Supplementary Materials to

Tumor necrosis factor-inducible gene 6 interacts with CD44, which is involved in fate-change of hepatic stellate cells

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Supplementary Materials and Methods

Quantitative real-time PCR

Total RNA, which had been stored at -80°C, was extracted with TRIZOL™. After assuring sufficient RNA quality and concentration, total RNA was used to synthesized cDNA using the SuperScript II First-strand Synthesis System (Invitrogen) following the manufacturer's instruction. The gene expression was evaluated by qRT-PCR analysis. mRNAs were quantified by real-time RT-PCR per the manufacturer's instructions (Eppendorf, Mastercycler Real-Time PCR). The sequences of primers for human and mouse are listed in Supplementary Table S1. The expression of values was normalized to the levels of human 40S ribosomal protein 9S mRNA. Samples were analyzed in duplicate according to the 2^-delta delta Ct method. All PCR products were directly sequenced for genetic confirmation by Macrogen Inc. (Seoul, Korea).

Immunoprecipitation assay

For immunoprecipitation assay, total proteins extracted from TSG-6-given pHSCs at 30 min, 1, 2, and 6 hours were immunoprecipitated at 4°C overnight with rabbit anti-CD44 primary antibody (ab157107; Abcam, Cambridge, MA, US)

and protein G Plus/Protein A agarose beads (Millipore, Darmstadt, Germany). They were washed three times with lysis buffer and boiled in 5× sample buffer for 10 min. Resulting clear supernatants were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the following steps were taken using the same method applied for Western blot.

Western blot assay

Total protein was extracted from cultured cells. Cells were washed twice with PBS and lysed in RIPA buffer (Thermo) containing protease inhibitor (Roche, Indianapolis, IN, USA) and centrifuged at 13,000 r.c.f. for 20 min. The supernatants containing whole protein extracts were used in subsequent analysis. To separate nuclear and cytosolic fraction from total protein, harvested cells were suspended in buffer A (10 mM HEPES, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF) and incubated on ice for 20 min. After adding 0.1% NP-40, lysated cells were incubated on ice for 20 min additionally. After centrifugation at 5,000 r.c.f. for 2 min, the supernatants were collected for cytosolic fraction. Then, pellets were resuspended with buffer C (20 mM HEPES, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM EGTA) and incubated on ice for 30 min. After

centrifugation at 13,000 r.p.m for 15 min, the supernatants were collected for nuclear fraction. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of total protein were separated by SDS-PAGE and transferred to PVDF (polyvinylidene difluoride, Millipore) membranes. Primary antibodies used in this study were as follows: rabbit anti-TSG-6 (sc-30140; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-CD44 (ab157107; Abcam, Cambridge, MA, US), rabbit anti-phospho-GSK3α/β (9331; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-GSK3β (9315; Cell Signaling Technology), rabbit non-phospho-β-CATENIN (8814; Cell Signaling Technology), mouse anti-TCF4 (05-511; Millipore), rabbit anti-YAP-1 (4912; Cell Signaling Technology), rabbit anti-TAZ (4883; Cell Signaling Technology), rabbit anti-Lamin B1 (ab16048; Abcam), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; MCA4739; AbD Serotec, Hercules, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit (ADI-SAB-300; Enzo Life Sciences, Farmingdale, NY, USA) or anti-mouse IgG (ADI-SAB-100; Enzo Life Sciences) was used as secondary antibody. Membranes were developed by chemiluminescence (ATTO Corporation, Tokyo, Japan). Blots obtained from three independent experiments were scanned, and a range of interest (ROI) around the

band of interest was defined. Band intensities were calculated using the CS analyzer 2.0 program (ATTO Corporation).

Immunofluorescent staining

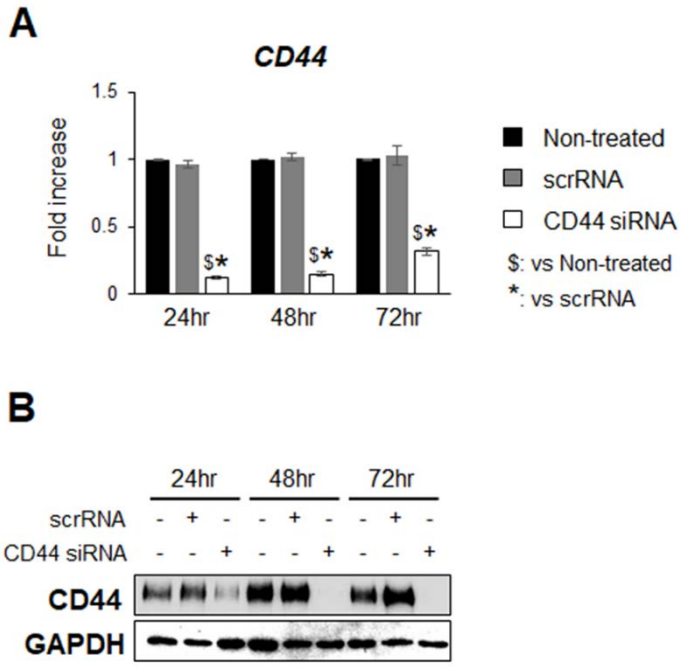
For immunofluorescent staining, cultured cells were washed with PBS, fixed and permeabilized with acetone and methanol, respectively. After fixation, they were washed three times with PBS to remove fixing solution and incubated with blocking solution for 30 min. Cells were incubated with primary antibodies, rabbit anti-CD44 (ab157107; Abcam), rabbit anti-non-phospho-β-CATENIN (8814; Cell Signaling Technology), rabbit anti-SOX9 (AB5535; Millipore) and rabbit anti-CK7 (ab181598; Abcam) at 4°C overnight. After washing with PBS, cells were incubated with Alexa Fluor 568-labelled goat anti-rabbit IgG (A-11011; Invitrogen) as secondary antibodies for 30 min at room temperature. 4',6-diamidino-2-phenylinole (DAPI; VectaShield, Burlingame, California, USA) was employed in the counterstaining procedure. Slides were viewed with a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany) or a Leica DMi8 fluorescence microscope (Leica Microscope Co., Wetzlar, Germany).

Supplementary Table

Supplementary Table S1. Primer list of qRT-PCR

Gene	Forward sequence	Reverse sequence
TGF-β	GAGGCTACCCTAGACACAAGG	GGGTGCCGTTGCTCATCATA
α-SMA	CTTCGCCCCCTTTAATAGTGC	TGAACTCCAACGTCAAGCGG
COL1α1	TTATAGAGCGATACAAGGGGGAG	CGCCGTCTGATTATCTTGATGAG
EPCAM	CTGGCCGTAAACTGCTTTGT	AGCCCATCATTGTTCTGGAG
CD133	TGGATGCAGAACTTGACAACGT	ATACCTGCTACGACAGTCGTGGT
C-KIT	TGCTTCACAGAAGACCATGC	GTGACCAACATGGAGTCGTG
CK7	CAGGATGTGGTGGAGGACTT	CATTGAGGGTCCTGAGGAAG
SOX9	GTACCCGCACTTGCACAA	TCGCTCTCGTTCAGAAGTCTC
CD44	AGCAACCAAGAGGCAAGAAA	GTGTGGTTGAAATGGTGCTG
9S	GACTCCGGAACAAACGTGAGGT	CTTCATCTTGCCCTCGTCCA

Supplementary Figure



Supplementary Figure S1. CD44 expression in pHSCs transfected with scramble or siRNA for CD44. (A) qRT-PCR and (B) western blot analysis for CD44 in pHSCs transfected with scrambled siRNA (scrRNA) or siRNA targeting CD44 (CD44 siRNA). CD44 expression in these cells was examined at 24, 48 and 72 hours after removing scr or CD44 siRNA. GAPDH was used as an internal control. Data shown represent one of three experiments with similar results. Mean±SD results are graphed ($p<0.05 versus non-treated, *p<0.05 versus scrRNA).