Supplemental Materials

Systemic TRAIL treatment ameliorates liver cirrhosis in rats by eliminating activated hepatic stellate cells

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MATERIAL AND METHODS:

Rat blood analysis

Blood was collected by cardiac puncture, placed at room temperature for 2 h and centrifuged at 3000 rpm for 20 min. Routine liver function tests analyzed in serum included glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin, alkaline phosphatase (ALP), total bilirubin, and direct bilirubin.

Cell culture and $TRAIL_{PEG}$ treatment

Human primary hepatic stellate cells (hHSCs) and stellate cell medium (SteCM) were obtained from ScienCell Research Laboratories (Carlsbad, CA). hHSCs were cultured in SteCM medium supplemented with 2% of FBS, 1% of stellate cell growth supplement, and 1% of

penicillin/streptomycin solution in poly-L-Lysine coated plates following the instructions by the company. LX-2 cells were maintained in DMEM (Sigma-Aldrich, Logan, UT) supplemented with 10% of FBS and 1% of penicillin/streptomycin solution. hHSCs were then sub-cultured into 6-well plastic culture plates for 2, 4, 7 and 10 days and harvested for analysis. The expression levels of TRAIL receptors (DR4, DR5, DcR1, and DcR2) and activation markers (α-SMA, TGF-β, collagen-1, PDGF-R, TIMPs and MMPs) in cultured stellate cells were determined by Western blot and quantitative real time PCR (qPCR) analyses. Cells on day 2 (qHSCs) and day 7 (aHSCs) were treated with or without 1 μg/mL of $TRAIL_{PEG}$ for 3 h and collected for analyses. For the death-inducing signaling complex **(**DISC) immunoprecipitation (DISC-IP) assay, qHSCs and aHSCs were treated with or without 1 μg/mL of Flag-TRAIL for 30 minutes or 2 μg/mL $TRAIL_{PEG}$ for 60 minutes followed by analysis. Additional methods under "Death-inducing signaling complex-immunoprecipitation" below. To examine TRAIL-induced apoptosis pathways, cells were pre-treated with Nec-1 (Necrostatin-1, 50 μM, Sigma) and zVAD-fmk or IETD-fmk (20 μM, MP Biomedicals, Solon, OH) for 1 h followed by $TRAIL_{PEG}$ treatment for an additional 3 h. Apoptotic cells were photographed under the Nikon Eclipse TS100 inverted microscope (Nikon Instruments Inc. Melville, NY) and cell viability was determined by MTT assays.

Pharmacokinetic (PK) analysis of His-iLZ-TRAIL and $TRAIL_{PEG}$

The PK of proteins were measured in cynomolgus monkeys. Male cynomolgus monkeys (4-5 kg, Korea Research Institute of Chemical Technology (KRICT, Daejeon, Korea) were fasted for 12 h before drug administration. His-iLZ-TRAIL and $TRAIL_{PEG}$ (12.5 μg/kg, protein-based) were i.v. administered, and blood samples (450 μL) were collected from the vein and mixed with 50 μL of sodium citrate (3.8% solution) followed by centrifugation at 2,500g for 15 min at 4°C. The

plasma samples were separated and stored at -70°C. Animal studies were carried out in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals and approved by KRICT. The concentration of TRAIL was determined by Human TRAIL/TNFSF10 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) and analyzed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) based on a four-parameter logistic standard curve derived from His-iLZ-TRAIL and $TRAL_{PEG}$, respectively. PK parameters were obtained by non-compartmental analysis from WinNonlin (Pharsight Corporation, Mountain View, CA) and summarized in Supplementary Table 1.

Human primary hepatocyte culture and $TRAIL_{PEG}$ treatment

Cryopreserved human primary hepatocytes, human hepatocyte plating medium, and thawing medium were obtained from TRL (Triangle Research Labs, LLC, Durham, NC). According to the manufacturer's instructions, cryopreserved hepatocytes were thawed in thawing medium and cultured in human hepatocyte plating medium in a 6-well plate of collagen type I Biocoat (BD Biosciences, San Jose, CA). Cells were cultured overnight and then treated with $TRAIL_{PEG}$ or recombinant human His-iLZ-TRAIL for 3 h. After cells were harvested, the expression of TRAIL receptors (DR4/DR5) and apoptosis markers were determined by Western blot analysis, and cell viability was analyzed by MTT assays. To induce steatosis, human primary hepatocytes were incubated with FFA (200 μM, Oleate: Palmitate=2:1) for 18 h. Then, $TRAIL_{PEG}$ or His-iLZ-TRAIL were treated for an additional 3 h and western blotting was performed to analyze the TRAIL-induced apoptotic markers.

Comparative quantitative real time PCR (qPCR)

Total RNA from cultured cells and rat liver tissues was extracted with TRIzol reagent (Life Technologies, Grand Island, NY) following the instruction provided by the company. RNA

concentration was measured spectrophotometrically using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). 1-2 µg of total RNA were reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription System (Life Technologies). Comparative quantitative RT-PCR (qPCR) was performed in duplicate or triplicate for each sample using fast SYBR Green Master Mix (Life Technologies) and StepOnePlus Real-Time PCR System (Life Technologies). The expression levels of target genes were normalized to the expression of GAPDH and calculated based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$). qPCR for rat liver samples was performed using RT2 qPCR Primer set (Qiagen, Valencia, CA); Col1a2 (PPR56530A), Acta2 (PPR59337B), Mmp-3 (PPR48487B), Col3a1 (PPR43017A), Mmp-13 (PPR45162A), Timp-1 (PPR48051C), Timp-3 (PPR06533A), Gapdh (PPR06557B), Tgf-b1 (PPR06430B), and Bmp7 (PPR46571A). Additional name and sequences of primers and primers for humans and rats are summarized in Supplementary Table 2.

Western blot analysis

Anti-DR4 (Abcam, Cambridge, MA, #13890 or Santa Cruz Biotechnology, Santa Cruz, CA, H-130), anti-DR5 (Abcam, #16329 for Rat, #47179 for Human), anti-Caspase-8 (Cell Signaling Technology, Danvers, MA, #9746), anti-cleaved PARP-1 (Cell Signaling Technology, #5625), anti-cleaved Caspase-3 (Cell Signaling Technology, #9664), anti-cleaved Caspase-9 (Cell Signaling Technology, #7237), anti-BCL-2 (Cell Signaling Technology, #2870), anti-BCL-XL (Cell Signaling Technology, #4668), anti-alpha SMA (Sigma, A2547), anti-FLIP (Cell Signaling Technology, #8510), anti-RIP1 (Cell Signaling Technology, #3493), anti-MMP-2 (Santa Cruz Biotechnology, sc-10736), anti-Collagen 1 (Abcam, #90395), anti-p-BCL2 (Cell Signaling Technology, #2875), anti-TGF-β (Abcam, #66043), anti-TIMP-1 (Millipore, Billerica, MA, MBA3300), anti-PDGFR-β (Santa Cruz Biotechnology, sc-432), anti-GAPDH (Santa Cruz

Biotechnology, sc-1694), and anti-β-actin (Santa Cruz Biotechnology, sc-47778) were used in western blot analysis. In general, cells were lysed by sonication in ice-cold PBS buffer containing protease inhibitor cocktail (1 mM PMSF and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A). Cell lysates were clarified by centrifugation and the supernatants were measured for protein concentration by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Liver tissues from fibrosis and cirrhosis animal models were grounded in liquid nitrogen (LN_2) with a mortar and pestle. Grounded liver tissues were suspended in lysis buffer and sonicated briefly in ice-cold PBS buffer containing protease inhibitor cocktail (1 mM PMSF and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A). Extracts were then clarified by centrifugation, and the supernatants were measured for protein concentration by Bradford assay. The samples were subjected to electrophoresis through SDS-polyacrylamide gels and transferred to nitrocellulose membrane. After blocking the membrane with 3% bovine serum albumin (BSA, Sigma), the membranes were incubated with the primary antibodies overnight. GAPDH or β-actin was used for protein loading control. Protein bands were detected using chemiluminescence detection system onto X-ray film quantified by Multi Gauge software (Fujifilm).

Knockdown experiment

DR4 (Santa Cruz Biotechnology, Santa Cruz, CA, sc-35218), DR5 (sc-40237) or control siRNA (sc-37007) were transfected into LX-2 cells for 48h using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. $TRAIL_{PEG}$ was treated for 8 h and apoptotic markers were analyzed with western blotting following methods above.

Death-inducing signaling complex-immunoprecipitation

Primary human HSCs were cultured to achieve 80% confluence for 2 and 7 days, treated with 2 μg/mL $TRAIL_{PEG}$ at 37°C for 60 min, and then lysed with DISC IP lysis buffer (30 mM Tris, pH

7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, with 1 mM PMSF, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin A). Cell lysates were incubated with DR4 and DR5 antibodies (Abcam) overnight. Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, sc-2003) were incubated for an additional three hours and subsequently washed three times with DISC IP lysis buffer, resolved on SDS-PAGE gels and subjected to western blot analysis.

Liver histology and immunohistochemistry of liver fibrosis

Three lobes of liver tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut at 4 mm thickness. The sections were then stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC) staining, anti-α-SMA (DakoCytomation, Carpinteria, CA) antibody was used to detect the α-SMA^+ aHSC. Histostain-Plus Kit (Life Technologies) was used for all procedures of IHC. Briefly, liver sections were deparaffinized, hydrated, quenched in 3% hydrogen peroxide solution for 20 min and washed. Slides were treated with blocking solution and sequentially applied to primary antibody for 1 h. After washing the liver sections three times with PBS, samples were applied with biotinylated secondary antibody, washed, and treated with enzyme conjugated reagent. Liver sections were washed three times with PBS and developed by 3, 3' Diaminobenzidine (DAB) as the chromogen/substrate (Vector Laboratories, Burlingame, CA). To detect collagen deposition, liver sections were stained with Sirius red staining solution (Sigma) and washed in 5% acetic acid water. Stained liver tissues were imaged under a light microscope (Olympus America, Waltham, MA). For quantification of fibrotic area or cirrhotic area, stained liver tissues were imaged randomly collecting 20-30 photos per individual rat, and positively stained tissue was analyzed using Image J software (NIH, Bethesda, MD).

Double Immunofluorescence staining

Paraffin sectioned human cirrhotic livers were used for immunofluorescence staining **(**IF) stain with α-SMA and either DR4 or DR5. Liver tissues were deparaffinized and hydrated. For antigen retrieval, tissues were heated in citrate buffer (Thermo Scientific, Fremont, CA) by microwave and sat on the bench to cool down. Slides were washed three times with PBS and treated with blocking solution (3% of normal horse serum, Vectastain ABC system, Vector Labs, Burlingame, CA) for 30 min at room temperature. The liver tissues were then applied to primary antibodies, rabbit anti-DR4 antibody (Santa Cruz Biotechnology, H-130) or rabbit anti-DR5 antibody (Abcam) with mouse anti-α-SMA antibody (Sigma) and incubated overnight at 4°C. Slides were washed three times with PBS and applied to anti-rabbit IgG conjugated with Alexa fluor 488 and anti-mouse IgG conjugated with Alexa fluor 555 (Cell Signaling) for 30 min incubation at room temperature. After washing the liver slides, samples were mounted with Fluorescence Mounting Medium with DAPI (Vector Labs) and captured under the Zeiss fluorescence microscope (Carl Zeiss Microscopy, LLC. Thornwood, NY). In Situ Cell Death Detection Kit, Fluorescein (Roche, Indianapolis, IN) was used to detect apoptotic cells in rat liver tissues. Briefly, deparaffinized and hydrated slides were incubated with proteinase K for 15 min at room temperature, then washed twice with distilled water and incubated with terminal deoxynucleotidyl transferase (TdT) labeling reaction mixture at 37°C for 1 h. Next, cells were treated with TdT stop buffer to stop the labeling reaction and washed twice with distilled water. The slides were added to Step-Fluor solution, incubated at room temperature for 20 min and washed twice with PBS. Slides were then added to 1:500 diluted anti-α-SMA antibody (Sigma), incubated at room temperature for 1h and washed three times with PBS. Samples were then treated with anti-mouse-IgG Alexa Fluor 555 antibody for 30 min at room temperature and washed three times with PBS under protection from light. Samples were mounted with Fluorescence Mounting Medium with DAPI

and captured under the Zeiss fluorescence microscope using a 495 nm filter for TUNEL and a 555 nm filter for α-SMA.

Colocalization analysis

Data were based on collected liver tissues from CCl_4/PBS and CCl_4/$TRAIL_{PEG}$ treated rats that were fluorescently stained with TUNEL (green) and α-SMA (red). Co-localization image analysis was performed using Image J plug-in JACoP.[1] The average of Pearson's, M1 and M2 coefficient values were analyzed for statistical significance by non-parametric t-test.

Cell viability by MTT assay

Cultured cells were treated with $TRAIL_{PEG}$ or His-iLZ-TRAIL for 3h. After incubation, 5 μg/mL of MTT solution was added to each well and incubated for 1 h. After removal of the medium, 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a microplate reader (Bio-Tek Instruments Inc, Winooski, VT). Quadruplicate wells were assayed for each condition.

Measurement of hepatic hydroxyproline content

Hydroxyproline assay was measured by a Hydroxyproline Assay Kit (Sigma, MAK008-1KT) according to the manufacturer's instructions. Briefly, liver tissues were homogenized and incubated for 3 h at 120°C in 12 N HCl. After hydrolysis, samples were incubated with 100 μL of the Chloramine T/Oxidation buffer mixture for 5 min, and then each sample was incubated with 100 μL of the diluted dimethyl aminobenaldehyde (DMAB) for 90 min at 60°C. The samples were measured at 560 nm absorbance using a microplate reader (Bio-Tek Instruments Inc).

Fluorescence-activated cell sorting (FACS) analysis of DR4 and DR5 expression

Primary human HSCs were cultured for 2 and 7 days, cells were harvested, washed twice with cold PBS and incubated for 30 min with APC-conjugated anti-human DR4 (#307207) or PE-conjugated anti-human DR5(#307405) antibody (BioLegend, San Diego, CA). APC or PE-conjugated isotype IgG1 antibody (#400119 or #400111, BioLegend) was used as an isotype control. Cell surface expression of TRAIL receptors were analyzed by flow cytometry with FACSCalibur (BD Biosciences, San Jose, CA). Histographical and mean fluorescence intensity (MFI) data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Reference

1. S. Bolte, F.P. Cordelières. A guided tour into subcellular cololcaization analysis in light microscopy. Journal of Microscopy 2006; 224(3): 213-232.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Western blot analysis of healthy and steatotic human primary hepatocytes induced by free fatty acid (FFA) treated with various concentrations (ug/mL) of $TRAIL_{PEG}$ and His-iLZ-TRAIL. Cl., Cleaved. Casp, Caspase.

Figure S2. TRAIL receptors, DR5 and DR4, are upregulated in human cirrhotic livers. Immunohistochemical analyses of DR5 and DR4 in human liver tissues. P.A., parenchymal area; F.S., fibrotic septa; HBV, hepatitis B virus; HCV, hepatitis C virus; ALC, alcoholic liver cirrhosis. Original magnification 100x. Red arrows indicate TRAIL receptor positive cells in P.A.

Figure S3. Serum alanine transaminase (ALT) (a) and aspartate transaminase (AST) (b) levels from CCl_4-induced liver fibrosis models (Figure 2A).

Figure S4. Biomarker analysis in CCl_4-induced liver fibrosis models. Band intensities from Figure 3B were quantified by densitometry and normalized to GAPDH. Data expressed as mean ± s.e.m. #$P < 0.05$ vs. olive oil + PBS groups. *$P < 0.05$, **$P < 0.01$ vs. CCl_4 + PBS groups.

Figure S5. Pearson's colocalization image analysis of fluorescently stained liver tissues (Fig. 3C) from CCl_4/PBS and CCl_4/$TRAIL_{PEG}$ treated rats. Liver tissues were stained with TUNEL (apoptosis, green) and α-SMA (aHSCs, red). Co-localization image analysis was performed using Image J plug-in JACoP[1] and the resulting Pearson's, M1 and M2 coefficients are shown. The average of coefficient values were analyzed for statistical significance by non-parametric t-test. ***$P < 0.001$.

Figure S6. Additional biomarker analysis in CCl_4-induced liver cirrhosis models. a) Digital image quantification of α-SMA or collagen staining (n = 4-6 rats per group). b) Serum albumin, direct bilirubin and alkaline phosphatase (Alk. Phos.) levels. c) Gene expression levels of Acat2

(a-SMA) and Bmp7. Data expressed as mean ± s.e.m. ##P < 0.01 vs. olive oil + PBS groups. *P < 0.05 vs. CCl_4 + PBS groups.

Figure S7. Western blot analyses of death receptors and TRAIL signaling molecules in culture-activated LX2 cell lines.

Figure S8. Analysis of culture-activated human primary human HSCs. a) qPCR analyses of decoy receptors (Dcr1 and Dcr2) and Tgfb mRNA during transition from the quiescent (day 2) to the culture-activated phenotype (days 4, 7, 10). b) FACS analyses of functional DR5 and DR4 expression on HSC surface in a quiescent (day 2) and activated (day 7) cell phenotype. MFI, mean fluorescence intensity.

Figure S9. Apoptotic signaling and effects in HSCs during activation. a) Western blot analysis of pro- and anti-apoptotic proteins in HSCs from quiescent (day 2) to culture-activated phenotypes (days 4, 7, 10). b) Anti-apoptotic protein expression in qHSCs (day 2 in culture) and aHSCs (day 7 in culture) treated with or without $TRAIL_{PEG}$. c) Representative photos of HSCs from quiescent (day 2) to culture-activated phenotypes (days 4, 7, 10) treated with $TRAIL_{PEG}$ for 3 h. d) Representative photos of culture-activated HSCs (day 7) treated with cisplatin, doxorubicin, H_2O_2 or incubated without serum for 24 h or incubated with $TRAIL_{PEG}$ for 3 h.

Figure S10. DISC immunoprecipitation (IP) studies. Western blot analysis of qHSCs (q, 2 days) and aHSCs (a, 7 days) treated with Flag-TRAIL (1 µg/mL, for 30 min) and then immunoprecipitated with anti-Flag (M2) antibody (right). Whole cell lysates (WCL) are indicated (left). H.C., heavy chain, L.C. light chain.

Supplementary Table 1. Pharmacokinetic parameters of TRAIL variants after intravenous injections in cynomolgus monkeys (n = 2).

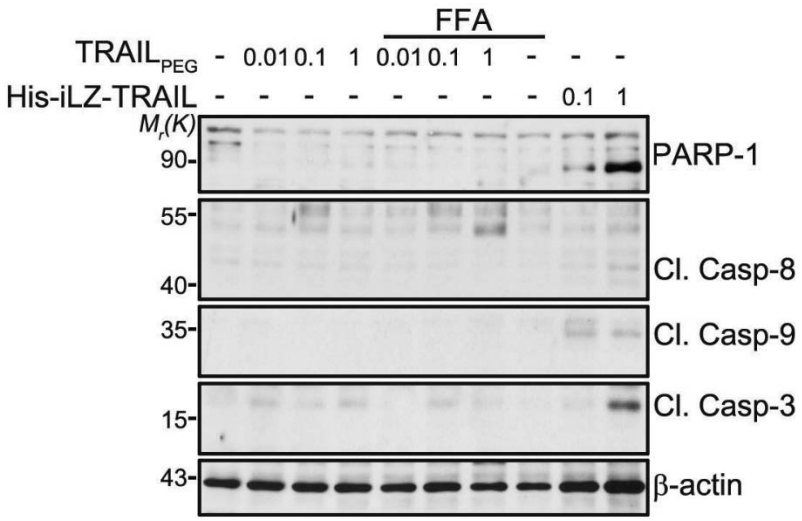
	His-iLZ-TRAIL	**TRAIL$_{PEG}$**
$t_{1/2}$ (h)[a]	0.9 ± 0.1	8.6 ± 1.8
AUC_{inf} (ng·h/mL)[b]	98.5 ± 24.2	2052.7 ± 698.5
CL (mL/h)[c]	0.130 ± 0.032	0.006 ± 0.002

[a]half-life; [b]area under the concentration-time curve; [c]clearance

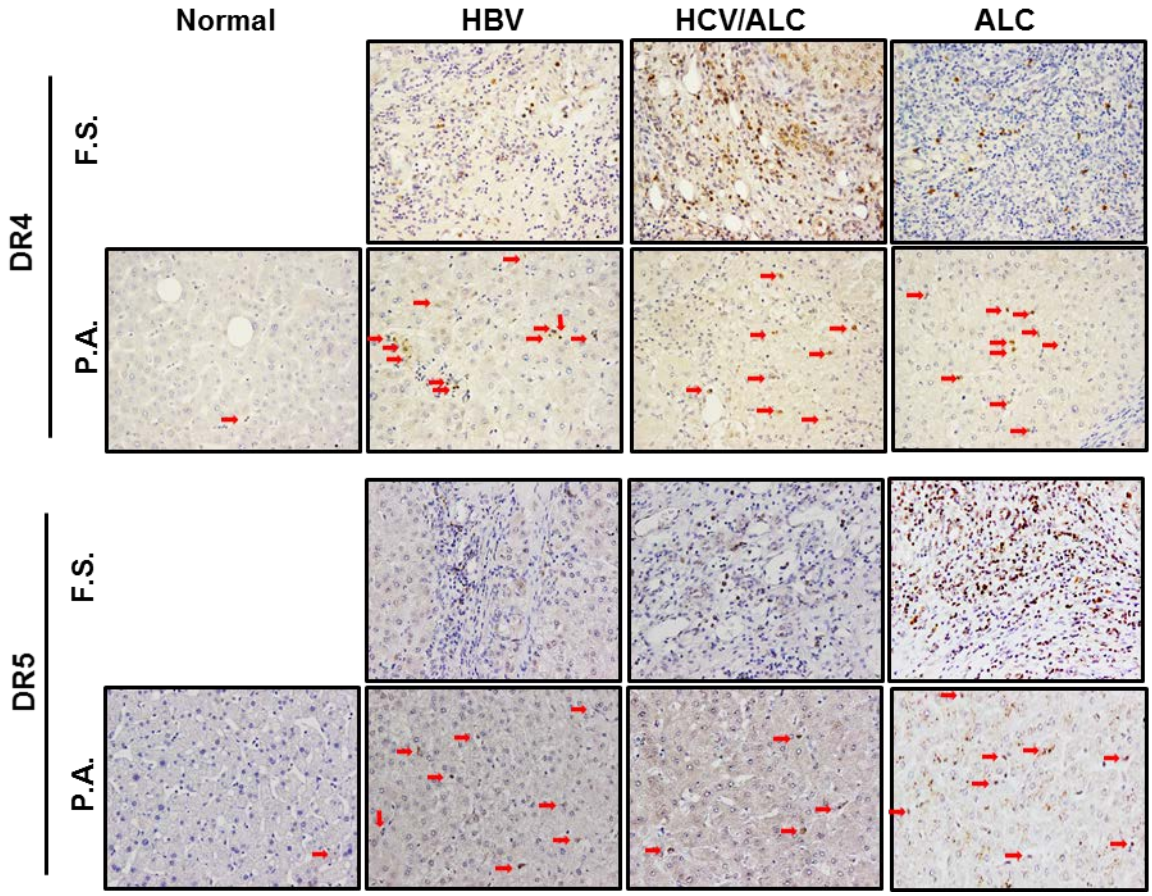
Supplementary Table 2.

Gene name	Forward (5’-3’)	Reverse (5’-3’)
Human		
TRAIL Receptor1/Dr4	TGTGACTTTGGTTGTTCCGTTGC	ACCTGAGCCGATGCAACAACAG
TRAIL Receptor2/Dr5	AAGACCCTTGTGCTCGTTG	AGGTGGACACAATCCCTCTG
TRAIL Receptor3/Dcr1	AAAGTTCCTGCACCATGACC	TGGCACCAAATTCTTCAACA
TRAIL Receptor4/Dcr2	GCTGAAGGGTGTCAGAGGAG	AGCCTGCCTCATCTTCTTCA
Acta-2	CCA GAG CCA TTG TCA CAC AC	CAG CCA AGC ACT GTC AGG
Tgfb1	CTT CCA GCC GAG GTC CTT	CCC TGG ACA CCA ACT ATT GC
Pdgf-r	CAG GAG AGA CAG CAA CAG CA	AAC TGT GCC CAC ACC AGA AG
Collagen-1A2	AGC AGG TCC TTG GAA ACC TT	GAA AAG GAG TTG GAC TTG GC
Mmp-2	GGA AAG CCA GGA TCC ATT TT	ATG CCG CCT TTA ACT GGA G
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
Rats		
Mmp-2	ACCAAGAACTTCCGACTATCCAATG	GTACCAGTGTCAGTATCAGCATCAG
Pdgf-r	ACACATCAAATACGCGGACA	GAGCACTGGTGAGT GTTGA

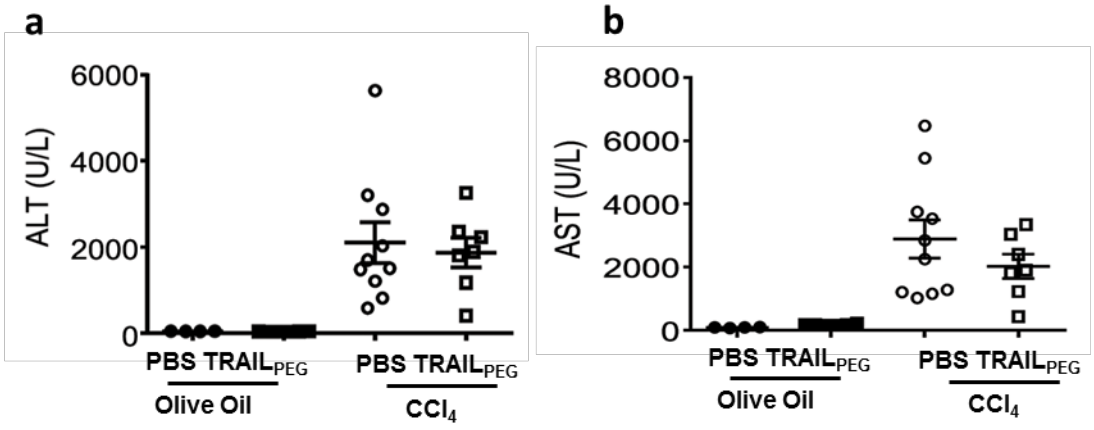
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



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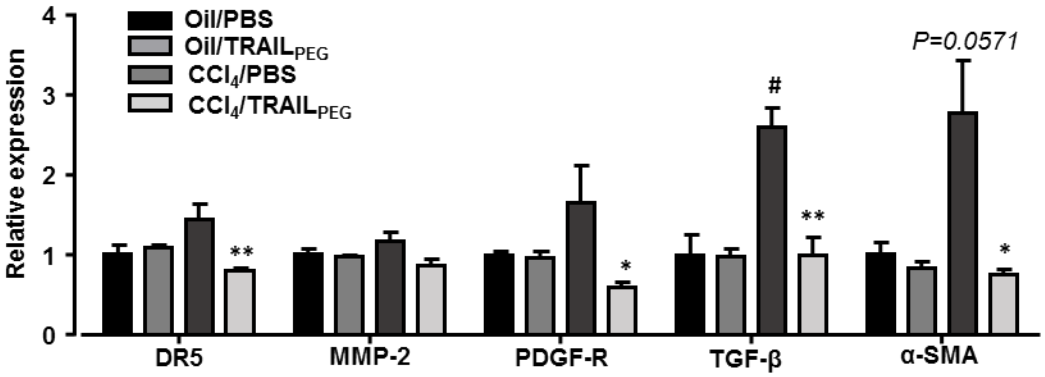

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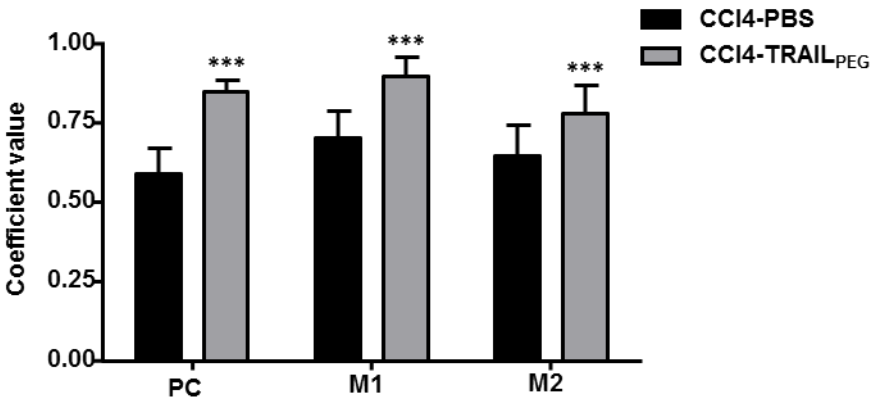

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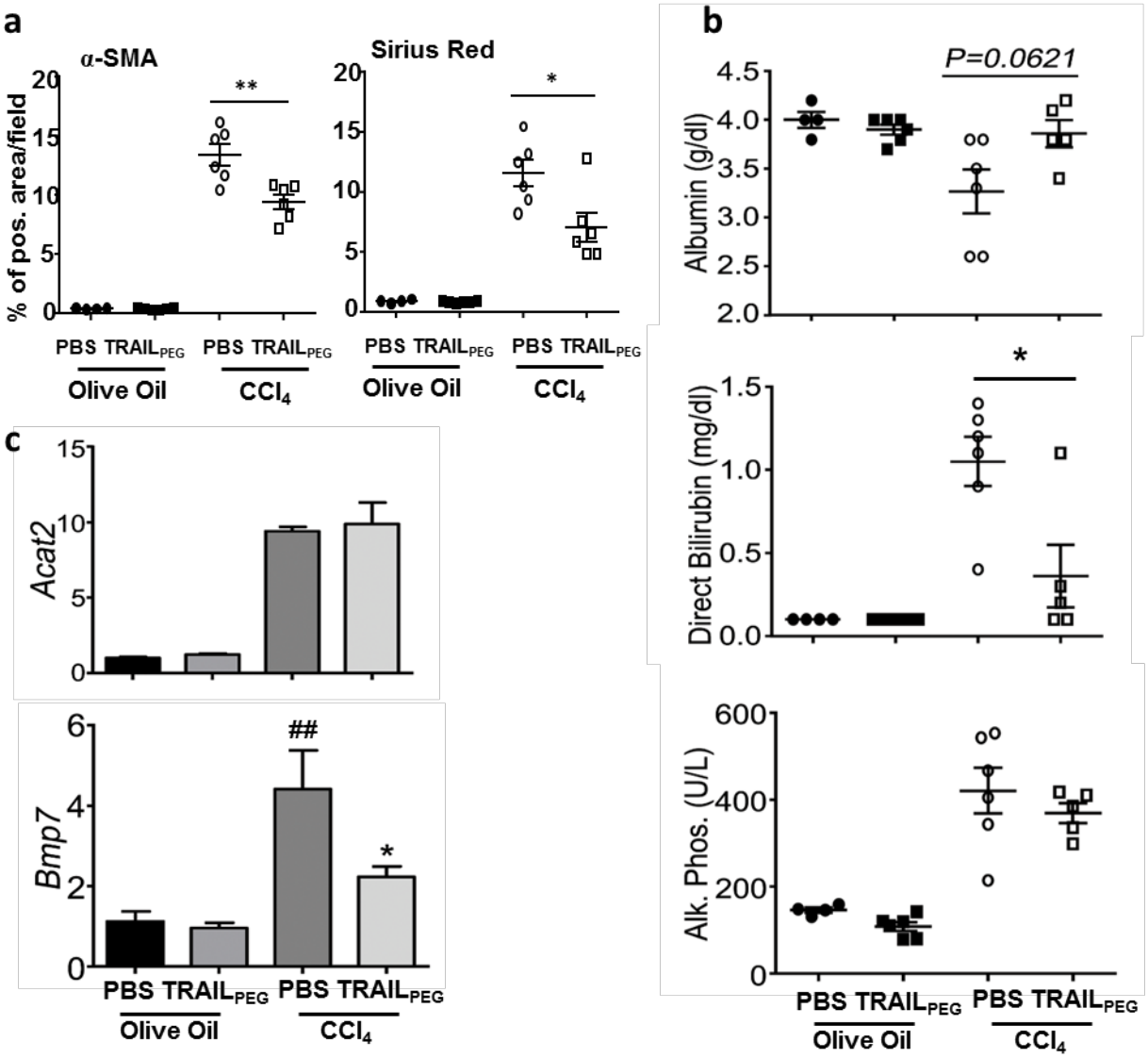
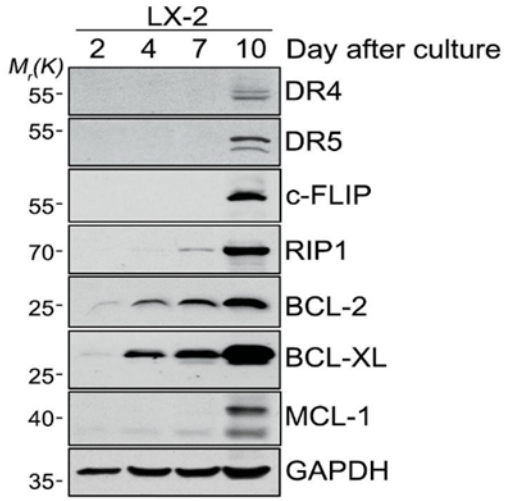


Supplementary Figure 7.



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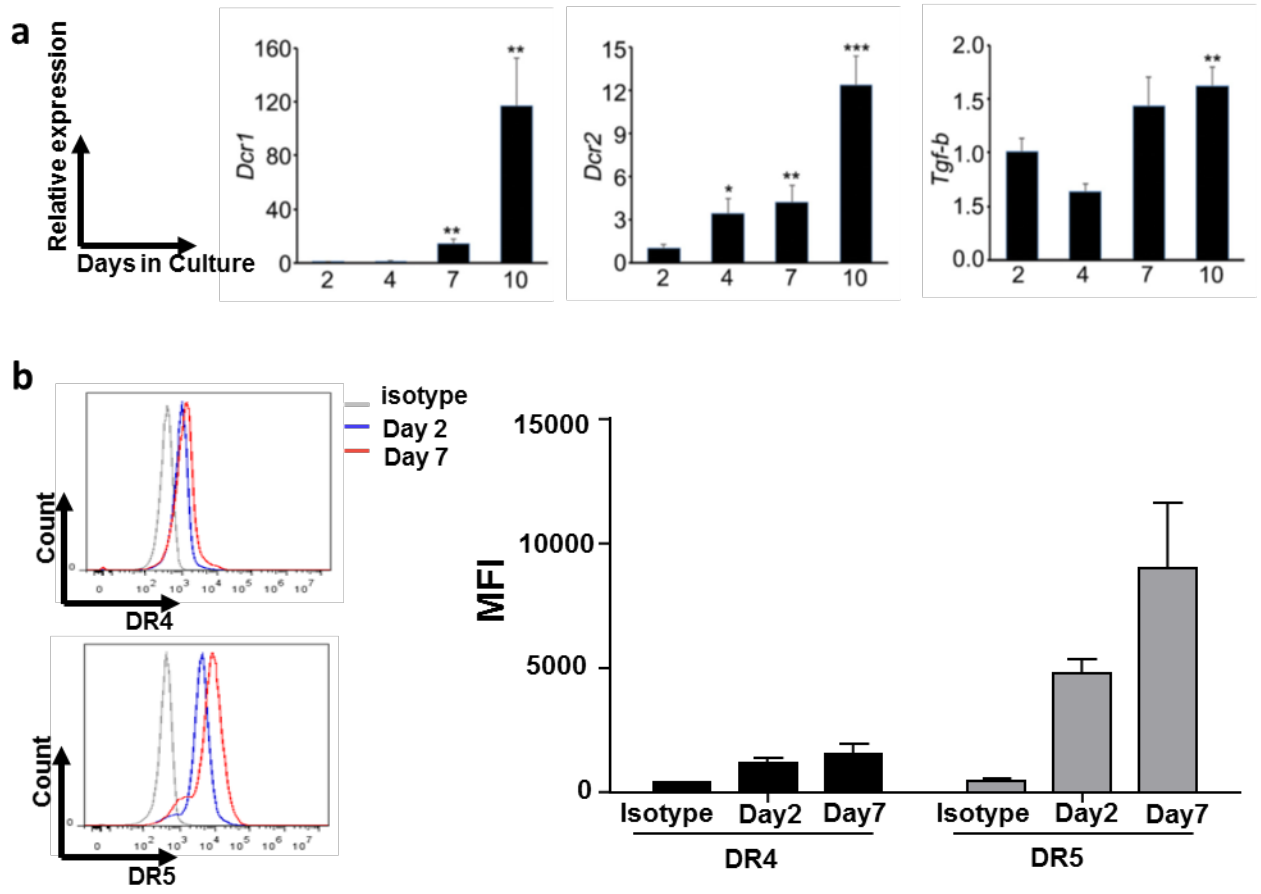
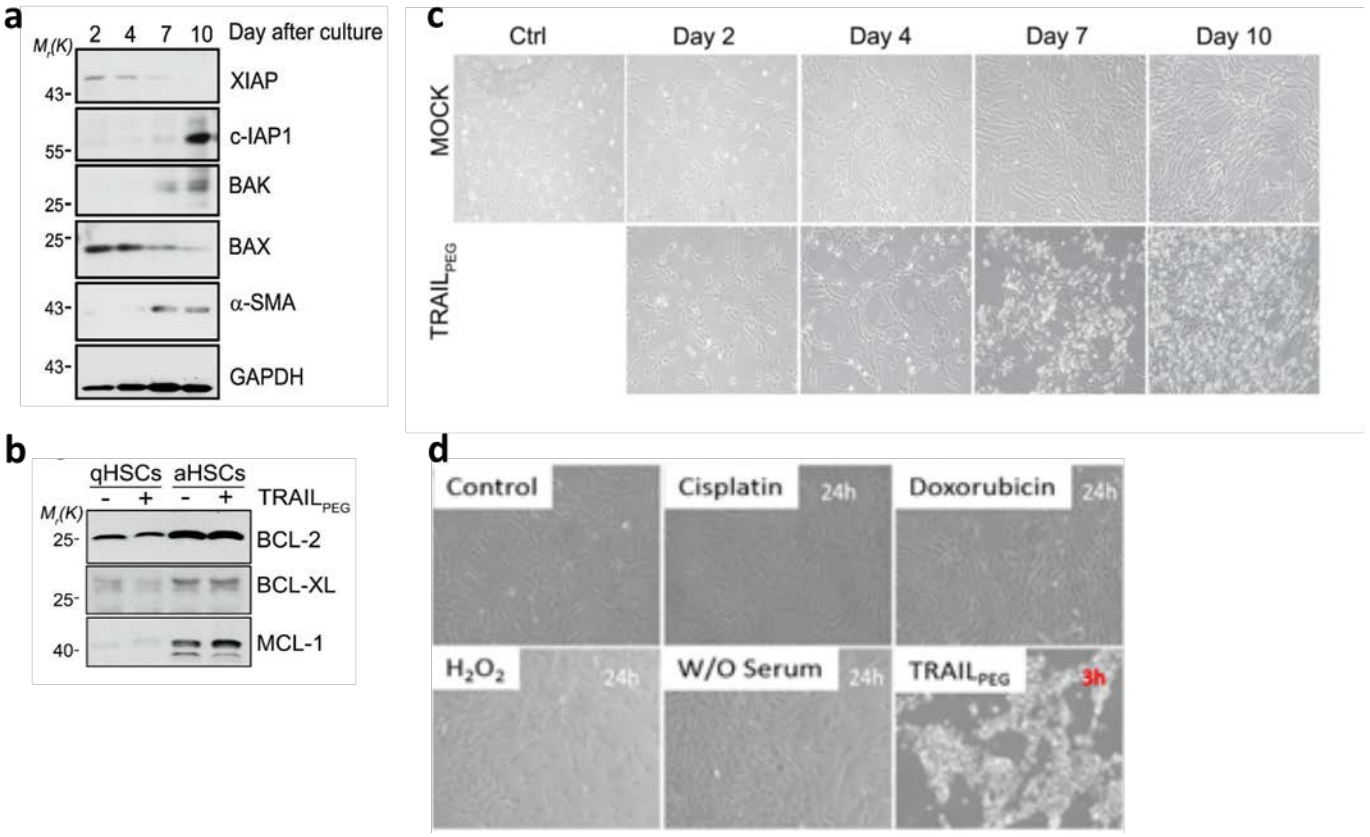


Supplementary Figure 9.



Supplementary Figure 10.