the lysates were clarified by centrifugation at 18,000 *g* to obtain soluble protein fractions. Streptavidin affinity purification was subsequently performed according to the manufacturer's instructions for the Pierce™ Streptavidin Magnetic Beads (Thermo Scientific, Cat. #88817). Briefly, 50 μL (0.5 mg) of magnetic beads were transferred into a 1.5-mL microcentrifuge tube and collected using a magnetic stand. After discarding the storage buffer, the beads were washed with 1 mL of Binding/Wash Buffer (Tris-buffered saline, TBS; Thermo Scientific, Cat. #28379) containing 0.1% Tween-20 by gentle inversion, followed by magnetic separation to remove the supernatant.

For pulldown assays, clarified cell lysates were incubated with 2–5 μL of 10 mM of biotinylated TDI01 or 10 mM biotin alone (negative control) overnight at 4°C with gentle mixing. The mixtures were then combined with pre-washed streptavidin magnetic beads and incubated for an additional 1 h at room temperature with rotation. Following incubation, the beads were washed five times with 1 mL of Binding/Wash Buffer to remove nonspecifically bound proteins. The beads were collected using a magnetic stand after each wash, and the final supernatants were retained for subsequent analyses.

LC-MS/MS analysis of pull-down samples

Protein-enriched magnetic beads from the pull-down assay were solubilized in 50 μL of 1% sodium deoxycholate (SDC). Disulfide bonds were reduced by adding dithiothreitol (DTT) to a final concentration of 10 mM and incubating at 37 °C for 60 min. Cysteine residues were alkylated with 50 mM iodoacetamide (IAA) at room temperature for 45 min in the dark. Proteolytic digestion was carried out by adding trypsin to a final concentration of 10 ng/μL and incubating at 37 °C overnight. The reaction was quenched with 2% trifluoroacetic acid (TFA), followed by centrifugation at 12,000 × *g* for 2 min. Peptides were desalted using StageTip C18 columns, lyophilized, and reconstituted in 0.1% TFA prior to LC-MS/MS analysis.

NanoLC-MS/MS was performed on an Orbitrap Exploris 240 mass spectrometer coupled to a Vanquish Neo UHPLC system (Thermo Scientific). Samples were introduced via an EASY-Spray nanosource and separated on an IonOpticks-TS analytical column (25 cm × 75 μm) maintained at a constant temperature using a Heater THOR Controller (IonOpticks). Chromatographic separation employed a binary solvent system consisting of 0.1% formic acid in water (Eluent A) and 0.1% formic acid in 80% acetonitrile/19.9% water (Eluent B). Peptides were eluted using the following gradient: 8–35% B over 52 min, 35–55% B over 8 min (total gradient: 60 min), followed by a column wash at 95% B for 9 min and re-equilibration to initial conditions.

Data-dependent acquisition (DDA) was conducted with full MS scans (*m/z* 400–1500) acquired at a resolution of 120,000. The automatic gain control (AGC) target was set to 300% in custom mode with a maximum injection time of 22 ms. Peptides were fragmented via higher-energy collisional dissociation (HCD) using a normalized collision energy of 30%.

Western blot

To verify the genetic deletion of *Rock2* in the EC, HSC, and hematopoietic cell-specific knockout mice, as well as in wild-type mice intravenously injected with an Alb-shRock2 AAV, liver tissues were collected and processed into single-cell suspensions. Hepatocytes, ECs, HSCs and CD45⁺ hematopoietic cells were isolated as described above. Total proteins were then extracted using RIPA lysis buffer (Beyotime Biotechnology, P0013B) with protease inhibitor cocktail (Bimake, B14011) and phosphatase inhibitor cocktail (Bimake, B15002). Antibodies, β-Actin (1:3000; catalog A5441, Sigma-Aldrich) and ROCK2 (1:1000, CST, Cat# 9029; 1:1000, Abcam, Cat#ab228000) were used for blot.

In addition, normal human precision-cut liver slices (hPCLSs) treated with 50 ng/ml Galectin-3 and fibrotic PCLSs subjected to the indicated treatments were analyzed using antibodies against TGFβ1 (1:1000; Proteintech, 18978-1-AP), Desmin (1:20000; Proteintech, 16520-1-AP), α-SMA (1:1000; Abcam, EPR5368), and Collagen I (1:1000; Abcam, ab6308).

Enzyme-linked immunosorbent assays (ELISA)

Normal human PCLSs were treated with vehicle (Ctrl) or Galectin-3. Fibrotic human PCLSs were treated with vehicle (Ctrl), Galectin-3, TDI01, or a combination of TDI01 and Galectin-3. After 5 days of treatment, culture supernatants were collected and submitted to Wuhan Servicebio Technology Co., Ltd., where enzyme-linked immunosorbent assays (ELISA) were performed to quantify CCL2, IL-10, IL-17, IL-18, IL-1β, IL-2, IL-4, IL-6, IL-8, TGFβ, and TNF-α levels.

QUANTIFICATION AND STATISTICAL ANALYSIS

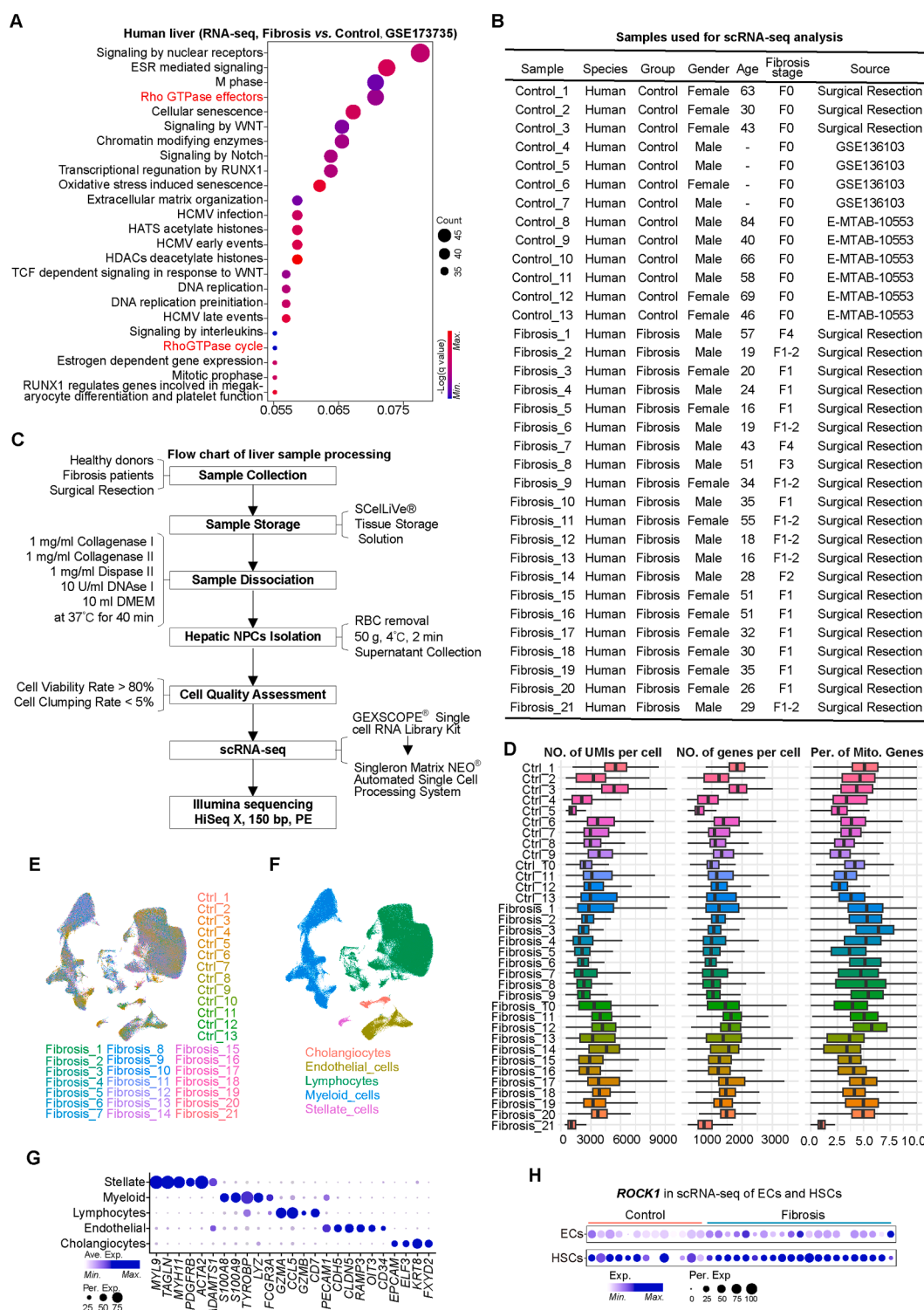
Statistical analyses were performed using GraphPad Prism (V8.4.3). Detailed statistical parameters and significance values are indicated in the figures and figure legends. Data are presented as mean $\pm$ SEM unless otherwise specified. Statistically significant was defined as $p < 0.05$, and was determined by unpaired or paired two-tailed Student's *t* tests, or one-way or two-way Analysis of Variance (ANOVA) where appropriate. Significance is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant, $p > 0.05$.

ADDITIONAL RESOURCES

Phase I Clinical Trial: Safety, tolerability, and single/multiple dose administration of TDI01 in healthy Chinese subjects; kinetic characteristics and food effects (ChiCTR2200058868; <https://www.chictr.org.cn/showproj.html?proj=166937>). Study protocols are provided in [Data S1](#).

Extended Clinical Trial: Exploratory study of TDI01 safety and efficacy in patients with hepatic fibrosis (ChiCTR2400082056; <https://www.chictr.org.cn/showproj.html?proj=220481>). Study protocols are provided in [Data S1](#).

Supplemental figures



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Figure S1. Upregulation of ROCK2 in the ECs and HSCs of human fibrotic livers, related to Figure 1

- (A) Dot plot displaying the DEGs identified between human fibrotic livers and normal livers (datasets GEO: GSE173735). Gene enrichment analysis was performed using the Reactome database. Sample size $n = 6$ for each group.
- (B) Human liver sample information for scRNA-seq analysis. Four healthy samples were sourced from GEO: GSE136103. 6 healthy samples were sourced from ArrayExpress under accession number E-MTAB-10553. The remaining samples were obtained from surgical resections.
- (C) Processing workflow for scRNA-seq of human livers.
- (D) Processed quality control information for individual scRNA samples, including unique molecular identifiers (UMIs) per cell (left), gene counts per cell (middle), and mitochondrial gene percentages (right).
- (E) Uniform manifold approximation and projection (UMAP) plot of scRNA-seq data for individual healthy and fibrotic livers.
- (F) UMAP plot showing the clusters of different cell types.
- (G) Dot plot quantifying expression of marker genes in cell types.
- (H) Dot plot showing *ROCK1* expression in the ECs and HSCs of human healthy and fibrotic livers (tumor free). $n = 13$ for the healthy control group and $n = 21$ for the fibrosis group.

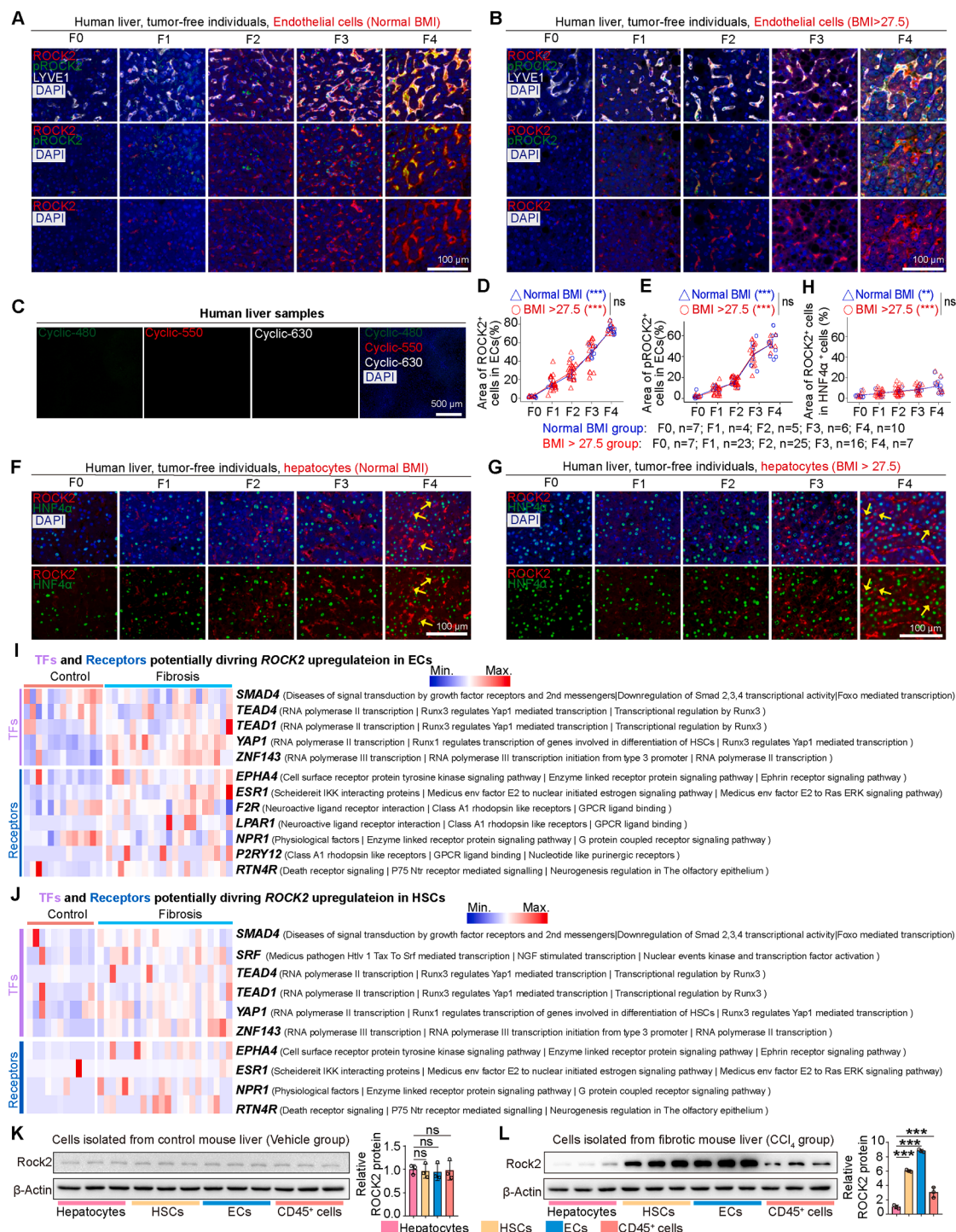


Figure S2. ROCK2 expression in the hepatocytes of human patients and mouse liver fibrosis models, related to Figure 1

(A–E) Stage-dependent upregulation of ROCK2 and pROCK2^{S1366} in the ECs of human fibrotic liver. Liver sections from patients with normal BMI (18.5 < BMI < 27.5) (A) and obese BMI (BMI > 27.5) (B) at fibrosis stages F0–SF4 were co-stained for ROCK2, pROCK2, and LYVE1. The negative control is shown in (C). The proportion of ROCK2⁺ (D) and pROCK2⁺ ECs (E) was quantified. In this figure, the interplay between BMI and fibrosis stages on ROCK2 expression was analyzed by two-way ANOVA. Progression of ROCK2 within fibrosis stages was analyzed by one-way ANOVA followed by Tukey's post hoc test. For normal BMI group, $n = 7$ (F0), $n = 4$ (F1), $n = 5$ (F2), $n = 6$ (F3), and $n = 10$ (F4). For obese BMI (>27.5) group, $n = 7$ (F0), $n = 23$ (F1), $n = 25$ (F2), $n = 16$ (F3), and $n = 7$ (F4). Scale bars, 100 μ m. In this figure, statistical difference is denoted as follows: ns, no significant; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean $\pm$ SEM in this figure.

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(F–H) ROCK2 expression in the hepatocytes of patients with normal BMI ($18.5 < \text{BMI} < 27.5$) (F) and obese BMI ($\text{BMI} > 27.5$) (G). ROCK2 was co-stained with hepatocyte marker HNF4 α . The proportion of ROCK2⁺ hepatocytes was quantified (H).

(I and J) Heatmap visualization of transcription factors (TFs)^{64–68} (upper) and receptors^{69–78} (below) that are upregulated in fibrotic liver ECs (I) and HSCs (J), which are also reported to induce ROCK2 expression. Data are derived from scRNA-seq. Each row represents a gene labeled with its symbol followed by three functional pathway annotations derived from the MSigDB database.

(K and L) Western blot analysis of ROCK2 in hepatocytes, HSCs, ECs, and CD45⁺ immune cells isolated from indicated mouse livers. Liver fibrosis was induced by repeated intraperitoneal injections of CCl₄ (2.5 $\mu\text{L/g}$) twice weekly for 5 weeks. Band intensities were quantified, and statistical difference was analyzed using one-way ANOVA.

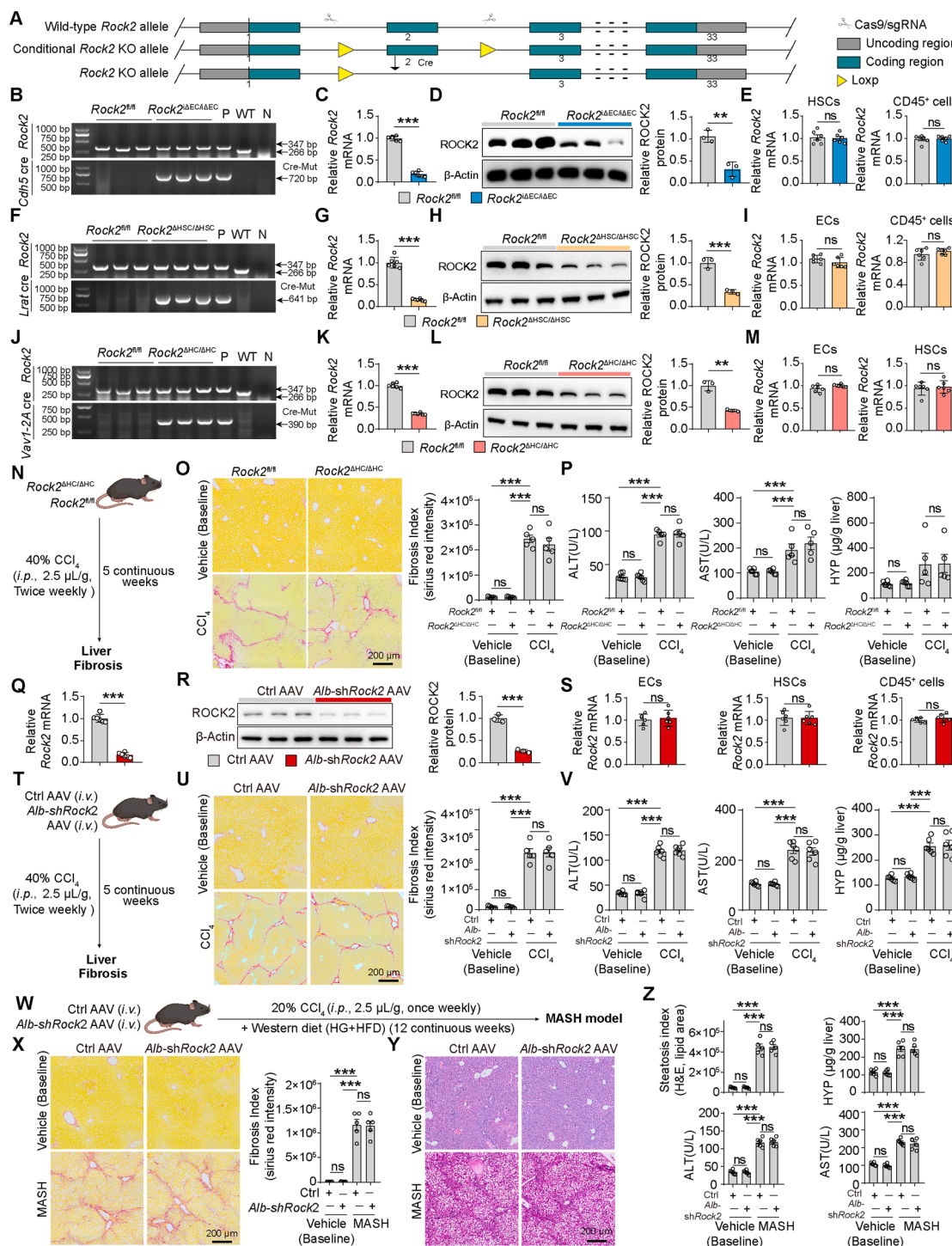


Figure S3. Characterization of mice lacking *ROCK2* in ECs, HSCs, HCs, and hepatocytes, related to Figure 2

(A) Restriction maps of the mouse WT *ROCK2* allele and steps leading to creation of floxed *ROCK2* allele. Exon2 of *ROCK2*-201 (ENSMUST00000020904.7) transcript containing 82-bp coding sequence is designed as the KO region. To enable efficient gene editing, loxP sites are inserted into the non-coding sequences flanking exon 2, thereby facilitating Cre-mediated recombination and subsequent excision of the targeted exon.

(B) Genotyping of EC-specific *ROCK2* conditional KO mice. Representative PCR-based genotyping results are shown for control floxed *ROCK2* (*Rock2*^{fl/fl}; Cre-negative) and EC-specific *ROCK2* KO (*Rock2*^{fl/fl}; *Cdh5*-Cre^{ERT2}) mice. The upper image shows the PCR genotyping analysis of *ROCK2* allele. The floxed *ROCK2* (*Rock2*^{fl/fl}) allele appears as a 347-bp band, and the 266-bp band corresponds to the WT *ROCK2* allele. The bottom image shows the PCR analysis of the

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Cdh5-Cre^{ERT2} transgene. The 720-bp band indicates mice carrying *Cdh5*-Cre^{ERT2} (Cre⁺), and this band is absent in either control *ROCK2*^{fl/fl} mice without Cre (Cre⁻; *ROCK2*^{fl/fl}) or WT mice. P, positive control; WT, wild-type control; N, negative control.

(C–E) *ROCK2* deletion specificity and efficiency in the liver ECs of *ROCK2*^{ΔEC/ΔEC} mice, as compared with control *ROCK2*^{fl/fl} mice. Real-time PCR and western blotting analysis of *ROCK2* mRNA (C) and *ROCK2* protein in liver ECs (D).

To compare the cell type-specific KO efficiency of *ROCK2* in *ROCK2*^{ΔEC/ΔEC} mice. *ROCK2* expression was assessed in the HSCs and CD45⁺ HCs (E) in control *ROCK2*^{fl/fl} and *ROCK2*^{ΔEC/ΔEC} mice. Statistical difference was determined using an unpaired Student's *t* test; *n* = 5 per group. ns, not significant, *p* > 0.05, ***p* < 0.01, and ****p* < 0.001.

(F) Genotyping of HSC-specific *ROCK2*-conditional KO mice. Representative PCR-based genotyping results for control (Cre-negative; *ROCK2*^{fl/fl}) and HSC-specific *ROCK2* KO (*ROCK2*^{fl/fl}; *Lrat*-Cre⁺) mice are presented. In the upper image, the 347-bp band corresponds to the floxed *ROCK2* (*ROCK2*^{fl/fl}) allele, and the 266-bp band corresponds to the WT *ROCK2* allele. The bottom image presents the PCR analysis results of mice carrying HSC-specific *Lrat*-Cre transgene. Mice carrying *Lrat*-Cre (Cre⁺) display bands at 641 bp, and this band is absent in either *Lrat*-Cre-negative control (Cre⁻; *ROCK2*^{fl/fl}) or WT mice. P, positive control; WT, wild-type control; N, negative control.

(G–I) The specificity of *ROCK2* KO in the HSCs was validated in *ROCK2*^{ΔHSC/ΔHSC} mice. Real-time PCR and western blot analysis were performed to measure *ROCK2* mRNA (G) and *ROCK2* protein (H) levels in the HSCs of control *ROCK2*^{fl/fl} and *ROCK2*^{ΔHSC/ΔHSC}. To compare the cell-type-specific KO efficiency of *ROCK2* in *ROCK2*^{ΔHSC/ΔHSC} mice, *ROCK2* mRNA expression in the liver ECs and CD45⁺ HCs (I) in control *ROCK2*^{fl/fl} and *ROCK2*^{ΔHSC/ΔHSC} mice was also assessed. Statistical significance was determined using an unpaired Student's *t* test; *n* = 5 per group. ns, not significant, *p* > 0.05, and ****p* < 0.001.

(J) Genotyping of HC-specific *ROCK2*-conditional KO mice. Representative PCR-based genotyping results are presented for control (Cre-negative; *ROCK2*^{fl/fl}) and HC-specific *ROCK2* KO (*ROCK2*^{fl/fl}; *Vav1*-2A-Cre) mice. Upper image shows the PCR analysis result of indicated *ROCK2* alleles. The 347-bp band corresponds to the floxed *ROCK2* (*ROCK2*^{fl/fl}) allele, and the 266-bp band corresponds to the WT allele. Bottom image shows the PCR analysis result of the *Vav1*-2A-Cre transgene. Mice carrying *Vav1*-2A-Cre exhibit a 390-bp band (Cre⁺), which is absent in mice without *Vav1*-2A-Cre (Cre⁻). P, positive control; WT, wild-type control; N, negative control.

(K–M) The specificity of *ROCK2* KO in HCs was confirmed in *ROCK2*^{ΔHC/ΔHC} mice, as compared with control *ROCK2*^{fl/fl} mice. Real-time PCR and western blotting were performed to quantify the expression of *ROCK2* mRNA (K) and *ROCK2* protein (L) in CD45⁺ HCs. In addition, real-time PCR was conducted to measure *ROCK2* mRNA levels in the ECs and HSCs (M) in *ROCK2*^{fl/fl} and *ROCK2*^{ΔHC/ΔHC} mice. Statistical significance was assessed using an unpaired Student's *t* test; *n* = 5 per group. ns, not significant, *p* > 0.05, and ****p* < 0.001.

(N–P) Experimental design evaluating the role of *ROCK2* in CD45⁺ HCs during liver fibrosis (N). Control *ROCK2*^{fl/fl} mice and *ROCK2*^{ΔHC/ΔHC} mice were used. Both vehicle- and CCl₄-treated groups were included to establish baseline and injury conditions, respectively. Compared with control *ROCK2*^{fl/fl} mice, *ROCK2*^{ΔHC/ΔHC} mice exhibited similar levels of liver injury and fibrosis after repeated CCl₄ challenge, as reflected by collagen deposition (O), serum AST and ALT levels, and hepatic HYP content (P). Statistical analysis was performed using one-way ANOVA, (*n* = 5 per group; ns, not significant, *p* > 0.05, and ****p* < 0.001).

(Q–S) Validation of hepatocyte-specific *ROCK2* knockdown in mice. To selectively knockdown *ROCK2* in hepatocytes, WT mice were intravenously injected with AAV carrying a hepatocyte-specific ALB promoter-driven shRNA against *ROCK2* (Alb-shRock2 AAV). Control mice received AAVs encoding a non-targeting shRNA. Real-time PCR and western blotting were performed to assess *ROCK2* mRNA (Q) and *ROCK2* protein levels in hepatocytes (R). In parallel, *ROCK2* expression was examined by real-time PCR in the ECs, HSCs, and CD45⁺ HCs (S) from control AAV- and Alb-shRock2 AAV-treated mice. Statistical analysis was performed using unpaired Student's *t* test; *n* = 6 per group. ns, not significant, *p* > 0.05; ****p* < 0.001.

(T–V) Experimental design to assess the role of hepatocyte *ROCK2* in liver fibrosis (T). WT mice received intravenous injections of control AAV or Alb-shRock2 AAV. Both groups were subjected to repeated CCl₄ administration for 5 weeks to induce fibrosis. Vehicle-treated groups served to establish baseline. Compared with mice treated with control AAV, mice with hepatocyte-specific *ROCK2* silencing exhibited similar levels of liver injury or fibrosis under both baseline and CCl₄-induced conditions, including collagen deposition (U), serum ALT and AST levels, and hepatic HYP content (V). Representative images and quantifications are shown. Statistical analysis was performed using one-way ANOVA, (*n* = 6 per group; ns, not significant, *p* > 0.05, and ****p* < 0.001).

(W–Z) Contribution of hepatocyte *ROCK2* to mouse MASH was assessed using AAV expressing hepatocyte-specific *ROCK2* shRNA (Alb-shRock2) or control AAV (W). After either baseline or MASH treatment, there is little difference in the tested parameters between control AAV and Alb-shRock2 AAV-treated mice, including collagen deposition (X), steatosis (Y), serum AST and ALT and liver HYP (Z) were comparable between groups. Statistical analysis was performed using one-way ANOVA (*n* = 6 per group; ns, not significant, *p* > 0.05, and ****p* < 0.001).

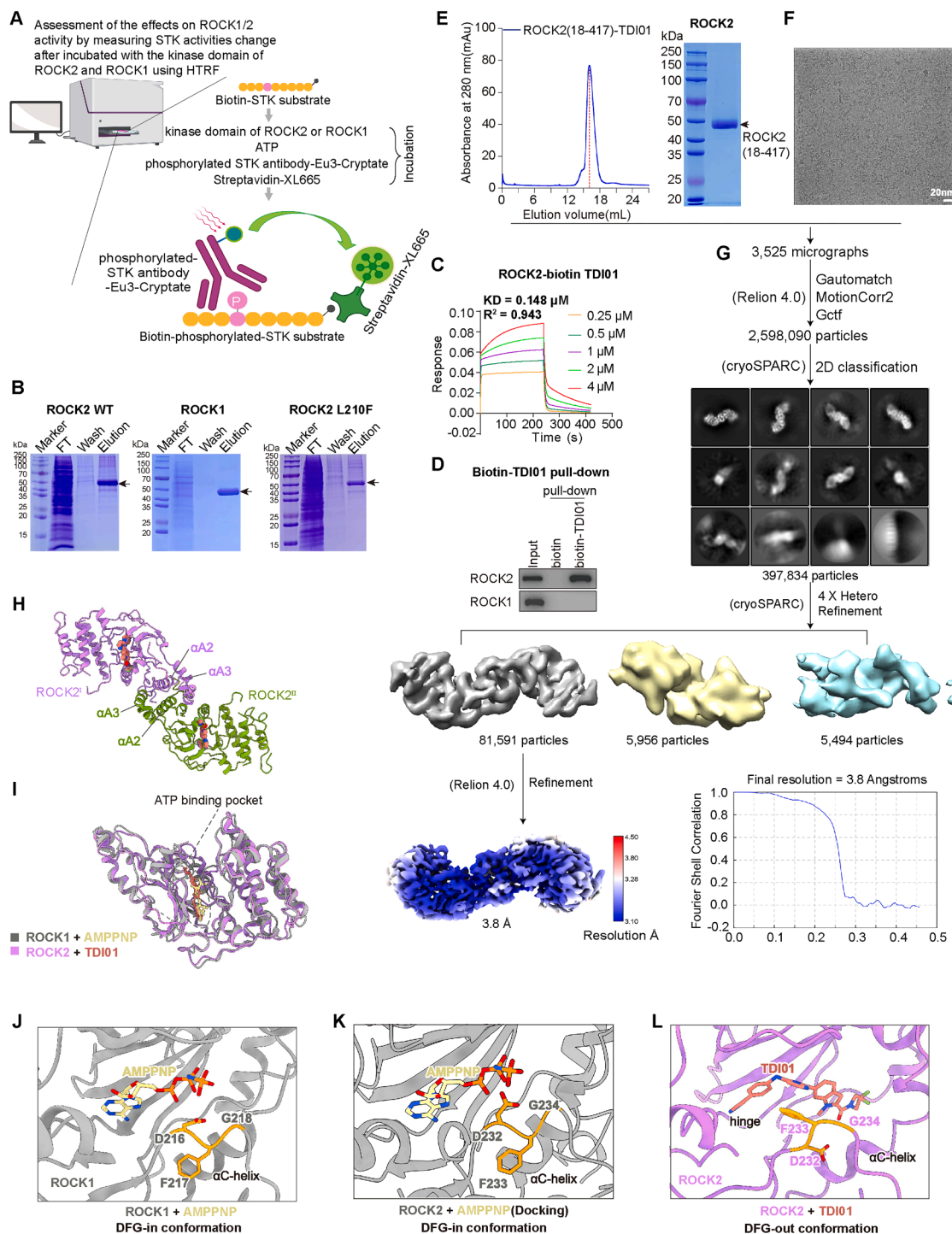


Figure S4. Identification of compound TDI01 as ROCK2-selective inhibitor and structural analysis of ROCK2-TDI01 complex by cryo-EM, related to Figure 4

(A) The flowchart illustrates the biochemical evaluation process of all synthesized compounds for ROCK inhibitory activity. The assays were conducted using HTRF technology to identify inhibitors that selectively inhibit ROCK2 activity without affecting ROCK1. This method is based on time-resolved fluorescence resonance energy transfer (TR-FRET) signal generation. STK substrate-biotin, a universal substrate phosphorylated by the KDs of both ROCK1 and ROCK2, was employed to independently assess the activity of each isoform through separate incubations. Detailed experimental procedures are provided in the [STAR Methods](#) section under “homogeneous time-resolved fluorescence (HTRF).” This relates to Figure 3.

(B) SDS-PAGE analysis of purified KDs of ROCK2 WT (left), ROCK1 (middle), and the L210F-mutant ROCK2 (right).

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- (C) BLI analysis of interaction between biotin-TDI01 and ROCK2 KDs.
 - (D) Western blot analysis of ROCK2 and ROCK1 binding by biotin-labeled TDI01 in human primary ECs.
 - (E) The size-exclusion chromatogram and SDS-PAGE analysis of purified ROCK2-TDI01 complex.
 - (F) Cryo-EM microscope image of ROCK2-TDI01 complex.
 - (G) The data processing pipeline for the ROCK2-TDI01 complex and the gold-standard Fourier shell correlation (FSC) curves of the final cryo-EM maps.
 - (H) Cartoon representation of ROCK2-TDI01 complex. The structure of ROCK2 KD adopts a typical kinase fold, consisting of a small N-terminal lobe and a larger C-terminal lobe connected by a flexible hinge. Dimerization of the ROCK2 molecules is mediated by two α helices (α A2 and α A3) from the N-terminal lobe of each protomer.
 - (I) TDI01 binds in the ATP-binding pocket of ROCK2. Structural comparison of ROCK2-TDI01 complex with ROCK1-AMPPNP complex (PDB: 2v55).
 - (J) ROCK1 exhibits the active DFG-in conformation in the ROCK1-AMPPNP complex (PDB: 2v55). ROCK1 is colored in gray. The ATP analog, AMPPNP, is colored in yellow.
 - (K) Molecular docking of ROCK2 with AMPPNP demonstrates that ROCK2 adopts the DFG-in conformation within the ROCK2-AMPPNP complex. ROCK2 is depicted in gray and AMPPNP is represented in yellow.
 - (L) ROCK2 adopts the inactive DFG-out conformation within the ROCK2-TDI01 complex. ROCK2 is depicted in purple, while TDI01 is shown in brown.

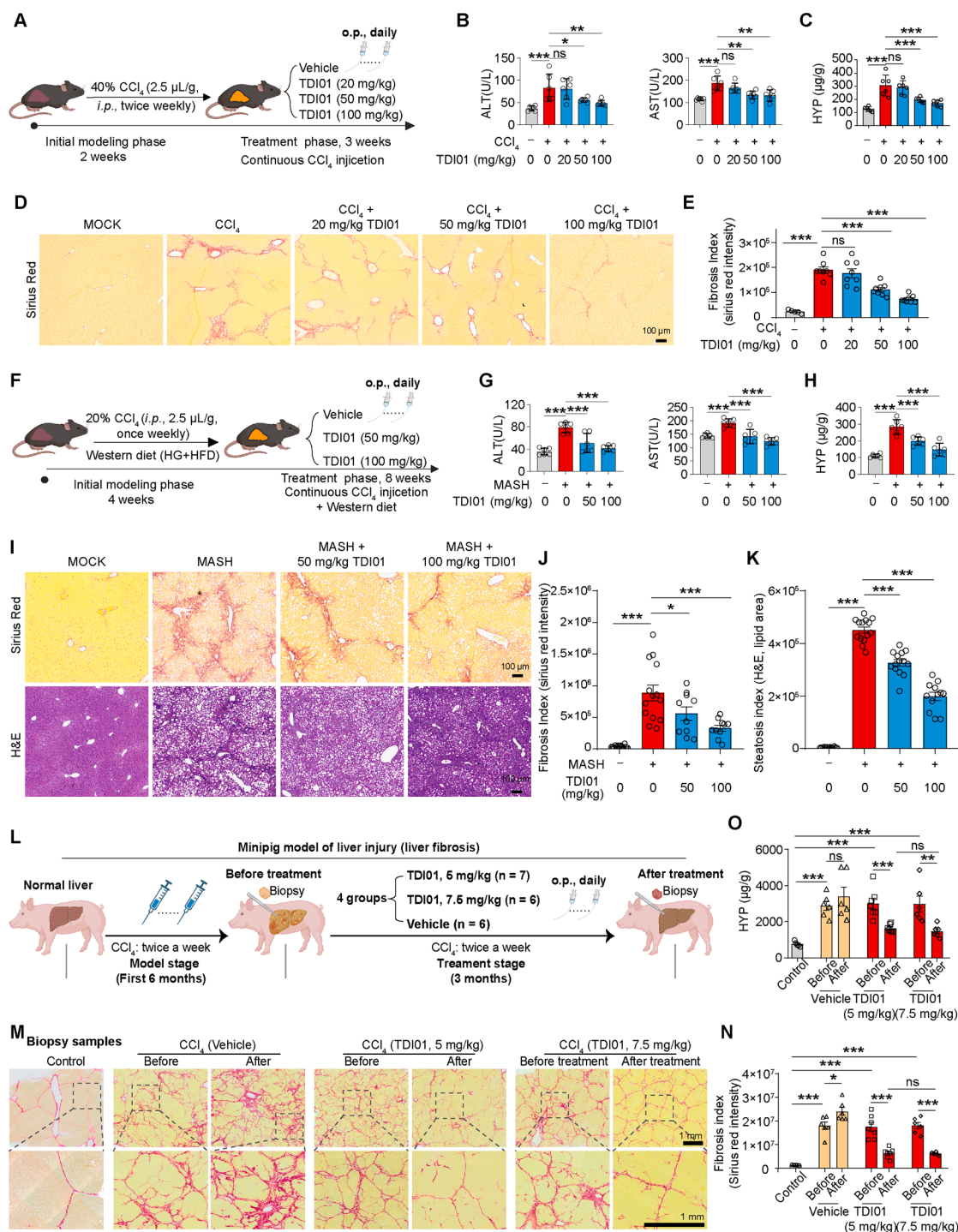


Figure S5. The anti-fibrotic efficacy of TDI01 in mouse and minipig liver fibrosis models, related to Figure 5

(A) Approaches to evaluate the therapeutic effects of TDI01 (administered orally at doses of 20, 50, and 100 mg/kg) in a mouse liver injury model induced by repeated intraperitoneal injections of 40% CCl₄ (2.5 μL/g, twice weekly). *n* = 5, MOCK group, *n* = 8 in vehicle and TDI01 treatment groups.

(B and C) The efficacy of TDI01 at different doses was assessed by analyzing serum levels of ALT and AST (B) and liver HYP content (C) in the mouse liver injury model. *n* = 6 per group.

Throughout this figure, statistical significance was determined using one-way ANOVA; ns, *p* > 0.05, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Data are presented as mean ± SEM.

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(D and E) The therapeutic effect of TDI01 at different doses was evaluated by assessing collagen deposition via Sirius red staining in the mouse injury model. Representative images and quantifications are shown. $n = 5$, MOCK group, $n = 8$ in vehicle and TDI01 treatment groups.

(F) Approaches to evaluate the therapeutic effects of TDI01 in mouse MASH model induced by low-concentration CCl₄ (20% CCl₄, 2.5 μ L/g, once weekly) intraperitoneal injection and a western diet (containing 21.1% fat, 41% sucrose, and 1.25% cholesterol, supplemented with a fructose [23.1 g/L] and glucose [18.9 g/L] solution). TDI01 with 50 or 100 mg/kg was given to mice 4 weeks after the induction phase of this MASH model. $n = 13$, MOCK group, $n = 14$ in vehicle group, and $n = 10$ in each TDI01 treatment group.

(G and H) Serum liver function indices, including ALT and AST (G), and liver HYP content (H) were measured in the indicated groups. $n = 6$ per group.

(I–K) Liver fibrosis was evaluated through Sirius red and H&E staining in the indicated mouse groups (I). Representative images are shown, with Sirius red quantification displayed in (J). The quantification of steatosis, based on H&E staining, is also presented in (K).

(L) The therapeutic effects of TDI01 were evaluated in a minipig liver fibrosis model, which was induced by biweekly intraperitoneal injections of CCl₄ (0.1 mL/kg, corresponding to 0.16 g/kg) for a duration of 6 months. Following this induction, liver biopsies were performed, revealing a distinct fibrotic phenotype characterized by collagen deposition. The minipigs were subsequently randomized into three treatment groups: vehicle, TDI01 (5 mg/kg), and TDI01 (7.5 mg/kg). These doses in minipig were derived from the tested doses in mouse liver fibrosis model. TDI01 was administered orally, and liver tissue samples were obtained at specified time points post treatment to facilitate a longitudinal comparison of the anti-fibrotic effects within the same animal.

(M and N) TDI01 treatment decreased the established liver fibrosis in the minipig liver injury model, as assessed by Sirius red staining of liver biopsy samples in the indicated groups before and after treatment (M). Quantification of collagen deposition is shown on the left side of (N). Statistical significance throughout the figure was determined using one-way ANOVA, followed by post hoc comparisons. * $p < 0.05$ and *** $p < 0.001$. Scale bar, 1 mm.

(O) Dot plot depicting HYP content in the liver biopsies from indicated groups. $n = 5$ in normal control group, $n = 7$ in TDI01 (5 mg/kg), and $n = 6$ in all other groups.

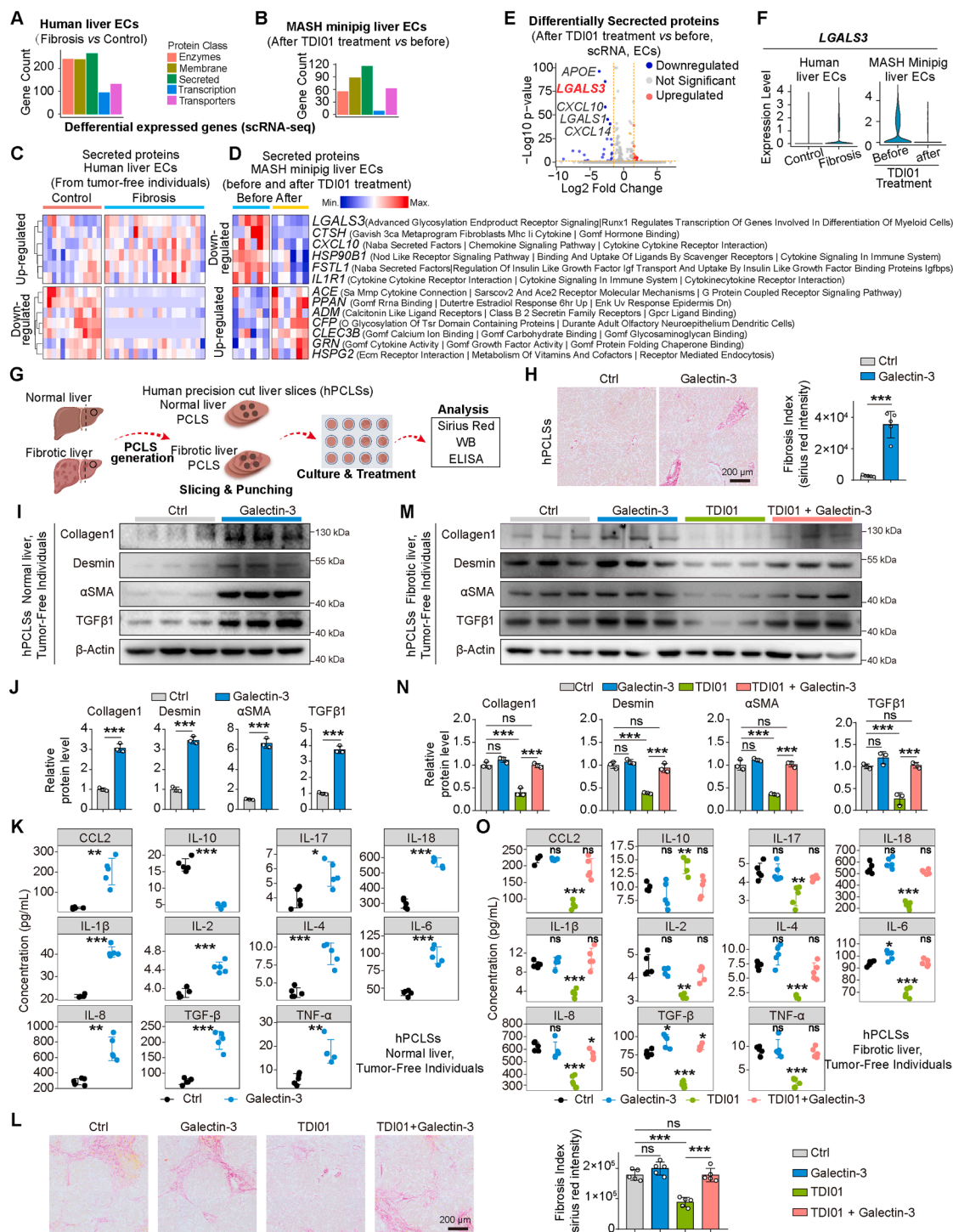


Figure S6. The anti-fibrotic effect of TDI01 is at least partially mediated by attenuating the production of pro-fibrotic galectin-3 from liver ECs, related to Figure 5

(A and B) Bar plots illustrating the indicated categories of DEGs in the liver ECs from human control and fibrotic livers (A), as well as from MASH minipigs before and after TDI01 treatment (B), including enzymes, membrane proteins, secreted proteins, TFs, and transporters. $n = 12$ in the human control group, $n = 21$ in the human fibrosis group, and $n = 6$ in minipig groups.

(C and D) TDI01 treatment normalized the production of secreted factors that are commonly dysregulated in both human and minipig fibrotic liver ECs. To investigate the pro-fibrotic crosstalk between liver ECs and perivascular HSCs, secreted proteins were compared in the ECs of control and fibrotic human livers (C) and MASH minipig livers before and after treatment (D). Heatmap illustrating the upregulation and downregulation of secreted proteins in indicated groups.

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Each row represents a gene labeled with its symbol followed by three functional pathway annotations derived from the MSigDB database. TDI01 treatment reversed the downregulation of regeneration-associated factors and the upregulation of fibrosis-associated factors commonly identified in both human and minipig fibrotic liver ECs. Among these DEGs, galectin-3/LGALS3 was found to be top gene that was upregulated in human and pig fibrotic liver ECs and suppressed by TDI01 treatment.

(E) Volcano plot illustrates the expression of genes corresponding to secreted proteins in the liver ECs of MASH minipigs after TDI01 treatment vs. before. Blue dots represent downregulated DEGs, and red dots indicate those upregulated ones. The selection criteria are an adjusted $p < 0.05$ and an absolute $\log_2$ fold change > 1.5 .

(F) Violin plot displaying the change of *LGALS3* expression in the liver ECs of indicated human groups (left) and MASH minipigs before and after treatment (right). $n = 12$ in the human control group, $n = 21$ in the human fibrosis group, and $n = 6$ in minipig groups.

(G) Strategy to define the relationship between ROCK2 inhibitor TDI01 and *LGALS3*/galectin-3 in human livers. Normal and fibrotic hPCLSs were obtained from patients and treated with 3 μ M TDI01 with or without 50 ng/mL galectin-3 addition.

(H) Representative Sirius red staining illustrating collagen deposition in normal hPCLSs following treatment with vehicle (control) or galectin-3. Quantitative analysis is shown on the right ($n = 5$). Statistical evaluation was performed using an unpaired two-tailed Student's t test; significance is denoted as *** $p < 0.001$.

(I and J) Western blot analysis of normal hPCLSs treated with galectin-3 demonstrated upregulation of collagen I, α -smooth muscle actin (α -SMA), desmin, and transforming growth factor β 1 (TGF- β 1) (I). Optical density was quantified using ImageJ ($n = 3$) (J). Statistical significance was assessed by unpaired two-tailed Student's t test; *** $p < 0.001$.

(K) ELISA quantification of pro-fibrotic and inflammatory cytokines in the culture supernatant of normal hPCLSs treated with vehicle (Ctrl) or galectin-3 ($n = 5$). Statistical significance was assessed by unpaired two-tailed Student's t test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(L) Representative Sirius red staining illustrating collagen deposition in fibrotic hPCLSs following indicated treatments. Quantitative analysis is shown on the right. Statistical evaluation was performed using one-way ANOVA ($n = 5$); significance is indicated as *** $p < 0.001$; ns, not significant.

(M and N) Western blot analysis of fibrotic PCLSs with indicated treatments. Protein levels of collagen I, α -SMA, desmin, and TGF- β 1 were analyzed, and representative image is shown (M). Densitometric quantification was performed using ImageJ and shown in (N) ($n = 5$). Statistical significance was determined by one-way ANOVA; *** $p < 0.001$; ns, not significant.

(O) ELISA of culture supernatants from fibrotic hPCLSs treated with vehicle (Ctrl), galectin-3, TDI01, or the combination of TDI01 and galectin-3 ($n = 5$). Statistical significance was evaluated using one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ns, not significant.

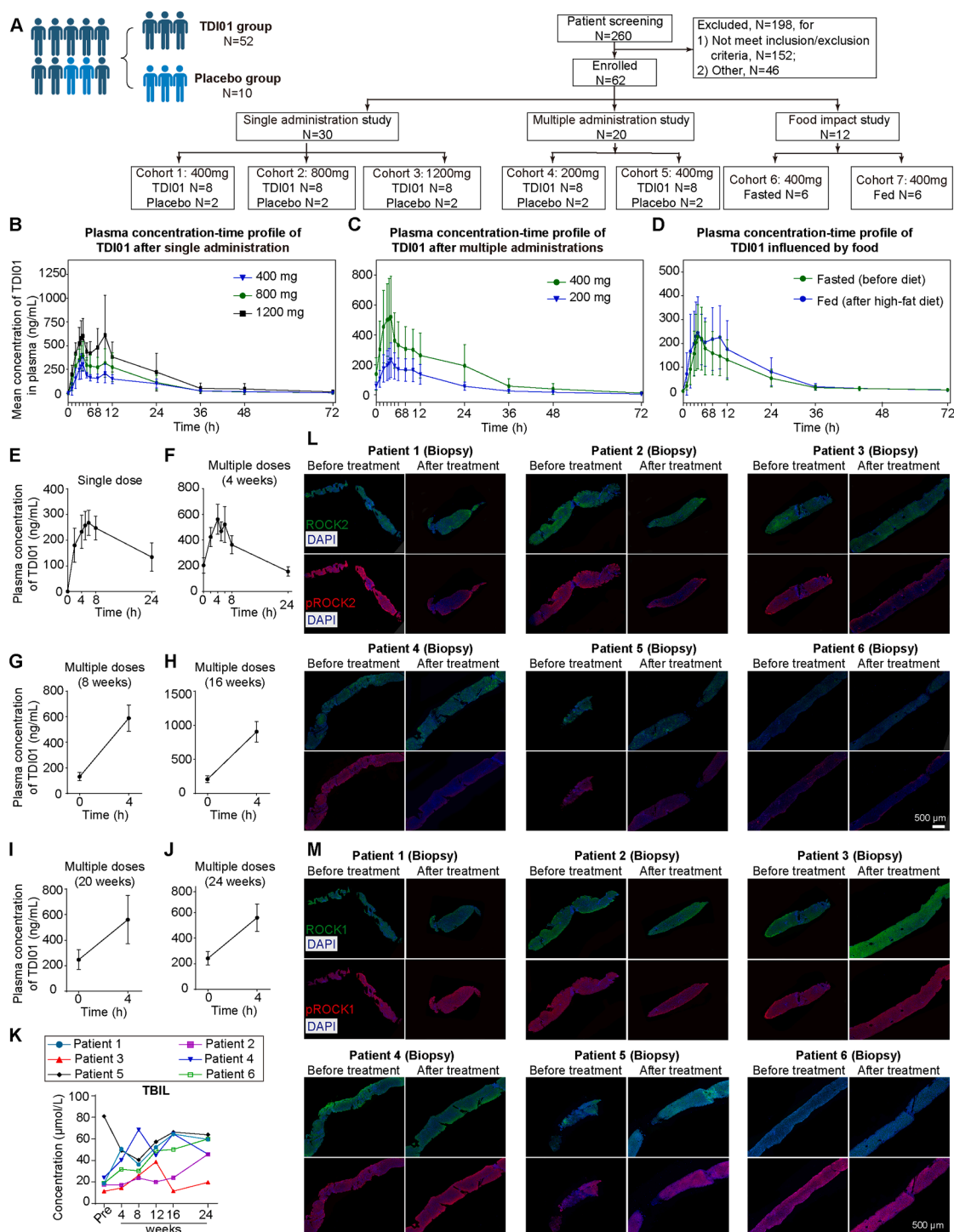


Figure S7. Phase 1 and extended clinical studies of TDI01 in healthy volunteers and patients with liver fibrosis, related to Figures 6 and 7
(A) Schematic outline of the phase 1 trial illustrating the placebo ($n = 10$) and TDI01 treatment ($n = 52$) groups (left). Consolidated Standards of Reporting Trials (CONSORT) flow diagram is shown. Trial number, ChiCTR2200058868. Protocols are provided in [Data S1](#). Participant information is provided in [Table S1](#).
(B–D) PK was evaluated across cohorts, including single oral doses of 400, 800, and 1,200 mg (B), multiple administrations at doses of 200 and 400 mg (C), as well as fasting and postprandial conditions following administration of 200 and 400 mg of TDI01 (D). For multiple-administration, individuals received once-daily dosing for 7 days. T_{max} , time to maximum concentration; $t_{1/2}$, half-lives; C_{max} , maximum concentration; AUC_{0-t} , area under the curve from time zero to the last measurable concentration.

(legend continued on next page)

(E) PK profiles of TDI01 in the plasma after indicated single oral dose of 200 mg in all the enrolled 6 patients in extended clinical studies. Related PK parameters are listed in [Table S3](#).

(F) PK profiles of TDI01 in the plasma after indicated multiple oral administrations after 4 weeks of 200 mg in the 6 patients. Related PK parameters are listed in [Table S3](#).

(G–J) PK profiles of TDI01 in the plasma at 0 and 4 h post administration, following indicated 8 (G), 16 (H), 20 (I), 24 (J) weeks of 200 mg treatment in patients 1–6.

(K) Longitudinal changes in the serum levels of TBIL were assessed in patients before treatment (pretreatment) and 4, 8, 16, and 24 weeks post treatment. One-way repeated measures ANOVA was applied for statistical analysis of the significant interaction between TDI01 treatment and time.

(L) TDI01 inhibits the ROCK2 phosphorylation without affecting its expression levels in patients. Multicolor immunohistochemistry was performed to assess the quantity of ROCK2 and pROCK2 in the liver biopsy specimens obtained from three patients before and after TDI01 treatment. The quantification is shown in [Figure 7E](#). Paired Student's *t* test (two-tailed) was conducted for statistical evaluation. *n* = 6 patients; **p* < 0.05; ns (no significant difference); *p* > 0.05.

(M) TDI01 treatment did not influence the expression and phosphorylation of ROCK1 in tested biopsy samples. Multicolor immunohistochemistry was conducted to compare the amounts of ROCK1 and pROCK1 in indicated liver biopsy specimens, the quantification is shown in [Figure 7F](#). Paired Student's *t* test (two-tailed) was conducted. *n* = 6; ns (no significant difference); *p* > 0.05.