Supporting Materials and Methods

Alcohol Dehydrogenase III Exacerbates Liver Fibrosis by Enhancing Stellate Cell Activation and Suppressing Natural Killer Cells in Mice

Hyon-Seung Yi,[1] Young-Sun Lee,[1] Jin-Seok Byun,[2] Wonhyo Seo,[1] Jong-Min Jeong,[1] Ogyi Park,[3] Gregg Duester,[4] Takeshi Haseba,[5] Sun Chang Kim,[6,7] Keun-Gyu Park,[8] Bin Gao,[3] and Won-Il Jeong[1,6]

Materials. CCl_4, OptiPrep, 4-MP and type I Collagenase were purchased from Sigma-Aldrich (St. Louis, MO). Percoll gradient and DNase I were obtained from GE Healthcare (Buckinghamshire, UK) and Roche (Indianapolis, IN), respectively.

Staining. Five-μm sections of paraffin embedded tissue blocks were stained with 0.1% Sirius Red (Sigma-Aldrich) to examine collagen deposition. For measuring of collagen deposition, 6 areas of each section were taken and analyzed using an Olympus BX51 microscope equipped with a CCD camera (Tokyo, Japan) and computer-assisted image analysis with DP2-BSW (Olympus). Immunohistochemistry was performed with anti-α-SMA antibody (Dako, Glostrup, Denmark). The antigen-antibody complexes were visualized with avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories, Burlingame, CA) and DAB (Invitrogen, Eugene, OR). To identify the apoptotic HSCs, double staining was performed with anti-α-SMA antibody (Dako, Glostrup, Denmark) and apoptosis detection kit (Roche). Immunofluorescence staining was performed with anti-α-SMA antibody (Dako, Glostrup, Denmark), anti-α-ADH3 antibody (Abcam, UK) and anti-desmin antibody (Dako, Denmark), then visualized with FITC-conjugated anti-goat IgG secondary antibody (Abcam, UK) or Alexa Fluor®594 conjugated anti-mouse IgG secondary antibody (Invitrogen). Finally, VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) as manufacturer's instructions. All of visual inspection was done using Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a CCD camera and computer-assisted image analysis with DP2-BSW (Olympus) at various magnifications.

Measurement of retinoids with HPLC. Cells were quantified and extracted as described.[1, 2] HPLC measurements were performed using a Hewlett–Packard 1100 HPLC equipped with a

Zorbax Eclipse 5 μm XDB-C18 analytical column (250 X 4.6 mm; Agilent Technologies Inc, Palo Alto, CA). A linear gradient solvent system: 5% acetic acid aqueous solution/MeOH from 55:45 to 35:65 in 40 min; the flow rate was 1 mL/min. Peaks were detected by UV absorption (330 nm for retinol and 360 nm for the others) with a diode array detector. Acitretin was used as internal standard. All standard reagents and solvents were purchased from Sigma. LC-MS measurements were performed for double checking of compounds on micromass/Waters LCT Premier Electrospray Time of Flight mass spectrometer coupled with a Waters HPLC system

Isolation of liver mononuclear cells (MNCs) and NK cells. Liver MNCs were isolated as described previously.[2-4] Mouse livers were pressed through cell strainer with 70um Nylon mesh. The liver cell suspension was suspended in RPMI-1640 medium and centrifugated at 400 RPM during 5 minutes for the elimination of hepatocytes. The supernatant was collected, washed in Phosphate Buffered Saline (PBS), and resuspended in 40% Percoll (Sigma) in RPMI-1640 medium. The cell suspension was centrifugated at 2400 RPM, 4°C for 30 minutes. The supernatant was removed with suction and MNCs were collected, washed in PBS, and resuspended in RPMI-1640 medium. For isolation of hepatic NK cells, CD3-liver MNCs were separated from liver MNCs by negative magnetic cell sorting (Miltenyi Biotec, Auburn, CA). Then NK1.1+CD3- cells were purified by positive magnetic cell sorting by anti NK1.1 monoclonal antibodies. Approximately 93% of the MACS-purified cells were NK1.1+CD3- cells.

Co-culture HSCs with liver NK cells. HSCs were cultured in medium including fetal bovine serum and horse serum on 12-well plates for 3days. On day 4, HSCs were cultured in serum-free medium and then the cells were co-cultured with liver NK cells for 3 hours. Before co-culturing, NK cells were treated with 4-methylpyrazole (4-MP) or distilled water for two hours.

RT-PCR and real-time PCR analyses. PCR analyses were performed and all primers used are listed in Table 1. Total RNA was isolated from liver cells using Trizol Reagent (Invitrogen, Eugene, OR) in accordance with the manufacturer's instructions. The same quantity of total RNA was reverse-transcribed to complementary DNA (dDNA) using amfirevertII cDNA synthesis Master Mix (Gendepot). Real-time PCR was performed using

SYBR Green Realtime PCR Master Mix (Toyobo). The comparative Ct method was used to quantify transcript which were normalized respect to β-actin. Results were analyzed by ΔΔCt method. Values were expressed as fold change in comparing with control.

***Western blotting*.** Liver tissue or cells were homogenized in RIPA buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, phosphotase and protease inhibitors) for extraction of the protein. Western blot analyses were performed with 50-80μg protein from liver or cells homogenates using anti-β-actin antibodies (Sigma-Aldrich), anti-α-SMA (Sigma), anti-TGF-ß1 (Cell signaling, USA), and anti-ADH3 antibodies (abcam, Cambridge, UK). Immunoreactive bands were visualized on nitrocellulose membranes using alkaline-phosphate-linked anti-rabbit antibody and the ECL detection system with a PhosphoImager (GE Healthcare, Piscataway, NJ).

Cytotoxicity Assay. Calcein-AM was purchased from Molecular Probes (Eugene, OR) as a 1 mg/mL solution in dry dimethyl sulfoxide. On day 4, activated hepatic stellate cells were used as target cells and labeled with 15 μM Calcein-AM for 30 minutes at 37°C with occasional shaking. Liver NK cells were isolated and used as effector cells as described previously. The effector cells and target cells (E:T =1:10) were mixed in 96-well plates. After co-culturing for 4 hours at 37°C in 5% CO2, the supernatant (75 μL) was harvested, and Calcein-AM fluorescence was measured using a microplate spectrofluorimeter (excitation filter: 484 ± 9 nm; brand-pass filter: 530 ± 9 nm). Specific lysis was calculated according to the following formula: [(test release – spontaneous release)/(maximum release – spontaneous release)] ×100.

***Fluorescence Activated Cell Sorting (FACS) Analyses*.** Isolated liver MNCs were stained using anti-CD45, anti-NK1.1, anti-Rae1γ, anti-CD3, anti-TRAIL (BD Biosciences), and anti-NKG2D antibodies (eBioscience). For blocking of non-specific antibody reactions, cells were pre-incubated with anti-mouse CD16/32 mouse Fc blocker (BD bioscience) before antibody treatment. The stained cells were analyzed using a BD™ LSR II Flow Cytometer (BD bioscience) and FlowJo software (Tree Star, Ashland, OR). For intracellular staining, PE-conjugated anti-IFN-γ antibody (BD) was used. Intracellular cytokine staining was performed after re-stimulation of cells with phorbol-myristate acetate/ionomycin/brefeldin A for 5 hours

using BD Cytofix/Cytoperm (BD Biosciences).

Small Interfering Ribonucleic Acid (siRNA) Targeting ADH3 and Transfection of HSCs or NK cells. Mouse ADH3 siRNA were designed and synthesized by a commercial company using the mouse nucleotide sequence for ADH3 mRNA from GenBank. The siRNAs did not cross-react with any other genes. The mouse ADH3 siRNA target sequences were as follows: 5'-AAUUCUUUGGCCUUUGCGAUU-3' (sense) and 3'-UCGCAAAGGCCAAAGAAUUUU-3' (antisense); 5'-CUGGAAUUGUGGAAAGUGUUU-3' (sense) and 5'-ACACUUUCCACAAUUCCAGUU-3' (antisense). HSCs were seeded into 12-well plates at a density of 7×10^4 cells/well. On Day 1, the cells were transfected with ADH3 siRNA or negative scramble siRNA using Lipofectamine™ RNAiMAX (Invitrogen) at various siRNA concentrations. The transfected cells were incubated in medium containing 10% fetal bovine serum, and then the cells were collected at the designated time points.

Table 1. Primers used in RT-PCR and Real-time PCR (Mouse)

Genes	Forward (5'-3')	Reverse (5'-3')	PCR product (base pairs)
ADH1	GGTCTGTCTGTCATCATTGG	TCTTTCCAGAACGAAGCAGGTC	489
ADH2	GAGCCCTCTCACAAACCTCTGTGG	CTACACACCCCAGGCCAAAGACAG	310
ADH3	GTGGGAGTAGCTGCTTCAGG	GTGCATCAGATCAAAGGCTTG	215
RDH1	CAACAGGTTCACATCCAGTACTC	GCAGCTGTGTAAACCATGTGGC	431
CRAD1	TGGGCTAAGTACAGACTT	CCTCCATCTTTCTTCTCA	278
CRAD2	CACAGAATATCTCTCTCTGC	GAGTCTCAAGAACCACATGC	1058
CRAD3	CTCCTCTTCTCTCAGTAG	TATACCCTGCAGCATGAC	455
α-SMA	CTGACAGAGGCACCACTGAA	GAAGGAATAGCCACGCTCAG	287
COL1A1	TCCTCCAGGGATCCAACGA	GGCAGGCGGGAGGTCTT	148
TGF-β1	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	182
PDGFRβ	ACGTACCCTACGACCACCAG	TCCATTGGAAGTTCACCACA	234
IL-6	TCCATCCAGTTGCCTTCTTG	TTCCACGATTTCCCAGAGAAC	166
MCP-1	TCAGCCAGATGCAGTTAACGC	TCTGGACCCATTCCTTCTTGG	184
Rae1α	AGGTTGTGAGCTGCTCGATT	GGGGTAGGATCCTTGATGGT	272
Rae1γ	ATTTGCATTTGCGATGTGAA	GCTGTATTGCCTGGCATTTT	378
RARα	AAGAGGGACTGCGTGAAGG	GAAGAAGGCGTAAGGGGGTA	155
RARβ	ACAAGTCATCGGGCTACCAC	CTGTGCATTCCTGCTTTGAA	248
RARγ	AGGTCACCAGAAATCGATGC	CTGGCAGAGTGAGGGAAAAG	211
RXRα	ATCGACACCTTCCTCATGGA	AGTAAAGATGGCGAGGATGG	204
RXRβ	CCCATTGACACCTTCCTCAT	GACTTGGAGACCAAGCGAAC	199
Granzyme	TCGACCCTACATGGCCTTAC	TGGGGAATGCATTTTACCAT	197
NKG2D	GCATTGATTCGTGATCGAAA	GCCACAGTAGCCCTCTCTTG	374
IFN-γ	AGACATCTCCTCCCATCAGCAG	TAGCCAAGACTGTGATTGCGG	158
TRAIL	CCCTGCTTGCAGGTTAAGAG	GGCCTAAGGTCTTTCCATCC	219
Perforin	GATGTGAACCCTAGGCCAGA	GGTTTTTGTACCAGGCGAGA	162
ACSL1	ACCAGCCCTATGAGTGGATTT	CAAGGCTTGAACCCCTTCTG	91
Catalase	CCCCTATTGCCGTTCGATTCT	TTCAGGTGAGTCTGTGGGTTT	211
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT	148

Table 2. Primers used in RT-PCR and Real-time PCR (Human)

Genes	Forward (5'-3')	Reverse (5'-3')	PCR product (base pairs)
ADH1a	AGTCATCCCACTCGCTATTCC	GTCCCCTGAGGATTGCTTACA	102
ADH1b	CCCGGAGAGCAACTACTGC	AACCAGTCGAGAATCCACAGC	224
ADH1c	CTCGCCCCTGGAGAAAGTC	GGCCCCCAACTCTTTAGCC	223
ADH3	TGCCAGAAGATAAGAGTCACTCA	GCTGGTTCCCATGTAATGCAAA	102
β-actin	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT	285

Supporting Figure Legends

Supporting Fig. 1. 4-MP treatment inhibits activation of HSCs. WT hepatocyte (Hep), WT HSCs, ADH1 knock-out ($ADH1^{-/-}$) HSCs and human HSC cell lines (LX-2 and hTERT) were isolated and cultured for various time points with or without 4-MP treatment. (A) Real-time PCR analyses of human HSC cell line LX-2 and hTERT. (B) Real-time PCR analyses of mouse HSCs and human hTERT after atRA (1μM) treatment for 24 hour. (C) Western blot analyses of freshly isolated and 4-day cultured HSCs (D4 HSCs) with or without 4-MP treatment. (D) Supernatant levels of IL-6 and MCP-1 in cultured WT HSCs. (E) Real-time PCR analysis of fatty acyl CoA synthetase in mouse HSCs with or without 4-MP treatment. (F) Cultured $ADH3^{-/-}$ HSCs were treated with 4-MP and then HSCs were subjected to real-time PCR analyses. (G,H) RT- and real-time PCR analyses of cultured $ADH1^{-/-}$ HSCs with or without 4-MP treatment. (I) Apoptotic HSCs were measured by TUNEL staining (original magnification, ×200). Data are representative of three independent experiments and are expressed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$ in comparison with the corresponding controls.

Supporting Fig. 2. 4-MP- or siRNA-mediated inhibition of ADH3 in NK cells suppresses IFN-γ production of NK cells and expression of COL1A1 and MCP-1 in HSCs. (A) Liver NK cells were isolated and then subjected to RT-PCR analyses. (B) NK cells were treated with retinol for 3 hours in the presence or absence of 4-MP and then the supernatants were subjected to IFN-γ measurement. (C) Cultured 4-day (D4) HSCs were co-cultured with NK cells treated with ADH3 siRNA or scramble siRNA for 3 hours and then they were subjected to real-time PCR analyses. Data are representative of three independent experiments and are expressed as the mean ± SEM. $*P < 0.05$ in comparison with the corresponding controls.

Supporting Fig. 3. Ablation of the ADH3 gene impedes CCl_4- and BDL-induced liver fibrosis. Liver fibrosis was induced by CCl_4 injection or BDL for 2 weeks in WT and $ADH3^{-/-}$ mice. (A) Normal liver tissues of WT and $ADH3^{-/-}$ mice were stained with Sirius Red and α-SMA antibody (original magnification, ´ 100). (B) Whole liver tissues of mice with CCl_4 treatment were subjected to Western blotting. (C) Isolated HSCs of mice with CCl_4 treatment were

subjected to real-time PCR analyses. (D) Serum levels of ALT, AST, total bilirubin and IFN-γ of mice with BDL. (E) Liver sections of mice with BDL were stained with Sirius red and α-SMA antibody (original magnification, ´ 100). (F, G) Whole liver tissues of mice with BDL were subjected to Western blotting and real-time PCR analyses respectively. (H) Isolated HSCs of mice with BDL were subjected to real-time PCR analyses. Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ in comparison with the corresponding controls.

Supporting Fig. 4. The analyses of liver MNCs using flow cytometry after reciprocal bone marrow transplantation between WT and ADH3-deficient mice. (A) The number of liver MNCs per gram of liver weight after transplantation. (B) Population of liver MNCs after transplantation. Data are expressed as the mean ± SEM.

References

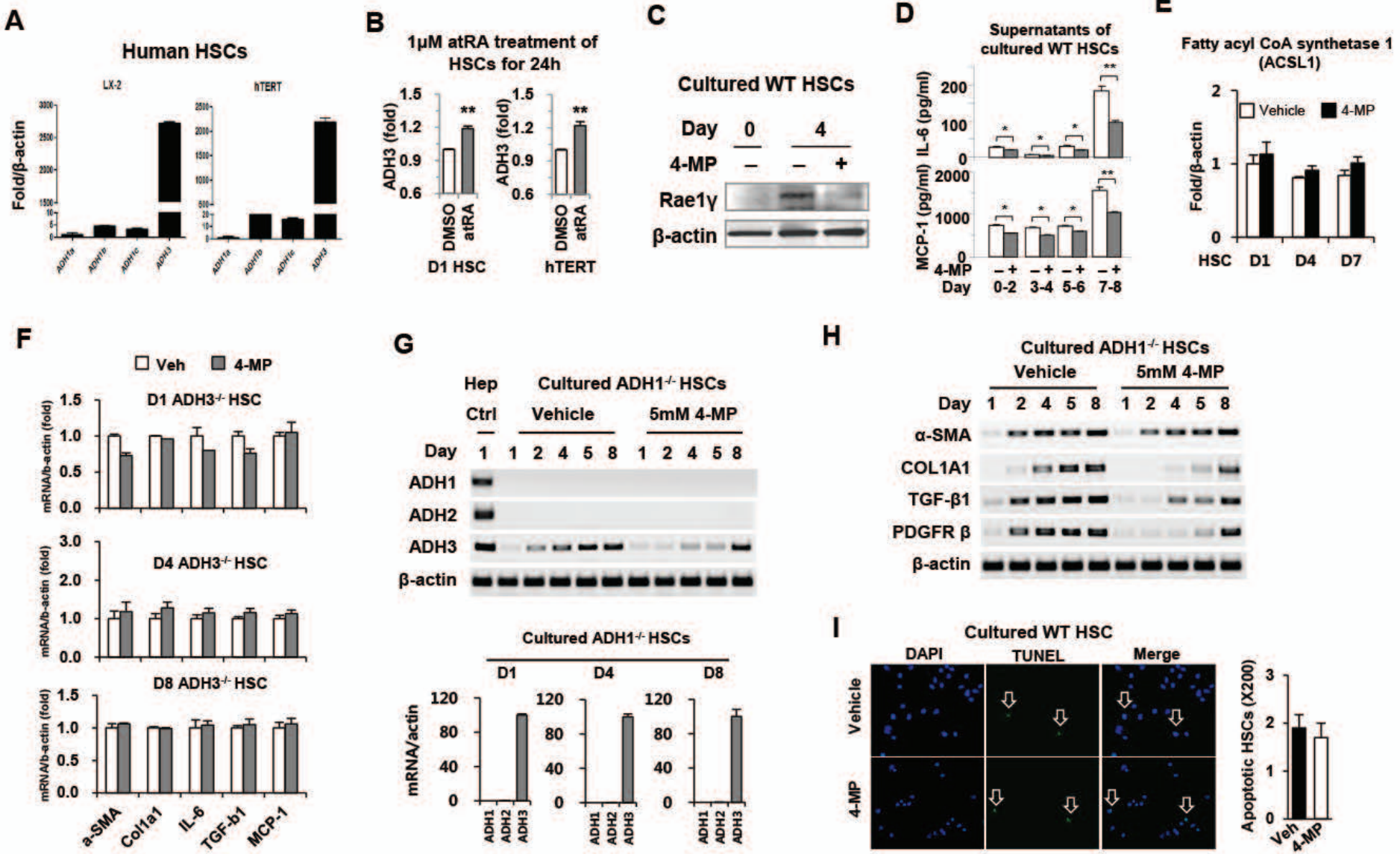
1. Radaeva S, Wang L, Radaev S, Jeong WI, Park O, Gao B. Retinoic acid signaling sensitizes hepatic stellate cells to NK cell killing via upregulation of NK cell activating ligand RAE1. Am J Physiol Gastrointest Liver Physiol 2007;293:G809-816.

2. Jeong WI, Park O, Suh YG, Byun JS, Park SY, Choi E, Kim JK, et al. Suppression of innate immunity (natural killer cell/interferon-gamma) in the advanced stages of liver fibrosis in mice. Hepatology 2011;53:1342-1351.

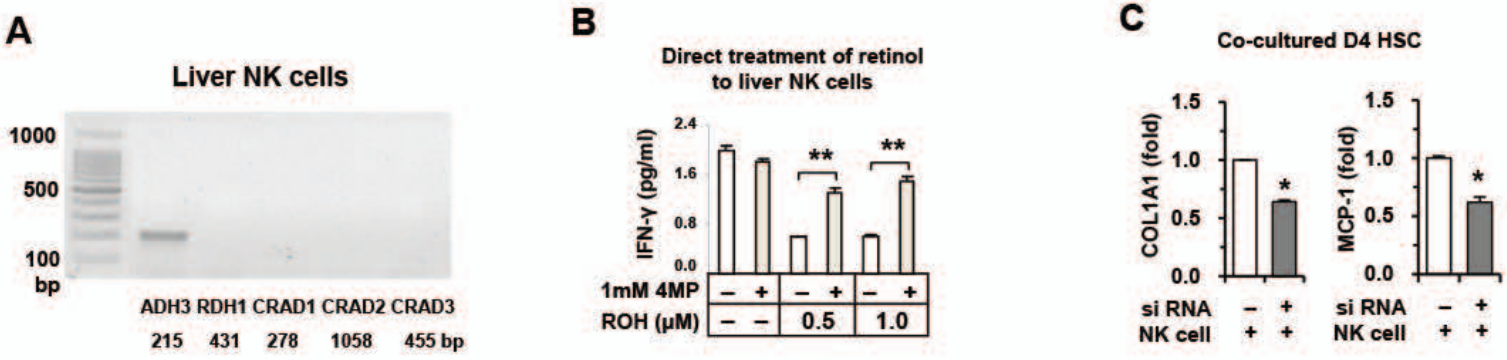
3. Radaeva S, Sun R, Jaruga B, Nguyen VT, Tian Z, Gao B. Natural killer cells ameliorate liver fibrosis by killing activated stellate cells in NKG2D-dependent and tumor necrosis factor-related apoptosis-inducing ligand-dependent manners. Gastroenterology 2006;130:435-452.

4. Jeong WI, Park O, Radaeva S, Gao B. STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity. Hepatology 2006;44:1441-1451.

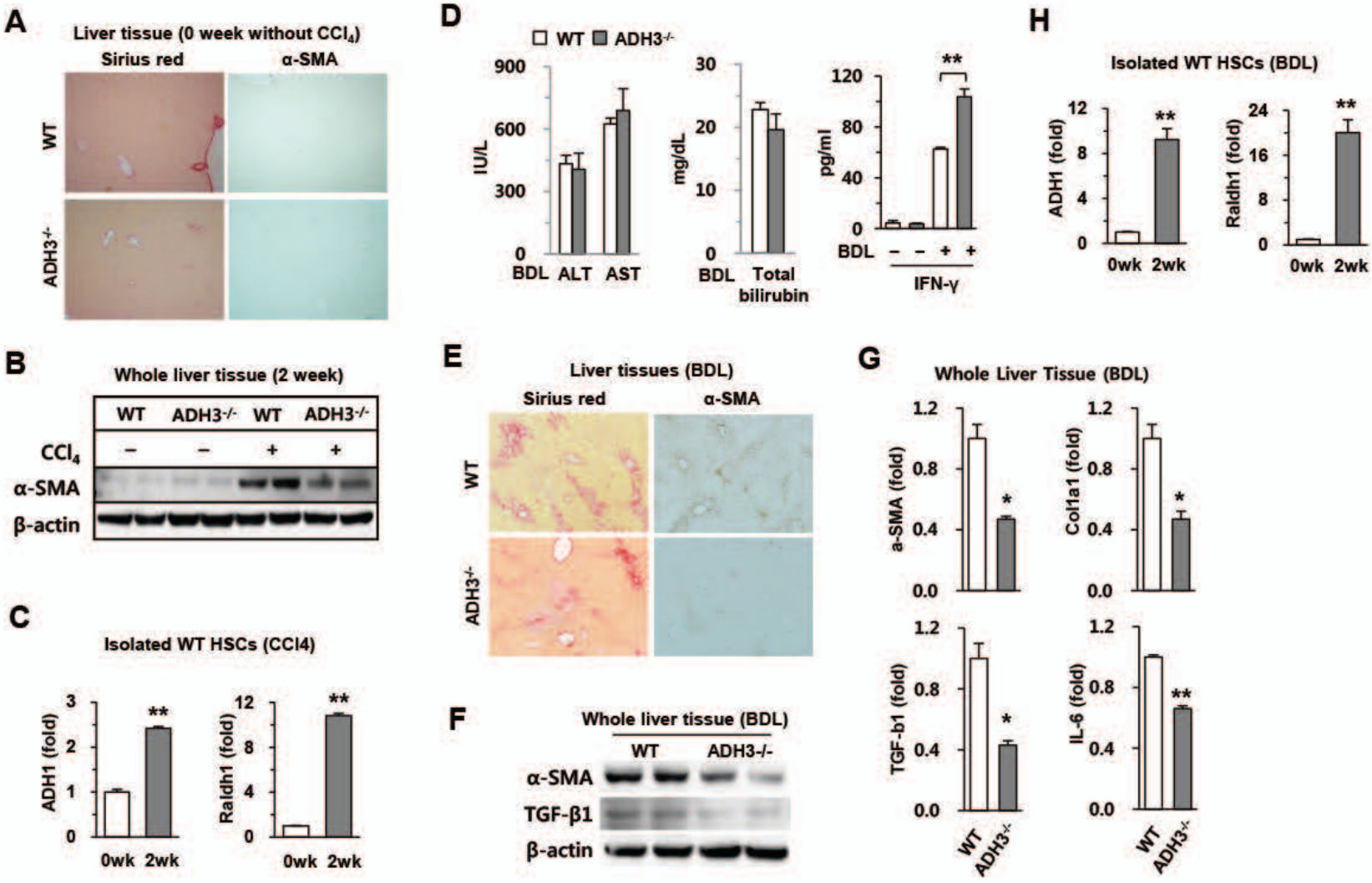
Supporting Fig. 1.



Supporting Fig. 2.



Supporting Fig. 3.



Supporting Fig. 4.