**CTGF mediates tumor-stroma interactions between hepatoma cells and hepatic stellate cells to accelerate HCC progression.**

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**Supplementary Materials and Methods**

**Single-sample gene set enrichment analysis (ssGSEA)**

We downloaded GSE14520 expression data from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) (Ref. PMID: 22202459), which contains normalized Affymetrix HT Human Genome U133A array data from 220 samples of non-tumor tissue. All probe set values for a gene were collapsed into a single vector of values by taking a median value. The ssGSEA projection was performed using the GenePattern platform (<http://www.broadinstitute.org/cancer/software/genepattern#)>.

**Cytokine array**

Secretome analysis was performed in LX-2 cells non-treated or treated with recombinant CTGF protein. Twenty-four hours after plating, LX-2 cells were serum starved for 12 hours. Cells were thereafter treated with or without 5 nM recombinant CTGF protein. Culture supernatant was collected 24 hours after the start of recombinant CTGF treatment and underwent secretome analysis (N = 1). Human XL Cytokine Array Kit (R&D systems, Minneapolis, MN, USA) was used for secretome analysis according to the manufacture’s protocol.

**Supplementary Figure Legends**

Supplementary Fig. 1. CTGF is mainly derived from tumor cells in liver tumors.

(A) Immunohistochemistry for AFP and CTGF in serial sections of human HCC tissues. (B) Double immunofluorescence staining for CTGF and α-SMA in human HCC tissues. (C) Immunohistochemistry for AFP and CTGF in serial sections of liver tumors from 8-month-old KrasG12D mice. (D) Double immunofluorescence staining for CTGF and α-SMA in 9-month-old KrasG12D mice. Arrowheads of the same color indicate the identical cells in serial sections (A, C).

Supplementary Fig. 2. CTGF expression is regulated through the Ras/Mek/Erk pathway.

(A-D) CTGF expression in liver cancer cell lines was evaluated after activation or inhibition of the Ras signaling pathway (N = 4 per group). Expression levels of CTGF and phosphorylated Erk and Akt and CTGF concentration in culture supernatants at the indicated time point after treatment with 100 ng/mL EGF in Kras wild-type Huh7 cells (A). Cells were serum-starved for 12 hours before the addition of EGF. Gene and protein expression of Ras and CTGF 72 hours after transfection of Kras siRNAs (#1 and #2) or control siRNA into Kras mutant-type HepG2 cells (B). Protein expression of p-Akt and p-Erk 3 hours after the addition of LY294002 (a PI3K inhibitor), U0126 (a Mek inhibitor) or FR180204 (an Erk inhibitor) at indicated doses (C). CTGF gene expression and cell viability in HepG2 cells 6 hours after the addition of inhibitors at indicated doses (D). Cell viability was evaluated using a WST-8 assay (N = 4 per group). (E) Correlation between the CTGF gene expression levels and single-sample gene set enrichment analysis (ssGSEA) enrichment scores of the Ras/Mek/Erk pathway in 220 samples of non-tumorous liver tissues. In bar graphs, bars and top whiskers represent mean values and standard deviations, respectively. \*p < 0.05 vs. the control.

Supplementary Fig. 3. CTGF expression and secretion levels in hepatoma cells.

CTGF mRNA and protein expression and concentration in the culture supernatant of hepatoma cell lines (A) and parental, mock-transfected, and CTGF-overexpressing PLC/PRF/5 cells (B). Cell lysates and culture supernatants were collected 72 hours after plating (N = 4-6 per group). In bar graphs, bars and top whiskers represent mean values and standard deviations, respectively. \*p < 0.05 vs. PLC/PRF/5 cells.

Supplementary Fig. 4. CTGF knock-down in HepG2 cells abolishes the growth-promoting effect by the co-existence of LX-2 cells.

HepG2 cell growth was evaluated using a WST-8 assay *in vitro* or in xenograft models at the indicated time points. In the cell culture experiments, HepG2 cells were incubated under monoculture or Transwell-co-culture with the same number of LX-2 cells. (A) The CTGF protein expression and the growth of HepG2 cells transfected with CTGF siRNAs (#1 and #2) or control siRNA under monoculture or Transwell co-culture with LX-2 cells. Total protein was collected 72 hours after siRNA transfection. Cells were re-plated 72 hours after siRNA transfection and incubated under monoculture or Transwell co-culture with LX-2 cells. (B) CTGF protein expression and in vitro growth of HepG2 cells transfected with CTGF shRNAs or control shRNA. Total protein was collected 24 hours after plating. (C) Xenograft models of HepG2 cells transfected with CTGF shRNAs or control shRNA co-injected with or without the same number of LX-2 cells. HepG2 cells were injected alone or co-injected with the same number of LX-2 cells into the left and right flanks of the mice, respectively. The mice were sacrificed, and the xenograft tumors were enucleated 22 days after inoculation. Representative images of enucleated tumors and sequential tumor volumes are presented as N = 4 (A, B) or 9-10 (C) per group. In the line graphs, plots with whiskers represent mean values ± standard deviations. \*p < 0.05 vs the control.

Supplementary Fig. 5. Inhibition of IL-6-STAT-3 pathway attenuates the growth-promoting effect by the co-existence of LX-2 cells.

For co-culture experiments, HepG2 cells were co-cultured with the same number of LX-2 cells using Transwell. Total protein was collected 24 hours after the start of incubation. Cell growth was evaluated by a WST-8 assay at the indicated time points (N = 4). (A) Protein expression and growth of HepG2 cells co-cultured with LX-2 cells with or without 1.0 μg/mL anti-IL-6 neutralizing antibody treatment. (B) Protein expression and growth of HepG2 cells transfected with STAT-3 siRNA or control siRNA under monoculture or co-cultured with LX-2 cells. HepG2 cells were re-plated 72 hours after siRNA transfection and incubated under monoculture or co-culture with LX-2 cells. Plots with whiskers indicate mean values ± standard deviations. \*p < 0.05 vs the control.

Supplementary Fig. 6. Protein expression of p-STAT-3 in liver cancer is decreased by the hepatocyte-specific CTGF knockout in KrasG12D mice.

Protein expression of p-STAT-3 in liver tumors in KrasG12D CTGF+/+, KrasG12D CTGF+/- and KrasG12D CTGF-/- mice at 8 months of age.

**Supplementary Tables**

Supplementary Table 1. Background characteristics of 93 HCC patients who underwent hepatectomy.

|  |  |
| --- | --- |
| Characteristics | Value |
| Age (years)† | 66 (33-84) |
| Gender (male/female) | 75/18 |
| Etiology (HBV/HCV/HBV+HCV/non-B, non-C) | 19/49/5/20  |
| Liver damage (A/B/C) | 49/43/1 |
| Background liver status (NL/CH/LC) | 6/56/31 |
| AFP (negative; < 20 ng/mL/positive; ≤ 20 ng/mL) | 39/54 |
| DCP (negative; < 40 mAU/mL/positive; ≤ 40 mAU/mL) | 23/70 |
| Tumor number (1-5/≤ 6) | 59/34  |
| Maximum tumor diameter (cm) | 3.9 (0.8-20.0) |
| Macroscopic tumor classification (SN-IM/SN/SN-EG/CMN/IF) | 1/32/27/16/17 |
| Portal invasion (negative/positive) | 71/22 |
| Intrahepatic metastasis (negative/positive) | 61/32 |
| TNM stage (I/II/III/IVa/IVb) | 12/42/21/17/1 |

HBV, hepatitis B virus; HCV, hepatitis C virus; NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; SN-IM, small nodular type with indistinct margin; SN, simple nodular type; SN-EG, simple nodular type with extranodular growth; CMN, confluent multinodular type; IF, infiltrative type.

The cut-off values for AFP and DCP were 20 ng/mL and 40 mAU/mL, respectively.

Age and maximum tumor diameter are presented as the medians (ranges).

Supplementary Table 2. Cytokines which showed more than 1.5-fold elevation after recombinant CTGF treatment in secretome analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| Analyte | Non-treated | rhCTGF | ratio |
| Chitinase 3-like 1 | 5137.8 | 22104.96 | 4.302416 |
| LIF | 4458.945 | 17145.29 | 3.845145 |
| HGF | 8652.54 | 28941.4 | 3.344844 |
| GDF-15 | 2583.715 | 8358.12 | 3.234923 |
| IL-23 | 6110.045 | 19322.68 | 3.162444 |
| Apolipoprotein A-I | 10664.76 | 31368.47 | 2.941321 |
| Angiopoietin-1 | 5143.31 | 14376.7 | 2.795223 |
| TGF-α | 2734.535 | 7592.74 | 2.776611 |
| Endoglin | 11224.22 | 30847.58 | 2.748306 |
| IL-13 | 2596.2 | 6939.44 | 2.672922 |
| Growth Hormone | 6911.485 | 18024.37 | 2.607886 |
| Lipocalin-2 | 13935.63 | 35925.08 | 2.577931 |
| IL-16 | 1974.77 | 4975.385 | 2.519476 |
| PDGF-AB/BB | 4763.685 | 11594.61 | 2.433958 |
| IFN-γ | 20270.94 | 47725.7 | 2.354391 |
| EGF | 2748.035 | 6382.62 | 2.322612 |
| C-Reactive Protein | 9116.25 | 20758.58 | 2.277096 |
| Fas Ligand | 7017.2 | 15967.9 | 2.275537 |
| Complement Factor D | 8879.005 | 19687.01 | 2.217254 |
| Cripto-1 | 4494.69 | 9873.53 | 2.19671 |
| RAGE | 4472.8 | 9617.645 | 2.150252 |
| IL-5 | 11699.27 | 25078.89 | 2.143629 |
| FGF basic | 9214.26 | 18727.82 | 2.032482 |
| ST2 | 9091.645 | 17877.58 | 1.966375 |
| IL-6 | 27427.55 | 52624.7 | 1.91868 |
| DPPIV | 3243.32 | 6150.46 | 1.896347 |
| IP-10 | 3354.565 | 6339.235 | 1.889734 |
| IGFBP-3 | 3259.69 | 6142.33 | 1.884329 |
| SHBG | 52583.1 | 99000.61 | 1.882746 |
| ICAM-1 | 9648.48 | 17008.39 | 1.762805 |
| Complement Component C5/C5a | 6481.285 | 10700.89 | 1.651045 |
| IL-33 | 3682.825 | 5866.655 | 1.592977 |
| IL-12p70 | 7213.035 | 11122.32 | 1.541975 |
| FGF-7 | 4723.095 | 7186.805 | 1.52163 |
| IL-27 | 26327.64 | 39673.48 | 1.506914 |
| CD31 | 10524.17 | 15837.68 | 1.504887 |